DIVERSITY OF RHIZOBIA NODULATING THE TREE LEGUMES *Acacia mangium* AND *Paraserianthes falcataria* AND THEIR INTERACTION WITH ARBUSCULAR MYCORRHIZAL FUNGI IN YOUNG SEEDLINGS

IRDIKA MANSUR

Department of Biosciences
University of Kent

A thesis submitted to the University of Kent
for the degree of Ph.D. in the Faculty of Science, Technology and Medical Studies

United Kingdom 2000

Declaration

As permitted under the University of Kent regulations, this thesis is written in the style of a set of publications. All chapters in this thesis, except chapter 3, are written in the style of *New Phytologist*. Chapter 3 is written in the style of *Molecular Ecology*. It is anticipated that several chapters will be submitted for publication in these journals in the near future. No part of this thesis has been submitted in support of an application for any other degree or qualification of the University of Kent or any other University or Institute of learning.

Irdika Mansur 3 February 2000

Acknowledgement

I would like to express my gratitude to my supervisors Dr J.C. Dodd for his continual support and guidance throughout my entire study at UKC, and Prof. P. Jeffries and Dr Y. Setiadi for his invaluable advise and encouragement. Without their understanding and attention, I would not have come this far. I also thank my supervisory committee, Profs. M. Tuite (Chairman) and R.G. Burns (member) for their invaluable advise and discussion.

I also would like to thank Dr J.P. Clapp (UKC) for his significant technical help and advise for the molecular techniques used in my study, Dr G. Cadisch (Wye College, University of London) for his help in doing the ^{15}N isotope analysis of my samples from the interaction experiments in his laboratory. I also thank Dr M. Vosatka for measuring hyphal lengths in the microcosm study.

Thanks to the UK-Indonesia "Biodiversity for Biotechnology Development Project" (1994-1999) funded by the UK Department for International Development (DFID), the British Council and the IIB (wwwbio.ukc.ac.uk/IIBMIRCEN/) for granting me the award of scholarship.

I would like to thank Prof. Goodfellow and Dr. Ward (University of Newcastle-upon-Tyne) for the PyMS training, and Drs. Surapareddy and Manian (HRI, Warwickshire) from whom I initially learned the PCR technique, and Dr Murphy (Wye College, University of London) for sharing his experience in DNA extraction. Mr Ray Newsham is thanked for advice for image analysis and general photography.

Many thanks also to everyone in PJ/JCD and ATB labs, Amanda, Claire, Virginie, Matthew, Helia, Jo, Pedro, Dr Stephen Heald, Tim, Kung'u, Noah, Denise, Mike, Carmen, Ipi, Claire, Alia and many others who came and went during my study, for their help and friendships. Also everyone in the Forest Biotechnology, IUC-IPB, Bogor for all the help during my research in Indonesia. I wish to acknowledge the back up from the British Council, particularly Dr Mawdsley and his staff, Ms. Anita Selwood, Ms. Anne Doherty, the Indonesian Student Association and The Islamic Society at UKC. I also thanks Ms. Hall for arranging a nice lunch in my viva. Special thanks to my mother, father, brothers and sisters, and in laws for the constant support and prayers for me.

Finally, but very importantly, I owe a great deal to my wife Novi and son Deio for their companionship, patience, prayers and help throughout the time of my study, and her significant help for preparing the final draft of this thesis.

Contents

Abbreviations i
Abstract ii

I. Diversity of rhizobia nodulating the tree legumes *Acacia mangium* and *Paraserianthes falcataria* and their interaction with arbuscular mycorrhizal fungi in young seedling

Description of *A. mangium* and *P. falcataria* I.3
Natural distribution *A. mangium* and *P. falcataria* in Indonesia ... I.4
Importance of the species within and outside Indonesia I.4
Nitrogen-Fixing Potential of *A. mangium* and *P. falcataria* I.10
Current Taxonomy of Rhizobia I.10
Studies of the Diversity of Rhizobial Strains Nodulating Leguminous Tree Species I.15
Tripartite Symbioses between Leguminous Plants, Rhizobia and Arbuscular Mycorrhizal Fungi I.17
Objectives of the Study I.19
References I.20

II. Phenotypic diversity of root-nodule bacteria isolated from tropical leguminous tree species *Acacia mangium* and *Paraserianthes falcataria* growing in Indonesia

Introduction II.1
Materials and Methods II.2
Origin of soil and root nodule samples II.3
Trapping of rhizobia from soil samples II.3
Isolation of rhizobia II.7
Plant nodulation tests II.7
Characterisation of bacterial isolates II.7
The Gram stain II.8
Relative growth rates on YMA and YMB II.8
Acid and alkaline reaction II.9
Morphological characterisation II.9
Carbon source utilisation II.9
Antibiotic resistances II.9
NaCl tolerance II.10
Host range tests II.10
Data analyses for the phenotypic data II.11
PyMS analyses II.11
Results II.12
Root nodule collection and trapping II.12
Plant nodulation tests II.12
Morphological characterisation II.12

Carbon source utilisation II.17
Antibiotic resistance II.17
NaCl tolerance II.21
Host range tests II.21
Cluster analysis II.21
PyMS analysis II.24
Discussion II.27
References II.32

III. Ribotyping of rhizobia nodulating *Acacia mangium* and *Paraserianthes falcataria* from different geographical areas in Indonesia using PCR-RFLP-SSCP analyses

Introduction III.1
Materials and Methods III.3
Origin of the Rhizobia Isolates III.3
Growing of Rhizobial Isolates and DNA extraction III.4
PCR amplification of 16S rRNA gene III.6
PCR-RFLP of 16S rRNA gene III.6
PCR-RFLP-SSCP of 16S rRNA gene III.7
16S rRNA gene sequence analysis III.8
Results III.8
PCR amplification of 16S rRNA gene III.8
PCR-RFLP analyses of 16S rRNA gene III.8
PCR-RFLP-SSCP analyses of 16S rRNA gene III.10
16S rRNA gene sequencing III.14
Confirmation of sequence differences detected by PCR-RFLP-SSCP III.17
Discussion III.21
References III.25

IV. Interactions between different species of arbuscular mycorrhizal fungi and rhizobia on the growth of *Acacia mangium* and *Paraserianthes falcataria*

Introduction IV.1
Materials and Methods IV.4
Mycorrhizal species and rhizobial isolates IV.4
Media preparation IV.4
Seed surface sterilisation and germination IV.4
Spore extraction and surface sterilisation IV.6
Rhizobial inoculum IV.6
Plant inoculation IV.6
^{15}N- isotope labelling IV.8
Harvesting and sample analyses IV.8
Calculation of nitrogen fixation IV.9
Experimental design IV.9
Statistical analyses IV.11
Results IV.11
Paraserianthes falcataria IV.11

Seedling height and stem diameter IV.11
Shoot and root biomass, leaf areas and root length IV.12
AMF colonisation and root nodule formation IV.17
Seedling N and P level, and Ndfa IV.22
Acacia mangium IV.22
Seedling height and stem diameter IV.22
Seedling biomass, leaf areas and root length IV.25
AMF colonisation and root nodule formation IV.25
Seedling N and P levels, and Ndfa IV.28
Cluster analyses IV.33
Discussion IV.33
References IV.39

V. Establishment of mycorrhizas of four different species of arbuscular mycorrhizal fungi observed using a Petri-dish observation chamber (PDOC)

Introduction V.1
Materials and Methods V.3
Media preparation V.3
Seed surface-sterilisation and germination V.3
Spore extraction and surface-sterilisation V.5
Spore germination and plant inoculation V.5
Measurement of growth parameters of AMF V.6
Results V.7
Spore germination V.7
Root colonisation V.8
The architecture of the extra-radical mycelia (ERM) V.8
Spore ontogeny V.12
Discussion V.16
References V.22

VI. Conclusion

Phenotypic and genotypic characteristics of rhizobia nodulating *A. mangium* and *P. falcataria* provenances from Indonesia VI.1
Distribution of rhizobial isolates in Indonesia nodulating *A. mangium* and *P. falcataria* VI.2
Interactions between AMF and rhizobia on growth of *A. mangium* and *P. falcataria* VI.3
Establishment of arbuscular mycorrhizas from four different genera of AMF VI.4
Application of AMF and rhizobia in Indonesian forestry VI.7
References VI.9

Publications
Appendices

Abbreviations

ALS	'Arbuscule-like' structures
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
AP-PCR	Arbitrarily primed-PCR
BAS	Branched absorbing structures
BSA	Bovine serum albumin
bv.	biovars
dbh	Diameter at breast height
DGGE	Denaturing gradient gel electrophoresis
dH_2O	De-ionised water
EBI	European bioinformatics institute
ERM	Extra-radical mycelia
IGS	Inter genic spacer
INVAM	International culture collection of arbuscular mycorrhizal fungi
LSD	Least significant difference
MDE	Mutation determination enhanchement
MS medium	MOPS buffer salt medium
NCBI	National centre for biotechnology information
Ndfa	Nitrogen derived from atmosphere
PAGE	Polyacrylamide gel electrophoresis
PB	Peptone broth
PCR	Polymerase chain reaction
PDM	Petri-dish microcosm
PyMS	Pyrolysis mass spectrometry
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
REP	Repetitive extragenic palindromic
RFLP	PCR-restriction fragment length polymorphisms
rRNA	Ribosomal RNA
SCCP	Single-strand conformational polymorphism
SDS	Sodium dodecyl sulfate
TY	Tryptone yeast
UPGMA	Unweighted pair-group method using arithmetic average
YMA	Yeast-extract mannitol agar
YMB	Yeast-extract mannitol broth

Abstract

Acacia mangium and *Paraserianthes falcataria* are important leguminous tree species in Indonesia and many other countries. These trees form symbiotic relationships with rhizobia and arbuscular mycorrhizal fungi (AMF). These symbioses enable the trees to grow in adverse soils with low N and P availability, which is typical of tropical soils. Despite the extensive use of *A. mangium* and *P. falcataria*, the rhizobia that nodulate these species have not been subjected to a comprehensive systematic analysis, nor have the synergistic benefit of the tripartite symbiosis been fully exploited to improve the growth and nutrient acquisition of the trees. In this study, fifty seven rhizobial strains nodulating either *A. mangium* (26 strains) or *P. falcataria* (31 strains), were isolated from diverse geographical areas in Indonesia and were studied for their phenotypic and genotypic relationships. Fifty one strains of non-nodulating root-nodule bacteria isolated from the root-nodules of *A. mangium* and *P. falcataria* were also subjected to the phenotypic characterisation. Five and seven representatives of the rhizobial strains from *A. mangium* and *P. falcataria*, respectively, were tested for their effectiveness to promote plant growth and N-fixation when co-inoculated with one of Indonesian isolate of the three species of AMF, *Glomus manihotis* BEG112, *Gigaspora rosea* BEG111, and *Scutellospora heterogama* BEG40. Results of the phenotypic characterisation studies showed that rhizobial strains nodulating *A. mangium* and *P. falcataria* exhibited the phenotypic characteristics of *Bradyrhizobium* and were generally distinct from the non-nodulating root-nodule bacteria. This was confirmed by the results obtained using PCR-RFLP-SSCP analysis on the 57 rhizobial strains and the sequence data of the 16S rRNA gene of representative strains. Furthermore, results from the molecular analysis showed that these strains could be divided into two distinct groups. One group was genotypically close to *Bradyrhizobium elkanii* and the other to *Bradyrhizobium japonicum*. Additionally, *A. mangium* was also nodulated by a strain which was closely related to *Mesorhizobium loti*. The strains of *B. elkanii* and *B. japonicum* nodulating *A. mangium* and *P. falcataria* were ubiquitous throughout the Indonesian archipelago. The data from the interaction experiment between AMF, rhizobial strains and *P. falcataria* showed that rhizobial strains nodulating this species varied in their capacity to promote plant growth and N fixation. These capacities changed considerably when each rhizobial strain was co-inoculated with different species of AMF. Under the conditions used, *A. mangium* seemed more selective for its AMF and rhizobial symbionts, compared with *P. falcataria*, showing optimal compatibility only with *Gi. rosea* BEG111 and *B. elkanii* strains. This was indicated by a significant improvement in growth and nutrient acquisition of the seedlings when inoculated with these microsymbionts alone or in combination, compared with uninoculated controls or with seedlings inoculated with *B. japonicum* strains and the other two AMF species in any combination. A novel microcosm system, the Petri-dish microcosm, was developed in this study to investigate the establishment of AMF species from different genera. The results showed that *Ac. tuberculata* BEG41, *G. manihotis* BEG112, *Gi. rosea* BEG111, and *Sc. heterogama* BEG40 differed considerably in their spore germination, root colonisation, the spread of the extra-radical mycelium (ERM), architecture of the ERM, and spore formation. These observations may suggest differences in their ecological strategy and requirements to complete their life-cycles, which may eventually affect the outcome of their interactions with rhizobial strains and host plants, in terms of nodule formation, N-fixation and plant growth. In conclusion, this work indicates that rhizobia nodulating *A. mangium* and *P. falcataria* are phenotypically diverse, and genotypically close to *B. elkanii*, *B. japonicum*, and *M. loti*. These rhizobial

strains were also functionally diverse (ability to promote plant growth and N-fixation), and the functional diversity increased when they co-habited roots with AMF. The importance of an appropriate strategy for screening effective combinations of AMF and rhizobial strains for different tree species, and the potential application in nursery production of trees in Indonesia, is also highlighted.

I. Diversity of rhizobia nodulating the tree legumes *Acacia mangium* and *Paraserianthes falcataria* and their interaction with arbuscular mycorrhizal fungi in young seedlings

Leguminous plants are important both economically and ecologically. Agricultural leguminous crops have a long history as cash-crops and as cheap sources of protein for human consumption. Forage crops are widely planted to improve the quality of animal feed. Leguminous plants have also been planted for green manures (Giller & Wilson, 1993). More recently, leguminous trees have received considerable attention for use in land stabilisation and rehabilitation (Haselwandter & Bowen, 1996). Leguminous plants, which include herbs, shrubs, woody vines and trees form symbiotic relationships with rhizobia, a closely related group of bacterial genera which fix nitrogen (N) from the atmosphere when in the symbiotic partnership. This symbiosis, known since 1877 (Baldwin & Fred, 1929), induces the formation of nodules on the root of the plants and allow the plants to fulfil their need for N regardless of the N status of the soil.

Danso *et al.* (1992) suggested that the benefit of N fixation from leguminous trees could be exploited to maintain productivity in zero/or low-input agriculture systems, such as agroforestry and silvopastoral systems. Inclusion of the tree improves soil fertility (Giller & Wilson, 1993), hence promoting the growth of associated plants, or the subsequent plants, grown in the area. For example, DeBell *et al.* (1997) demonstrated a growth improvement of *Eucalyptus saligna* grown in combination with the leguminous tree *Paraserianthes falcataria.* The contribution of the *P. falcataria* to the growth of *E. saligna* was equal or greater than that achieved through N fertiliser application.

Leguminous trees play an important ecological role in the N cycle due to their ability to fix N from the atmosphere. Nitrogen which is released into the atmosphere through denitrification (Patra *et al.*, 1996; Kawashima *et al.*, 1996) becomes unavailable for non-N fixing organisms. The leguminous trees fix the N from the atmosphere for use in metabolism and incorporate it in their tissues. After the plant dies, the fixed N is then released into the environment through several mechanism as shown in Table 1. In order to gain the maximum benefit from the rhizobium-legume symbiosis, three strategies can be applied: selective breeding of leguminous plants, selection of appropriate rhizobial strains, and the improvement of environmental factors (Giller & Wilson, 1993).

Table 1. Mechanisms by which the N fixed in leguminous plants are transferred to the soil or plants growing with leguminous trees (Giller & Wilson, 1993).

1. Below-ground plant parts
a. Root and nodule senescence
b. Rhizodeposition
- root exudates
- active secretion
- passive loss
- sloughed off cells
c. Direct transfer between roots by interconnected mycorrhizal strands
2. Above-ground plant parts
a. Fallen leaves
b. Leachates
c. Ammonium loss and uptake by associated plants
d. Plant residues

Acacia mangium and *P. falcataria* are two nitrogen-fixing leguminous tree species which have been widely planted in Indonesia and many other countries. These species are chosen because of their ability to fix N from the atmosphere and their fast growth. However, few studies of nitrogen fixation and the rhizobia associated with these trees have been conducted (Umali-Garcia *et al.*, 1988; Galiana *et al.*, 1990; Turk & Keyser 1992; Galiana *et al.*, 1994, 1998; Nuswantara *et al.*, 1997).

Both trees also form a second symbiosis with arbuscular mycorrhizal fungi (AMF). This symbiosis enhances nutrient uptake, especially phosphate and has been reported to alter the rhizobium-legume interactions (Dela Cruz *et al.*, 1988). Unfortunately, in most studies involving rhizobium-legume interactions, the complementary significance of AMF is often ignored.

Description of *Acacia mangium* and *Paraserianthes falcataria*

Acacia mangium is a member of the family Leguminosae, sub-family Mimosoideae, and tribe Acaciae. The tree can grow to a height of 25-35 m and the diameter can be over 60 cm (Pinyopusarerk *et al.*, 1993). In a provenance trial conducted in Indonesia, the trees grew up to 8-11 m within 2 years (Toumela *et al.*, 1996). This tree species is tolerant of low-fertility acid soils with high Al content (Cole *et al.* 1996; Watanabe *et al.*, 1998).

Paraserianthes falcataria formerly known as *Adenanthera falcataria*, *Albizia falcataria*, *Albizia moluccana*, *Albizia fulva*, *Albizia eymæ*, and *Albizia falcata* (Nielsen *et al.*, 1983), is a member of the family Leguminosae, sub-family Mimosoideae, and tribe Ingeae. Like *A. mangium* the species is not nutrient-demanding for growth, and grows well in adverse sites such as acid and highly aluminium (Al)-saturated soils (Evensen *et al.*, 1995; Singh *et al.*, 1995). For example, Otsamo *et al.* (1996) have reported that *P. falcataria* could grow on *Imperata cylindrica* grassland in Indonesia in degraded soils with a low level of available N and P (Santoso *et al.*, 1996). *Paraserianthes falcataria* is a fast-growing species which can grow up to 5-8 m height within two years (Otsamo *et al.*, 1997), and potentially to 40 m in height and 80 cm in diameter (Martawijaya *et al.*, 1989). Normally, the trees are harvested when they are 7-years old, with a diameter at breast height (dbh) of 30-40 cm.

The natural distribution *A. mangium* and *P. falcataria* in Indonesia

Acacia mangium and *P. falcataria* are indigenous to Indonesia and are naturally distributed on the Eastern part of Indonesia. *Acacia mangium* can be found growing naturally in the Maluku and Irian Jaya provinces. It grows naturally at low elevations, from just above sea level to up to 700 m, in acid soils with pHs ranging from 4.5–6.5 and usually of low fertility. The mean monthly temperature of its natural habitat ranges from a minimum between 13-21°C and a maximum between 25-32°C, with average annual rainfall of 1446-2970 mm (Pinyopusarerk *et al.*, 1993). Outside Indonesia this species is also native to Australia and Papua New Guinea (Pinyopusarerk *et al.*, 1993).

Paraserianthes falcataria is found wild in the provinces of South Sulawesi, Maluku, and Irian Jaya (Nielsen *et al.*, 1983; Martawijaya *et al.*, 1989). The tree grows at low altitudes up to 1600 m above sea level (Nielsen *et al.*, 1983) at a temperature range of 22-29°C (Atmosuseno, 1994). The tree is also indegenous to Papua New Guinea, the Solomon Islands, and the Bismarck Archipelago (Nielsen *et al.*, 1983).

The importance of the species within and outside Indonesia

Acacia mangium and *P. falcataria* are both important leguminous trees in Indonesia. They have been planted outside their natural distributions for various purposes, such as for plantation forests, land rehabilitation and conservation, and agroforestry. *Acacia mangium* and *P. falcataria* have been chosen as the main species for industrial forest plantations (Danaatmadja, 1990). Trees growing in nurseries and plantation forests are shown in Fig. 1-4. Otsamo *et al.* (1997) have reported that these tree species are among the 8 outstanding tree species which have been used to reforest *I. cylindrica* grasslands. They exhibit high survival rates (90-100%) and growth. In the Kediri forest district *P. falcataria* has been used to replace teak (*Tectona grandis*) plantations on the areas which are no longer suitable for teak due to decreased soil fertility.

Acacia mangium and *P. falcataria* have been used for nursing trees (Otsamo *et al.*, 1996; Otsamo, 1998) to support the growth of indigenous dipterocarp species which are not able to tolerate open grassland conditions. These fast-growing trees are grown prior to

Figure 1. Seedlings of *Acacia mangium* grown in the nursery of Kiani Hutani Lestari Co., East Kalimantan, Indonesia.

Figure 2. *Acacia mangium* plantation in East Kalimantan, Indonesia.

Figure 3. *Paraserianthes falcataria* seedlings grown in a nursery in Kediri forest district, East Java, Indonesia.

Figure 4. *Paraserianthes falcataria* plantation in Kediri forest district, East Java, Indonesia.

Figure 5. An agroforestry system with *Paraserianthes falcataria* as the main tree species and pineapples are planted as understorey crop. During the rainy season chili plants and maize also planted under the trees.

Figure 6. *Paraserianthes falcataria* planted in a garden forest in East Kalimantan (a), and a ready-to-harvest *Paraserianthes falcataria* tree grown in a garden forest in West Java (b), Indonesia.

the planting of the dipterocarp seedlings to provide shading for the seedlings. The presence of the nursing trees increases the development of the evergreen woody vegetation which then enhances the secondary succession towards the natural forest flora. It also reduces the competition by grass and the risk of fire (Kuusipalo *et al.*, 1995).

Paraserianthes falcataria has also been used for agroforestry (Fig. 5) which is usually incorporated during the establishment of plantation forests to reduce the cost of planting and maintenance during the first two years. Farmers living around the forest are invited to plant agricultural crops, such as chilli plants, maize, upland rice, ground-nut, pineapple and soybean, in the forest land, and *A. mangium* or *P. falcataria* seedlings are provided by the forest company who manage the forest area.

Paraserianthes falcataria is very commonly planted in garden forests (Fig. 6; Smiet, 1990). Since only low maintenance (no need for fertiliser applications, weeding, or soil treatments) is needed, and light interception by the canopy of this tree is low (Otsamo, 1998), it allows the understorey crops to grow well. Furthermore, since it can be harvested as early as 5 years after planting, *P. falcataria* is an ideal species for this use. The planting of this species in the garden is profitable for the farmer because, in the short-term, the farmer can harvest the branches for firewood and the leaves for green manure or fodder. In the longer term, the farmers can benefit by selling the timber.

The planting of *A. mangium* and *P. falcataria* for other purposes has also been reported in Indonesia, such as for land rehabilitation to suppress soil erosion (Amas & Purwanto, 1992), for hedgerows (Evensen *et al.*, 1994), and as forage trees (Panjaitan *et al.*, 1993). In West Java, *A. mangium* and *P. falcataria* are also planted on tea and coffee plantations as shade trees as well as for windbreaks.

Despite their relatively isolated natural distributions, these species have been planted in many other tropical countries. Tables 2 and 3 show that both species are currently distributed throughout the tropical and sub-tropical regions of Africa, America, Asia and Australia under a range of environmental conditions. This shows the incredible versatility of these tree species.

Table 2. Distribution and use of *A. mangium* outside Indonesia

No.	Country	Planting purpose	References
1.	Australia	Natural and Plantation	Pinyopusarerk *et al.* (1993)
2.	Bangladesh	Plantation forest	Hosain *et al.* (1995)
3.	Brazil	Plantation forest	Moriera *et al.* (1993)
4.	Cameroon	Agroforestry	Duguma & Tonye (1994)
5	China	Plantation forest	Wang *et al.* (1991)
6.	Congo	Plantation Forest	Bernhard-Reversat (1993)
7.	Costa-rica	Pasture rehabilitation	Gonzalez & Fisher (1994)
8.	Fiji	Energy plantation	Bell & Evo (1983)
9.	Hawaii	Land rehabilitation	Cole *et al.* (1996)
10.	India	Shade tree	Alex (1995)
11.	Laos	Land rehabilitation	Sylavong & Turnbull (1991)
12.	Malaysia	Plantation forest	Majid & Paudyal (1992)
13.	Papua New Guinea	Natural forest	Pinyopusarerk *et al.* (1993)
14.	Sierra Leone	Plantation forest and	Bakarr & Janos (1996)
15.	Sri Lanka	Plantation forest	Weerawardane *et al.* (1991)
16.	Thailand	Plantation forest	Chittachumnonk *et al.* (1991)
17.	The Philippines	Agroforestry	Sato & Dalmacio (1991)
18.	Vanuatu	Community project	Neil (1987)
19.	Vietnam	Agroforestry	Kardel (1993)
20.	Zaire	Provenance trial	Khasa *et al.* (1995)

Table 3. Distribution and use of *P. falcataria* outside Indonesia

No.	Country	Planting purpose	Reference
1.	Bangladesh	Plantation forest	Baksha (1991)
2.	Brazil	Plantation forest	Moriera *et al.* (1993)
3.	Cameroon	Agroforestry	Duguma & Tonye (1994)
4.	China	Plantation forest, shade	Zou (1993), Cao & Lou
5.	Hawaii	Plantation forest	Binkley *et al.* (1992)
6.	India	Agroforestry	Singh *et al.* (1995)
7.	Malawi	Agroforestry	Maghembe & Prins (1994)
8.	Malaysia	Plantation forest	Tan (1984)
9.	New Caledonia	Rehabilitation of mining	Nasi (1994)
10.	Nigeria	Forage trees	Larbi *et al.* (1996)
11.	Papua New Guinea	Natural forest	Nielsen *et al.* (1983)
12.	Samoa	Alley cropping	Rogers & Rosecrance (1992)
13.	Singapore	Afforestation programmes	Lee *et al.* (1993)
14.	Solomon Islands	Natural forest	Nielsen *et al.* (1983)
15.	The Philippines	Agroforestry	Sato & Dalmacio (1991)
16.	Zambia	Alley cropping	Matthews *et al.* (1992)

The Nitrogen-Fixing Potential of *A. mangium* and *P. falcataria*

Umali-Garcia *et al.* (1988) found that inoculation with isolates of rhizobia significantly increased height, %N, and N content of *A. mangium* seedlings compared with uninoculated controls. In an acid soil (Oxisol, pH 5.8) the effect of inoculation was greater when the pH was increased to 6.5 by liming. Galiana *et al.* (1998) found that after inoculation with effective strains of rhizobia, the beneficial effects could still be observed up to 39 months after inoculation. The symbiotic *A. mangium* fix between 100-300 kg N ha^{-1} yr^{-1} atmospheric N, measured using ^{15}N isotopic techniques (Sanginga *et al.*, 1995). Thus, the potential of *A. mangium* to improve the N status of soil is high (Mishra *et al.*, 1994; Bernhard-Reversat, 1993).

Compared with *A. mangium*, *P. falcataria* has received less attention in relation to its symbiotic relationship with rhizobia, mainly because of the importance of the former species for plantation forests and the other uses (Table 2). Umali-Garcia *et al.* (1988) found that inoculation of *P. falcataria* with effective isolates of rhizobia increased the height, biomass and N content of seedlings by 24%, 55% and 25%, respectively compared with controls receiving N fertiliser equal to 100 kg N ha^{-1} or 41%, 122% and 35%, respectively compared with controls without N fertiliser. Binkley (1997) determined that percentage of N within seedlings of *P. falcataria* fixed from the atmosphere was 95%, which was higher than that measured in mature trees (55%) (People *et al.*, 1991). The contribution of *P. falcataria* in the enrichment of soil-N has been investigated by Garcia-Montiel & Binkley (1998) who showed that the N availability was increased under *P. falcataria* stands by 2.6-9 fold.

Current Taxonomy of Rhizobia

Rhizobia are beneficial soil bacteria that have been extensively studied. The bacteria were first isolated by Beijerinck in 1888 who proposed the name *Bacillus radicicola.* The genus was renamed *Rhizobium* by Frank in 1889 (Elkan, 1992). Since then the systematics have changed considerably.

Five species were recognised by Baldwin & Fred (1929) and grouped into a single genus, *Rhizobium* (Table 4). The classification was based upon "cross-inoculation groups', morphology, cultural characters, physiological properties, and the serological reactions of the bacteria. This classification was accepted until 1980, when a new genus *Bradyrhizobium* was recognised (Jordan, 1980) for slower growing rhizobia. Moreover, in the 1984 edition of Bergey's Manual of Systematic Bacteriology (Volume 1) Jordan (1984) reclassified the Rhizobiaceae. He retained the 2 genera, *Rhizobium* and *Bradyrhizobium*, but only 4 species as he combined three species formerly described as *R. leguminosarum*, *R. trifolii* and *R. phaseoli* into a single species *R. leguminosarum*, with three biovars, *viciae*, *trifolii*, and *phaseoli* (Table 5).

Rhizobium contains the fast-growing rhizobia with doubling time of 2-3 hours, that are able to use a wide range of carbohydrates, are susceptible to antibiotics, and produce acid. The genus was known to nodulate most of the temperate legumes. In contrast, *Bradyrhizobium* is a genus of slow-growing rhizobia that nodulate most of the tropical legumes. They have a doubling time of 5-6 hours, use only limited numbers of carbohydrates, are more resistant to antibiotics, and produce alkali (Jordan, 1984).

In the last decade, rhizobial taxonomy has experienced a rapid change. New genera and species have been recognised and transfers of species from one genus to another have also been made (Table 6). Since the classification of the rhizobia in the 1984 edition of Bergey's Manual of Systematic Bacteriology (Volume 1) (Jordan, 1984), three more new genera of rhizobia have been recognised: *Azorhizobium*, *Mesorhizobium*, and *Sinorhizobium* (Young & Haukka, 1996), and one genus *Allorhizobium* has been proposed (DeLajudie *et al.*, 1998), and 23 species have been recognised or proposed (Table 6). This is apparently as a result of more leguminous species and cultivars being studied, over a larger area, especially from tropical regions.

Although the cross-inoculation grouping of rhizobia, as demonstrated by Fred & Baldwin (1929), has been rejected in the newer taxonomies of rhizobia, it cannot be denied that the rhizobial-host symbiosis is somewhat specific (Lieven-Antoniou & Whittam, 1997; Santamaria *et al.*, 1997; Swelim *et al.*, 1997). Therefore, the more legume species and cultivars that are studied, the greater the chance of revealing new species or maybe even genera of rhizobia. One species of legume can be nodulated effectively by more than one species of rhizobia from the same or different genera, e.g.

Table 4. The taxonomic classification of the genus *Rhizobium* according to Baldwin & Fred (1929).

Species	**Cross inoculation group**
R. leguminosarum Frank	*Lathyrus* spp., *Pisum* spp., *Vicia* spp. and *Lens*
R. trifolii Dangeard	*Trifolium* spp.
R. phaseoli Dangeard	*Phaseolus vulgaris, Ph. angustifolia,* and *Ph.*
R. meliloti Dangeard	*Melilotus* spp., *Medicago* spp. and *Trigonella*
R. japonicum Kirchner	*Glycine max*

Table 5. The taxonomic classification of rhizobia according to Jordan (1984).

Genera	**Species**
Bradyrhizobium	*B. japonicum*
Rhizobium	*R. leguminosarum* bv. *trifolii*
	R. leguminosarum bv. *phaseoli*
	R. leguminosarum bv. *viciae*
	R. meliloti
	R. loti

Table 6. The current recognised genera and species of rhizobia.

Genera	Species	Original host/Cross inoculation group	Reference
Allorhizobium	*Al. undicola*	*Neptunia natans*	DeLajudie *et al.* (1998)
Azorhizobium	*Az. caulinodans*	*Sesbania rostrata*	Dreyfus *et al.* (1988)
Bradyrhizo-bium	*B. elkanii*	*Glycine* spp.	Kuykendall *et al.* (1992)
	B. japonicum	*Glycine* spp.	Jordan (1980)
	B. liaoningense	*Glycine* spp.	Xu *et al.* (1995)
Mesorhizo-bium	*M. amorphae*	*Amorpha fruticosa*	Wang *et al.* (1999)
	M. ciceri	*Cicer arientinum*	Nour *et al.* (1994), Jarvis *et al.* (1997)
	M. huakuii	*Astragalus sinicus*	Chen *et al.* (1991), Jarvis *et al.* (1997)
	M. loti	*Lotus corniculatus*	Jarvis *et al.* (1982), Jarvis *et al.* (1997)
	M. mediter-raneum	*Cicer arientinum*	Nour *et al.* (1995), Jarvis *et al.* (1997)
	M. plurifarium	*Acacia senegal, Acacia tortilis, Prosopsis juliflora*	DeLajudie *et al.* ((1998))
	M. tianshanense	*Glycyrrhiza palidiflora*	Chen *et al.* (1995), Jarvis *et al.* (1997)
Rhizobium	*R. etli*	*Ph. vulgaris*	Segovia *et al.* (1993)
	R. galegae	*Galega orientalis*	Lipsanen & Lindstrom (1988)
	R. gallicum	*Ph. vulgaris*	Amarger *et al.* (1997)
	R. giardinii	*Ph. vulgaris*	Amarger *et al.* (1997)
	R. hainanense	*Desmodium sinuatum*	Chen *et al.* (1997)
	R. huautlense	*Sesbania herbacea*	Wang *et al.* (1998)
	R. legumino-sarum bv. *phaseoli*	*Ph. vulgaris*, *Ph. angustifolia*, and *Ph. multiflorus*	Jordan (1984)
	R. legumino-sarum bv. *trifolii*	*Trifolium* spp.	Jordan (1984)
	R. legumino-sarum bv. *viciae*	*Lathyrus* spp. , *Pisum* spp. *Vicia* spp. and *Lens* spp.	Jordan (1984)
	R. mongolense	*Medicago ruthenica*	VanBerkum *et al.* (1998)
	R. tropici	*Ph. vulgaris*, *Leucaena* spp.	Martinez-Romero *et al.* (1991)

Table 6. Continued

Genera	Species	Original host/Cross inoculation group	Reference
Sinorhizobium	*S. fredii*	*Glycine* spp.	Scholla & Elkan (1984); Chen *et al.* (1988)
	S. medicae	*Medicago* spp.	Rome *et al.* (1996)
	S. meliloti	*Melilotus*, *Medicago*, and *Trigonella*	DeLajudie *et al.* (1994)
	S. saheli	*Sesbania* spp.	DeLajudie *et al.* (1994)
	S. teranga	*Sesbania* spp, *Acacia* spp.	DeLajudie *et al.* (1994)
	S. xinjiangensis	*Glycine* spp.	Chen *et al.* (1988)

Phaseolus vulgaris can be nodulated by 5 different species of *Rhizobium* (*R. leguminosarum* bv. *phaseoli*, *R. tropici*, *R. etli*, *R. gallicum*, and *R. giardinii*), whereas soybeans can be nodulated by five species belong to two genera of rhizobia: *S. fredii*, *S. xinjiangensis*, *B. japonicum*, *B. elkanii*, and *B. liaoningense* (Table 6).

The introduction of DNA fingerprinting techniques, particularly those based on the polymerase chain reaction (PCR) have also made significant contributions to the changes in rhizobial taxonomy (Kahindi *et al.*, 1997). They provide a simple and rapid tool for the separation of phenotypically indistinguishable isolates, to reveal genetic relationships between isolates, and to detect new species (Laguerre *et al.*, 1994; Selenska-Pobell *et al.*, 1995; Paffetti *et al.*, 1996).

Studies of the Diversity of Rhizobial Strains Nodulating Leguminous Tree Species

Until now, the rhizobia that nodulate *A. mangium* and *P. falcataria* have not been assigned into any particular species. At a generic level, these rhizobia belong to *Bradyrhizobium* (Souvannavong & Galiana, 1991; Moriera *et al.*, 1993). Souvannavong & Galiana (1991) characterised 43 isolates of rhizobia isolated from root nodules of *A. mangium* growing in its natural range in Australia, China, Congo, French Guyana, Ivory coast, and Senegal. All belonged to the genus *Bradyrhizobium* based on their phenotypic characteristics. More recently, 9 rhizobia were isolated from *A. mangium* growing in Indonesia, (Nuswantara *et al.*, 1997) and shown to be closely related to *B. elkanii*. However, more studies involving a greater number of isolates of rhizobia isolated from different areas, including the natural habitat of the trees, are still needed. Information on the rhizobia nodulating *P. falcataria* is limited, and studies are necessary to reveal the characteristics of the microsymbionts. Characterisation and identification of both groups of rhizobia are also needed to determine their specificity and nitrogen-fixing effectiveness (Turk & Keyser 1992; Lesueur *et al.*, 1996). This information is also needed to develop a strategy to conserve the diversity within the rhizobial population (Spoerke *et al.*, 1996) and to select the best strains for the inoculation of a particular plant in a specific area.

Recently, the rhizobia that nodulate tropical leguminous trees have received more attention, which has led to the discovery of new species, such as *S. saheli* and *S. teranga* isolated from leguminous tree species *Acacia* and *Sesbania* growing in West Africa, and *R.*

huautlense isolated from *Sesbania herbacea* in Mexico (Table 6). Rhizobia isolated from tropical regions tolerate acid and alkaline soils, and may tolerate high temperatures and high salinity (Lesueur *et al.*, 1993; Surange *et al.*, 1997; Hashem *et al.*, 1998). Surange *et al.* (1997) found that rhizobia isolated from the leguminous tree species *Albizia lebbeck* could survive temperatures up to 50°C, and that isolates of *Sesbania formosa*, *Acacia farnesiana*, and *Dalbergia sissoo* from India were adapted well to soils of pH 12. All strains isolated from these tree species tolerated salt concentrations up to 5% (w/v). Lesueur *et al.* (1993) have found that rhizobia isolated from *A. mangium* were able to tolerate a low soil pH of 4.5, and Al concentrations as high as 100 µM.

Studies of rhizobia nodulating leguminous trees presented above (Dupuy *et al.*, 1994; Haukka *et al.*,1996; Nuswantara *et al.*, 1997; Marsudi *et al.*, 1999) support the fact that rhizobia nodulating leguminous trees are genetically diverse. Some tree species may be nodulated by more than one taxon, while others are more specific. Anyango *et al.* (1995) described rhizobia from acid soils which were mainly broad host-range isolates.

Examination of the diversity of partial 16S ribosomal RNA gene sequences within strains of rhizobia isolated from root nodules of *A. senegal* and *Prosopsis chilensis* trees growing in Kenya and Sudan, showed that the symbionts belonged to *Rhizobium* and *Sinorhizobium* (Haukka *et al.*,1996). Dupuy *et al.* (1994) found that rhizobia isolated from *A. albida* growing in Senegal had close relationships with *B. elkanii* and *B. japonicum*. Marsudi *et al.* (1999) studied the diversity of rhizobia nodulating *Acacia saligna* from Australia, and found that this species was nodulated by *R. leguminosarum* bv. *phaseoli*, *R. tropici*, *B. japonicum*, and *Bradyrhizobium* spp. (*Lupinus*). Turk & Keyser (1992) found that *A. mangium* and *P. falcataria* were in the same cross-inoculation group, since their *Bradyrhizobium* spp. could nodulate either plant.

In practice, understanding the genetic diversity of rhizobia that nodulate a particular tree species is not the only solution to obtain the maximum benefit from N-fixation (Giller & Wilson, 1993). Therefore, a study of the functional diversity of the bacteria should go along with the study of the genetic diversity. Although many strains successfully nodulate the tree, it may be only one compatible isolate is necessary to establish effective N-fixation (Kahindi *et al.*, 1997). Despite the extensive use of *A. mangium* and *P. falcataria* in Indonesia and in other countries, studies of the rhizobia nodulating these tree species are still limited. In Indonesia at least, inoculation of these species with rhizobia has not become a routine practice. However, seedlings in the nursery readily become nodulated by

indigenous rhizobia. An important question needs to be answered: are the trees able to form symbiotic relationships with a broad range of rhizobial taxa, or are the rhizobia that are specific for these trees ubiquitous?

Tripartite Symbioses between Leguminous Plants, Rhizobia and Arbuscular Mycorrhizal Fungi

The tripartite symbiosis between rhizobia, AMF, and nitrogen-fixing agricultural crops (Chaturvedi & Kumar, 1991; El-Ghandour *et al.*, 1996; Soliman *et al.*, 1996), tropical forage legumes (Arias *et al.*, 1991), and pasture crops (Azcón *et al.*, 1991; Leopold & Hofner, 1991) have been reported to have positive effects on plant growth, nodule formation, nitrogen fixation, and phosphate uptake. Leguminous trees also commonly form a symbiotic relationship with AMF (Colonna *et al.*, 1991; Kaushik & Kaushik, 1995; Haselwandter & Bowen, 1996). The presence of both microsymbionts in the root is indicated externally by the presence of nodules and the external structures of AMF, such as extra radical mycelium (ERM) and spores. Inside the roots, the structures of AMF such as intra-radical hyphae, arbuscules, and in some cases vesicles can also be observed after root staining.

Arbuscular mycorrhizal fungi benefit plant growth by promoting plant nutrient acquisition, especially P (Bolan, 1991; Fitter *et al.*, 1996), N (Azcón *et al.*, 1991; Azcón & Barea, 1992), Cu and Zn (Tarafdar & Rao, 1997); they can also increase resistance to plant pathogens (Liu, 1995; Sharma *et al.*, 1995; Singh & Singh, 1995), and increase tolerance to heavy metals (Hashem, 1995) and salinity (Azcón & El Atrash, 1997). Recently, the use of AMF has also been reported to improve the survival of micropropagated leguminous trees during transplantation (Naqvi & Mukerji, 1998; Subhan *et al.*, 1998).

The presence of AMF also affects the legume-rhizobial symbiosis beneficially by increasing nodulation and nitrogen fixation in soils of low fertility (Singh & Singh, 1993; Haselwandter & Bowen, 1996). Azcón *et al.* (1991) suggested that the improvement of the nodulation and nitrogen fixation in the mycorrhizal plants was due to the improvement of the P status of the plants. Murakamimizukami *et al.* (1991) have also found that colonisation by AMF increased indole acetic acid content in nodules which then promoted nodule growth.

One of the problems of the legume-rhizobial symbiosis is that it is sensitive to available N in the soil. High N availability will inhibit nodulation and N fixation (Abdel-Wahab *et al.*, 1996). High N availability can result from the decomposition of litter produced by the legume itself (Postgate, 1998). In the tripartite symbiosis, where the soil fertility is poor and the N availability is low, rhizobia will nodulate the legume and fix N. However, when the N availability in the soil increases due to the decomposition of litter produced by the legume, AMF will enhance the uptake of N by the legume (Azcón *et al.*, 1991; Azcón & Barea, 1992). Therefore, the rapid uptake of the available N mediated by the mycorrhiza will prevent the deleterious effect of N on nodulation and N-fixation by rhizobia. With this kind of interaction, the N cycling between rhizobia-legume is more efficient. The legume, thus, not only obtains N from direct N-fixation, but also from the mineralised N via uptake by AMF.

The benefit of the tripartite symbiosis to AMF has also been reported (Biro *et al.*, 1993; Xie *et al.*, 1995; Barea *et al.*, 1996). Xie *et al.* (1995) have found that plant flavonoids mediate induced stimulation of colonisation by AMF in soybean roots. Xie *et al.* (1998) later found that the Nod factor, lipo-oligosacharides secreted by rhizobia that induce nodule formation (Price *et al.*, 1996), also promoted sporocarp production by *Glomus mosseae*. Barea *et al.* (1996) found that inoculation with rhizobia increased the number of mycorrhizal entry points in alfalfa roots, thus increasing colonisation by AMF.

Preliminary studies on the tripartite symbiosis have indicated that there may be a need for the selection of compatible combinations of the microsymbiont to obtain a maximum benefit from the 2 symbionts (Azcón *et al.*, 1991). Azcón *et al.* (1991) used six strains of *Rhizobium* (currently *Sinorhizobium*) *meliloti* and three *Glomus* spp., and found that symbiotic efficiency (promotion of plant growth, and N and P acquisition) depended on the particular combination of *Rhizobium* and *Glomus*, indicating selective and specific-compatibility. Other experiments using different plant and AMF species combinations, and rhizobial strains also have given similar results (Ianson & Linderman, 1993; Ruiz-Lozano & Azcón, 1993).

Despite the intensive planting of leguminous trees in adverse areas, where the growth of seedlings has been limited by the supply of P from soil (Binkley, 1997), the benefit of the tripartite symbiosis has not been adequately explored (Azis & Sylvia, 1993). Recent studies have shown that the tripartite symbiosis on leguminous trees can give the same beneficial effects as those demonstrated in agricultural crops (Dodd *et al.*, 1990; Baker *et*

al., 1995; Stamford *et al.*, 1997). Therefore, these studies need to be extended to other important leguminous tree species, including *A. mangium* and *P. falcataria*, by using a wide range of AMF species and rhizobial strains. An study of these two species using a single rhizobial strain and four AMF isolates, *Glomus fasciculatum*, *Gigaspora margarita*, *Scutellospora persica* and *Sclerocystis clavispora*, showed a positive response of these species to dual inoculation (De la Cruz *et al.*, 1988).

Objectives of the Study

In view of the lack of knowledge about the rhizobia that nodulate *A. mangium* and *P. falcataria* and their interactions with AMF as mentioned above, the objectives of this study are:

1. To isolate, characterise and identify the rhizobia from *A. mangium* and *P. falcataria* growing in different geographical regions in Indonesia using morphological and physiological characterisation, pyrolysis mass spectrometry (PyMS) of the whole cells, and PCR-based DNA fingerprinting.
2. To study the host range of the rhizobial strains obtained from these tree species by inoculating the strains onto other species of leguminous plants.
3. To study the variation in plant growth response and ability to fix N following inoculation of tree legumes with these rhizobial strains.
4. To study the variation in interactive effects between the rhizobial strains and AMF species from different genera in the greenhouse.
5. To study the different patterns of establishment of AMF species from different genera through the development of a microcosm-based observation chamber.

Because of the slow-growing nature of these trees, relative to most agricultural crops, these objectives were not tackled in a chronological sequence. Several aspects were investigated concurrently.

References

Abdel Wahab AM, Zahran HH, Abd-Alla MH. 1996. Root-hair infection and nodulation of four grain legumes as affected by the form and the application time of nitrogen fertilizer. *Folia Microbiologia* **41**: 303-308.

Alex YJ. 1995. Mangium "the wonder shade tree". *Indian Coffee* **59**: 25-26.

Amarger N, Macheret V, Laguerre G. 1997. *Rhizobium gallicum* sp.nov. and *Rhizobium giardinii* sp.nov., from *Phaseolus vulgaris* nodules. *International Journal of Systematic Bacteriology* **47**: 996-1006.

Amas K, Purwanto. 1992. Run-off and soil erosion under various land covers in Kadipaten, West Java. *Buletin Penelitian Hutan* **547**: 1-11.

Anyango B, Wilson KJ, Beynon JC, Giller KE. 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L in 2 Kenyan soils with contrasting pHs. *Applied* and *Environmental Microbiology* **61**: 4016-4021.

Arias I, Koomen I, Dodd JC, White RP, Hayman DS. 1991. Growth-responses of mycorrhizal and non-mycorrhizal tropical forage species to different levels of soil phosphate. *Plant and Soil* **132**: 253-260.

Atmosuseno BS. 1994. *Sengon: budidaya, kegunaan and prospek.* Penebar Swadaya. Jakarta.

Azcón R, El-Atrash F. 1997. Influence of arbuscular mycorrhizae and phosphorus fertilisation on growth, nodulation and N_2 Fixation (^{15}N) in *Medicago sativa* at four salinity levels. *Biology and Fertility of Soils* **24**: 81-86.

Azcón R, Barea JM. 1992. Nodulation, N_2 fixation (^{15}N) and N-nutrition relationships in mycorrhizal or phosphate–amended alfalfa plants. *Symbiosis* **12**: 33-41.

Azcón R, Rubio R, Barea JM. 1991. Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N-fixation (^{15}N) and nutrition *Medicago sativa* L. *New Phytologist* **117**: 339-404.

Azis T and Sylvia DM. 1993. Utilisation of vesicular-arbuscular mycorrhizal fungi in the establishment of nitrogen-fixing trees. In Suba Rao NS and Rodriguez-Barrueco C, eds. *Symbiosis in nitrogen-fixing trees.* Oxford and IBH Publishing Co.PVT.Ltd. 167-194.

Bakarr MI and Janos DP. 1996. Mycorrhizal associations of tropical legume trees in Sierra Leone, West Africa. *Forest Ecology and Management* **89**: 89-92.

Baker A, Sprent JL, Wilson J. 1995. Effects of sodium-chloride and mycorrhizal infection on the growth and nitrogen fixation of *Prosopsis juliflora. Symbiosis* **19**: 39-51.

Baksha MW. 1991. Incidence of bark-eating caterpillar on moluccana koroi (*Paraserianthes falcataria*). *Bangladesh Journal of Forest Science* **20**: 44-48.

Baldwin IL, Fred EB. 1929. Nomenclature of the root-nodule bacteria of the Leguminosae. *Journal of Bacteriology* **17**: 141-50.

Barea JM, Tobar RM, Azcon-Aguilar C. 1996. Effect of genetically modified *Rhizobium meliloti* inoculant on the development of arbuscular mycorrhiza, root morphology, nutrient uptake and biomass accumulation in *Medicago sativa. New Pytologist* **134**: 361-369.

Bell TIW, Evo T. 1983. Fuelwood plantations and the Fijian village community. *Fiji Pine Research Paper – Fiji Pine Commission Fiji Forestry Department* No **12**, 6 pp.

Bernhard-Reversat F. 1993. Dynamics of Litter and organic matter at the soil litter interface in fast growing tree plantations on sandy ferralitic soils (Congo). *Acta Oecologica International Journal of Ecology* **14**: 179-195.

Binkley D. 1997. Bioassays of the influence of *Eucalyptus saligna* and *Albizia falcataria* on soil nutrient supply and limitation. *Forest Ecology and Management* **91**: 229-234.

Binkley D, Dunkin KA, deBell D, Ryan MG. 1992. Production and nutrient cycling in mixed plantations of *Eucalyptus* and *Albizia* in Hawaii. *Forest Science* **38**: 393-408.

Biro B, Voros I, Kovespechy, Saegi J. 1993. Symbiont effect of rhizobium bacteria and vesicular arbuscular mycorrhizal fungi on *Pisum sativum* in recultivated mine spoils. *Geomicrobiology Journal* **11**: 275-284.

Bolan NS. 1991. A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* **134**: 189-207.

Cao PR, Lou SM. 1994. Studies on the allelopathy of tea plant *Camellia sinensis* (L.) Kuntze. *Journal of South China Agricultural University* **15**: 129-133.

Chaturvedi C, Kumar A. 1991. Nodulation and nitrogenase activity in gram (*Cicer arietinum* L.) as influenced by *Rhizobium* and V-A Mycorrhiza interaction. *National Academy Science Letters-India* **14**: 289-292.

Chen WX, Yan GH, Li JL. 1988. Numerical taxonomy study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic Bacteriology* **38**: 392-397.

Chen WX, Li GS, Qi YL, Wang ET, Yuan HL, Li JL. 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus. International Journal of Systematic Bacteriology* **41**: 275-280.

Chen WX, Wang E, Wang SY, Li YB, Chen XQ. 1995. Characteristics of *Rhizobium tianshanense* sp.nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, Peoples Republic of China. *International Journal of Systematic Bacteriology* **45**: 153-159.

Chen WX, Tan ZY, Gao JL, Li Y, Wang ET. 1997. *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *International Journal of Systematic Bacteriology* **47**: 870-873.

Chittachumnonk P, Sirilak S, Turnbull JE. 1991. Performance of *Acacia* species in Thailand. Advances in tropical *Acacia* research. Proceedings of an international workshop held in Bangkok, Thailand, 11 – 15 February 1991. *ACIAR Proceedings Series* **35**: 153-158.

Cole TG, Yost RS, Kablan R, Olsen T. 1996. Growth potential of twelve *Acacia* species on acid soil in Hawaii. *Forest Ecology and Management* **80**: 175-186.

Colonna JP, Thoen D, Ducousso M, Badji S. 1991. Comparative effects of *Glomus mosseae* and P fertiliser on foliar mineral composition of *Acacia sinegal* seedlings inoculated with *Rhizobium*. *Mycorrhiza* **1**: 35-38.

Danaatmadja O. 1990. Industrial forest plantation in West Java. *Duta Rimba* **16**: 39-45.

Danso SKA, Bowen GD, Sanginga. 1992. Biological nitrogen fixation in trees in agro-ecosystems. *Plant and Soil* **141**: 177-196.

DeBell DS, Cole TG, Whitesell CD. 1997. Growth development, and yield in pure and mixed stands of *Eucalyptus* and *Albizia. Forest Science* **43**: 286-298.

Dela Cruz RE, Manalo ML, Aggangan NS, Tambalo JC. 1988. Growth of three legume trees inoculated with VA mycorrhizal fungi and rhizobium. *Plant and Soil* **108**: 111-115.

DeLajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins MD, Dreyfus B, Kersters K, Gillis M. 1994. Polyphasic taxonomy of rhizobia-emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb.nov., *Sinorhizobium saheli* sp.nov., and *Sinorhizobium teranga* sp. nov. *International Journal of Systematic Bacteriology* **44**: 715-733.

DeLajudie P, LaurentFulele E, Willems A, Torck U, Coopman R, Collins MD, Kersters K, Dreyfus B, Gillis M. 1998a. *Allorhizobium undicola* gen.nov., sp.nov., nitrogen fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *International Journal of Systematic Bacteriology* **48**: 1277-1290.

DeLajudie P, Willems A, Nick G, Moreira F, Molouba F, Hoste B, Torck U, Neyra M, Collins MD, Lindstrom K, Dreyfus B, Gillis M. 1998b. Characterisation of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *International Journal of Systematic Bacteriology* **48**: 369-382.

Dodd JD, Arias I, Koomen I, Hayman DS. 1990. The management of vesicular-arbuscular mycorrhizal populations in acid-infertile soils of savana ecosystem. I. The effect of pre-crpping and VAMF inoculation on plant growth and nutrition in the field. *Plant and Soil* **122**: 229-240.

Dreyfus B, Garcia JC, Gillis M. 1988. Characterization of *Azorhizobium caulinodans* gen.nov., sp.nov., a stem-nodulating nitrogen fixing bacterium isolated *from Sesbania rostrata. International Journal of Systematic Bacteriology* **38**: 89-98.

Duguma B, Tonye J. 1994. Screening of multipurpose tree and shrub species for agroforestry in the humid lowland of Cameroon. *Forest Ecology* and *Management* **64**: 135-143.

Dupuy N, Willems A, Pot B, Dewettinck D, Vanderbruaene I, Maestrojuan G, Dreyfus B, Kersters K, Collin MD, Gillis M. 1994. Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida. International Journal of Systematic Bacteriology* **44**: 461-473.

El Ghandour IA, El Sharawi MAD, Abdel-Moniem EM. 1996. Impact of vesicular arbuscular mycorrhizal fungi and *Rhizobium* on the growth and P, N, and Fe uptake by faba-bean. *Fertilizer Research* **43**: 43-48.

Elkan GH. 1992. Taxonomy of the rhizobia. *Canadian Journal of Microbiology* **38**: 446-450.

Evensen CL, Dierolf TJ, Yost RS. 1994. Growth of four tree species managed as hedgerows in response to liming on an acid soil in West Sumatra, Indonesia. *Agroforestry Systems* **27**: 297-222.

Evensen CL, Dierolf TJ, Yost RS. 1995. Decreasing rice and cowpea yields in alley cropping on a highly weathered oxisol in West Sumatra, Indonesia. *Agroforestry Systems* **31**: 1-19.

Fitter AH, Newsham KK, Merryweather JW. 1996. Diversity and function of arbuscular mycorrhizas in natural ecosystems. In Azcón-Aguilar C, Barea JM, eds. *Mycorrhizas in integrated systems from genes to plant development, Proceedings of the fourth European symposium on mycorrhizas.* European Commission. Brussels. 58-62.

Galiana A, Chaumont J, Diem HG, Dommergues YR. 1990. Nitrogen-fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp. *Biology and Fertility of Soils* **9**: 261-267.

Galiana A, Prin Y, Mallet B, Gnahoua GM, Poitel M, Diem HG. 1994. Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium* strains under

field conditions: Long-term effect on plant growth and persistence of the introduced strains in soil. *Applied* and *Environmental Microbiology* **60**: 3974-3980.

Galiana A, Gnahova GM, Chaumont J, Lesueur D, Prin Y, Mallet B. 1998. Improvement of nitrogen fixation in *Acacia mangium* through inoculation with rhizobium. *Agroforestry Systems* **40**: 297-307.

Garcia-Montiel DC, Binkley D. 1998. Effect of *Eucalyptus saligna* and *Albizia falcataria* on soil processes and nitrogen supply in Hawaii. *Oecologia* **113**: 547-556.

Giller KE, Wilson KJ. 1993. *Nitrogen fixation in tropical cropping systems.* C.A.B. International, Wallingford.

Gonzales JE, Fisher RF. 1994. Growth of native forest species planted on abandoned pasture land in Costa-rica. *Forest Ecology* and *Management* **70**: 159-167.

Haselwandter K, Bowen GD. 1996. Mycorrhizal relations in trees for agroforestry and land rehabilitation. *Forest Ecology and Management* **81**: 1-17.

Hashem AR. 1995. The role of mycorrhizal infection in the resistance of *Vaccinium macrocarpon* to manganese. *Mycorrhiza* **5**: 289-292.

Hashem FM, Swelim DM, Kuykendall LD, Mohamed AI, Abdel Wahab SM, Hegazi NI. 1998. Identification and characterisation of salt- and thermo-tolerant *Leucaena* nodulating rhizobium strains. *Biology and Fertility of Soils* **27**: 335-341.

Haukka K, Lindstrom K, Young JPW. 1996. Diversity of partial 16S ribosomal RNA sequences among and within strains of African rhizobia isolated from *Acacia* and *Prosopsis*. *Systematic and Applied Microbiology* **19**: 352-359.

Hosain MK, Islam SA, Islam QN. 1995. Selection of plantation tree species for degraded sal forest areas of Bangladesh. *Bangladesh Journal of Forest Science* **24**: 5-10.

Ianson DC, Linderman RG. 1993. Variation in the response of nodulating pigeonpea (*Cajanus cajan*) to different isolates of mycorrhizal fungi. *Symbiosis* **15**: 105-119.

Jarvis BDW, Pankhurst CE, Patel JJ. 1982. *Rhizobium loti*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* **32**: 378-380.

Jarvis BDW, Van Berkum P, Chen WX, Nour SM, Fernandez MP, Cleyet-Marel JC, Gillis M. 1997. Transfer of *Rhizobium loti*, *R. huakui*, *Rhizobium ciceri*, *Rhizobium mediterraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen.nov. *International Journal of Systematic Bacteriology* **47**: 895-898.

Jordan DC. 1980. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen.nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology* **32**: 136-139.

Jordan DC. 1984. Family III. Rhizobiaceae Conn 1938, 321AL. In Krieg NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology*, vol. 1. William & Wilkins, Baltimore, London, England. 234-254.

Kahindi JHP, Woomer P, George T, Moreira FMD, Karanja NK, Giller KE. 1997. Agricultural intensification, soil biodiversity and ecosystem function in the tropics : The role of nitrogen-fixing bacteria. *Applied Soil Ecology* **6**: 55-76.

Kardel O. 1993. A theoretical study for North Vietnam of alternative agroforestry systems to pure cassava. *Agroforestry Systems* **21**: 251-262.

Kaushik JC, Kaushik N. 1995. Interaction between vesicular-arbuscular mycorrhizal fungi and *Rhizobium* and their influence on *Albizia lebbeck* (L.) Benth. In Adholeya A, Singh S, eds. *Mycorrhizae: biofertilizer for the future, Proceedings of the third national conference on mycorrhiza.* Tata Energy Research Institute, New Delhi. 212-215.

Kawashima H, Bazin MJ, Lynch JM. 1996. Global N_2O balance and nitrogen fertilizer. *Ecological Modeling* **87**: 51-57.

Khasa PD, Li P, Vallee G, Magnussen S, Bousquet J. 1995. Early evaluation of *Recosperma auriculiforme* and *R. mangium* provenance trials on four sites in Zaire. *Forest Ecology and Management* **78**: 99-113.

Kuusipalo J, Adjers G, Jafarsidik Y, Otsama A, Toumela K, Vuokko R. 1995. Restoration of natural vegetation in degraded *Imperata cylindrica* grassland-understorey development in forest plantation. *Journal of Vegetation Science* **6**: 205-210.

Kuykendall LD, Saxena B, Devine TE, Udell SE. 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp.nov. *Canadian Journal of Microbiology* **38**: 501-505.

Laguerre G, Allard M-R, Revoy F, Amarger N. 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Applied and Environmental Microbiology* **60**: 56-63.

Larbi A, Smith JW, Adekunle IO, Kurdi IO. 1996. Studies on multipurpose fodder trees and shrubs in West Africa. *Agroforestry Systems* **33**: 29-39.

Lee SK, Goh KC, Yee SO. 1993. Afforestation as an interim measure to improve reclaimed lands in Singapore. *Land Degradation and Rehabilitation* **4**: 1-10.

Leopold HJ, Hofner W. 1991. Improvement of clover yield and quality by inoculation with VAM fungi and *Rhizobium* bacteria. *Angewandte Botanik* **65**: 23-33.

Lesueur D, Diem HG, Dianda M, LeRoux C. 1993. Selection of *Bradyrhizobium* strains and provenances of *Acacia mangium* and *Faidherbia albida* : relationship with their tolerance to acidity and aluminium. *Plant and Soil* **149**: 159-166.

Lesueur D, Tassin J, Enilorae MP, Sarraih JM, Peltier R. 1996. Study of *Calliandra calothyrsus- Rhizobium* nitrogen-fixing symbiosis. In Evans O, ed. *International workshop on the genus Calliandra, Bogor, 23 – 27 January 1996.* Winrock International. 62-76.

Lieven-Antoniou CA, Whittam TS. 1997. Specificity in the symbiotic association of *Lotus corniculatus* and *Rhizobium loti* from natural populations. *Molecular Ecology* **6**: 629-639.

Lipsanen P, Lindstrom K. 1988. Infection and root nodule structure in the *Rhizobium galegae* sp. nov. *Galega* sp. symbiosis. *Symbiosis* **6**: 81-95.

Liu R -J. 1995. Effect of vesicular-arbuscular mycorrhizal fungi on *Verticillium* wilt of cotton. *Mycorrhiza* **5**: 293-297.

Maghembe JA, Prins H. 1994. Performance of multipurpose trees for agroforestry two years after planting at Makoka, Malawi. *Forest Ecology and Management* **64**:171-182.

Majid NM, Paudyal BK. 1992. Pruning trial for *Acacia mangium* Willd plantation in peninsular Malaysia. *Forest Ecology and Management* **47**: 285-293.

Marsudi NDS, Glenn AR, Dilworth MJ. 1999. Identification and characterisation of fast- and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna. Soil Biology and Biochemistry* **31**: 1229-1238.

Martawijaya A, Kartasujana I, Mandang YI, Prawira SA, Kadir K. 1989. *Atlas kayu Indonesia. 2nd ed.* Research and Development Agency. The Ministry of Forestry. Bogor – Indonesia.

Martinez Romero E, Segovia L, Mercante FM, Franco AA, Graham P, Pardo MA. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *International Journal of Systematic Bacteriology* **41**: 417-426.

Matthews RB, Lungu S, Volk J, Holden ST, Solberg K. 1992. The potential of alley cropping in improvement of cultivation systems in the high rainfall areas of Zambia. 2. Maize production. *Agroforestry Systems* **17**: 241-261.

Mishra TK, Nandi A, Singh AK, Banerjee SK. 1994. Distribution of organic matter and nutrients in different tree components of two *Acacia* species and subsequent nitrogen enrichment in lateritic soil. *Annals of Forestry* **2**: 52-59.

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. 1993. Characterisation of rhizobia isolated from different divergence groups of tropical leguminosae by comparative polyacrylamide gel electrophoresis of their total protein. *Systematic and Applied Microbiology* **16**: 135-146.

Murakamimizukami Y, Yamamoto Y, Yamaki S. 1991. Analyses of indole acetic-acid and absisic-acid contents in nodules of soybean plants bearing VA mycorrhizas. *Soil Science and Plant Nutrition* **37**: 291-298.

Naqvi NS, Mukerji KG. 1998. Mycorrhization of micropropagated *Leucaena leucocephala* (Lam.) de Wit. *Symbiosis* **24**: 103-113.

Nasi R. 1994. Dossier : New Caledonia. Rehabilitation of mines sites : an example of research in favor of the environment. *Bois et Forests des Tropiques* **242**: 5-57.

Neil PE. 1987. Notes on potential multipurpose and community forest tree species. *Forest Research Report Vanuatu* No: 1-87.

Nielsen I, Guinet P, Baretta-Kuipers T. 1983. Studies in the Malaysian, Australian and Pacific Ingeae (Leguminosae-Mimosoideae): the genera *Archidendropsis*, *Wallaceodendron*, *Paraserianthes*, *Pararchidendron* and *Serianthes* (part 2). *Bulletin du Museum National d'Histoire Naturelle, B Adansonia* **5**: 335-360.

Nour SM, Fernandez MP, Normand P, Cleyetmarel JC. 1994. *Rhizobium ciceri* sp.nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *International Journal of Systematic Bacteriology* **44**: 511-522.

Nour SM, Cleyetmarel JC, Normand P, Fernandez MP. 1995. Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp.nov. *International Journal of Systematic Bacteriology* **45**: 640-648.

Nuswantara S, Fujie M, Sukiman HI, Yamashita M, Yamada T, Murooka Y. 1997. Phylogeny of bacterial symbionts of the leguminous tree *Acacia mangium*. *Journal of Fermentation and Bioengineering* **84**: 511-518.

Otsamo R. 1998. Effect of nurse tree species on early growth *Anisoptera marginata* Korth (Dipterocarpaceae) on an *Imperata cylindrica* (L.) Beauv. grassland site in South Kalimantan, Indonesia. *Forest Ecology and Management* **105**: 303-311.

Otsamo R, Adjers G, Hadi TS, Kuusipalo J, Otsamo A. 1996. Early performance of 12 shade tolerant tree species interplanted with *Paraserianthes falcataria* on *Imperata cylindrica* grassland. *Journal of Tropical Forest Science* **8**: 381-394.

Otsamo A, Adjers G, Hadi TS, Kuusipalo J, Vuokko R. 1997. Evaluation of reforestation potential of 83 tree species planted on *Imperata cylindrica* dominated grassland – A case study from South Kalimantan, Indonesia. *New Forest* **14**: 127-143.

Paffetti D, Scotti C, Gnocchi S, Fancelli S, Bazzicalupo M. 1996. Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varietas. *Applied and Environmental microbiology* **62**: 2279-2285.

Panjaitan M, Sturr WW, Jessop R. 1993. Growth of forage tree legumes at four agro-climatic sites in Indonesia. *Journal of Agriculture Science* **120**: 311-317.

Patra AK, Burford JR, Rego TJ. 1996. Volatilization losses of surface-applied urea nitrogen from Fertisols in the Indian semi-arid tropics. *Biology and Fertility of Soils* **22**: 345-349.

People MB, Bergersen FJ, Turner GL, Sampet C, Rerkasem B, Bhromsiri A, Nurhayati DP, Faizah AW, Sudin MN, Norhayati M, Herridge DF. 1991. Use of natural enrichment of 15N in plant available soil N for the measurement of symbiotic N2 fixation. In IAEA/FAO, *Stable isotopes in Plant Nutrition, Soil Fertility and Environmental Studies*. IAEA, Vienna. 117-130.

Pinyopusarerk K, Liang SB, Gunn BV. 1993. Taxonomy, distribution, biology, and use as an exotic. In Awang K, Taylor D, eds. *Acacia mangium: growing and utilisation*. Winrock International and The Food and Agriculture Organisation of the United Nations, Bangkok, Thailand. 1-19.

Postgate J. 1998. *Nitrogen fixation* , 3rd ed. Cambridge University Press, Cambridge, UK.

Price NPJ, Talmont F, Wieruszeski J-M, Promé D, Promé J-C. 1996. Structural determination of symbiotic nodulation factors from the broad host-range *Rhizobium* species NGR234. *Carbohydrate Research* **289**: 115-136.

Rogers S, Rosecrance R. 1992. Coppice management of *Paraserianthes falcataria* in Western Samoa. *Nitrogen Fixing Tree Research Report* **10**: 178-179.

Rome S, Fernandez MP, Brunel B, Normand P, CleyetMarel JC. 1996. *Sinorhizobium medicae* sp.nov., isolated from annual *Medicago* spp. *International Journal of Systematic Bacteriology* **46**: 972-980.

Ruiz-Lozano JM, Azcón R. 1993. Specificity and functional compatibility of VA mycorrhizal endophytes in association with *Bradyrhizobium* strains in *Cicer arietinum*. *Symbiosis* **15**: 217-226.

Sanginga N, Vanlauwe B, Danso SKA. 1995. Management of biological N_2 fixation in alley cropping system : estimation and contribution to N balance. *Plant and Soil* **174**: 1-2, 119-141.

Santamaria M, Carzo J, Leon-Barrias M, Gutierrez-Navarro AM. 1997. Characterisation and differentiation of indigenous rhizobia isolated from Canarian shrub legumes of agricultural and ecological interest. *Plant and Soil* **190**: 143-152.

Santoso D, Adiningsih S, Mutert E, Fairhurst T, Van Noordwijk M. 1996. Soil fertility management for reclamation of *Imperata* grasslands by smallholder agroforestry. *Agroforestry Systems* **36**: 181-202.

Sato A, Dalmacio RV. 1991. Maize production under an intercropping system with fast-growing tree species : a case in the Philippines. *Japan Agricultural Research Quarterly* **24**: 319-326.

Scholla MH, Elkan GH. 1984. *Rhizobium fredii* sp.nov., a fast growing species that effectively nodulates soybeans. *International Journal of Systematic Bacteriology* **34**: 484-486.

Segovia L, Young JPW, Martinez-Romero E. 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp.nov. *International Journal of Systematic Bacteriology* **43**: 374-377.

Selenska-Pobell S, Gigova L, Petrova N. 1995. Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. *Journal of Applied Bacteriology* 79: 425-431.

Sharma MP, Bhargava S, Varma MK, Adholeya A. 1995. Effect of vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum* on survival and development of

root-knot nematode *Meloidogyne incognita* in tomato. In Adholeya A, Singh S, eds. *Mycorrhizae: biofertilizer for the future, Proceedings of the third national conference on mycorrhiza.* Tata Energy Research Institute, New Delhi. 115-118.

Singh HP, Singh TA. 1993. The interaction of rock-phosphate , *Bradyrhizobium*, vesicular-arbuscular mycorrhizae and phosphate-solubilising microbes on soybean grown in a sub-Himalayan molisol. *Mycorrhiza* 4: 37-43.

Singh RS, Singh D. 1995. Effect of *Glomus aggregatum* inoculation and P-amendments on *Fusarium oxysporum* f.sp. *ciceri* causing chickpea wilt. In Adholeya A, Singh S, eds. *Mycorrhizae: biofertilizer for the future, Proceedings of the third national conference on mycorrhiza.* Tata Energy Research Institute, New Delhi. 119-123.

Singh BP, Dhyani SK, Chauhan DS, Prasad RN. 1995. Effect of multipurpose tree species on chemical properties of an acid Alfisol in Meghalaya. Indian Journal of Agricultural Science **65**: 345-349.

Smiet AC. 1990. Agroforestry and fuel-wood in Java. *Environmental Conservation* **17**: 235-238.

Soliman S, El-Ghandour IA, Khandra-Abbady A. 1996. Effect of nitrogen and phosphorous supply, and rhizobium and VAM fungus inoculations on dinitrogen fixation in soybeans. *Folia Microbiologia* **41**: 197-200.

Souvannavong O, Galiana A. 1991. *Acacia mangium-Rhizobium* symbiosis: selection and propagation of the host plant and the microorganism. In Taylor DA, MacDicken KG, eds. *Research on multipurpose tree species in Asia.* Winrock International. Bangkok, Thailand. 216-222.

Spoerke JM, Wilkinson HH, Parker MA. 1996. Non-random genotypic associations in a legume-*Bradyrhizobium* mutualism. *Evolution* **50**: 146-154.

Stamford NP, Ortega AD, Temprano F, Santos DR. 1997. Effect of phosphorus fertilization and inoculation of *Bradyrhizobium* and mycorrhizal fungi on growth of *Mimosa caesalpiniaefolia* in an acid soil. *Soil Biology* and *Biochemistry* **29**: 959-964.

Subhan S, Sharmila P, Saradhi PP. 1998. *Glomus fasciculatum* alleviates transplantation shock of micropropagated *Sesbania sesban. Plant Cell Report* **17**: 268-272.

Surange S, Wollum AG, Kumar N, Nautiyal CS. 1997. Characterization of *Rhizobium* from root nodules of leguminous tree growing in alkaline soils. *Canadian Journal of Microbiology* **43**: 891-894.

Swelim DM, Hashem FM, Kuykendall LD, Hegazi NI, Abdul Wahab SM. 1997. Host specificity and phenotypic diversity of *Rhizobium* strains nodulating *Leucaena, Acacia* and *Sesbania* in Egypt. *Biology and Fertility of Soils* **25**: 224-232.

Sylavong L, Turnbull JW. 1991. Australian Acacias in Laos. Advances in tropical *Acacia* research. Proceedings of an international workshop held in Bangkok, Thailand, 11 – 15 February 1991. *ACIAR Proceedings Series* **35** : 227-228.

Tan KC. 1984. The response of *Paraserianthes falcataria* to row thinning. *Malaysian Forestry* 47: 322-334.

Tarafdar JC, Rao AV. 1997. Response of arid legumes to VAM fungal inoculation. *Symbiosis* **22**: 265-274.

Toumela K, Otsamo A, Kuusipalo J, Vuokko R, Nikles G. 1996. Effect of provenance variation and singling and pruning on early growth of *Acacia mangium* Willd – plantation on *Imperata cylindrica* (L.) Beauv. dominated grassland. *Forest Ecology and Management* **84**: 241-249.

Turk D, Keyser HH. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Canadian Journal of Microbiology* **38**: 451-460.

Umali-Garcia M, Libuit JS, Baggayan RL. 1988. Effects of rhizobium inoculation on growth and nodulation of *Albizia falcataria* (L.) Fosh. and *Acacia mangium* Willd. in the nursery. *Plant and Soil* **108**: 71-78.

VanBerkum P, Beyene D, Bao GP, Campbell TA, Eardly BD. 1998. *Rhizobium mongolense* sp.nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *International Journal of Systematic Bacteriology* **48**: 13-22.

Wang MR, Fang YL, Turnbull JW. 1991. The history of *Acacia* introductions to China. Advances in tropical *Acacia* research. Proceedings of an international workshop held in Bangkok, Thailand, 11 – 15 February 1991. *ACIAR Proceedings Series* **35**: 64-66.

Wang ET, VanBerkum P, Beyene D, Sui XH, Dorado O, Chen WX, Martinez-Romero E. 1998. *Rhizobium huautlense* sp.nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *International Journal of Systematic Bacteriology* **48**: 687-699.

Wang ET, VanBerkum P, Sui XH, Beyene D, Chen WX, Martinez-Romero E. 1999. Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *International Journal of Systematic Bacteriology* **49**: 51-56

Watanabe T, Osaki M, Tadano T. 1998. Effects of nitrogen source and aluminium on growth of tropical tree seedling adapted to low pH soils. *Soil Science and Plant Nutrition* **44**: 655-666.

Weerawardane NDR, Vivekanandan K, Turnbull JW. 1991. Acacia species and provenance trials in uplands of Sri Lanka. Advances in tropical *Acacia* research. Proceedings of an international workshop held in Bangkok, Thailand, 11 – 15 February 1991. *ACIAR Proceedings Series* **35**: 166-169.

Xie ZP, Staehelin C, Vierheilig H, Wiemken A, Jabbouri S, Broughton WJ, Vogellange R, Boller T. 1995. Rhizobial nodulation factors stimulate mycorrhizal colonisation of nodulating and non-nodulating soybeans. *Plant Physiology* **108**: 1519-1525.

Xie ZP, Muller J, Wiemken A, Broughton WJ, Boller T. 1998. Nod factors and tri-iodobenzoic acid stimulate mycorrhizal colonisation and affect carbohydrate partitioning in mycorrhizal roots of *Lablab purpureus*. *New Phytologist* **139**: 361-366.

Xu LM, Ge C, Cui Z, Li J, Fan H. 1995. *Bradyrhizobium liaoningense* sp.nov., isolated from the root nodules of soybeans. *International Journal of Systematic Bacteriology* **45**: 706-711.

Young JPW, Haukka KE. 1996. Diversity and phylogeny of rhizobia. *New Phytologist* **133**: 87-94.

Zou SQ. 1993. Cultivation of *Amomum villosum* in tropical forests. *Forest Ecology and Management* **60**: 157-162.

II. Phenotypic diversity of root-nodule bacteria isolated from tropical leguminous tree species *Acacia mangium* and *Paraserianthes falcataria* growing in Indonesia

Introduction

Acacia mangium and *Paraserianthes falcataria* are two economically important leguminous tree species grown in Indonesia and many other countries. These multipurpose tree species are fast-growing and are commonly planted to produce timber, firewood and fodder, and also for land reclamation. *Acacia mangium* and *P. falcataria* form symbiotic relationships with rhizobia, which enable the trees to fix nitrogen (N) from the atmosphere (Umali-Garcia *et al.*, 1988; Galiana *et al.*, 1990; Turk & Keyser, 1992; Galiana *et al.*, 1994; Fremont *et al.*, 1999). Despite this knowledge about their symbiotic potential, information about the phenotypic characteristics of the microsymbionts is lacking. These tree species have been reported to be nodulated mainly by *Bradyrhizobium* (Turk & Keyser, 1992; Moreira *et al.*, 1993, 1998), although the presence of *Rhizobium* in the root nodules has also been reported (Padmanabhan *et al.*, 1990; Fremont *et al.*, 1999). Nuswantara *et al.* (1997) reported that rhizobia isolated from *A. mangium* from Indonesia were closely related to *Bradyrhizobium elkanii* based on 16S ribosomal RNA gene sequences. This study, however, used a limited number (9) of strains, which might not reflect the diversity of rhizobia nodulating *A. mangium* in Indonesia. They did not find fast-growing rhizobia associated with *A. mangium*. In contrast, there is no information regarding the rhizobia nodulating *P. falcataria* in Indonesia. Therefore, more extensive studies of rhizobia nodulating these tree species from wide geographical regions in Indonesia are needed.

Phenotypic characterisation (cultural, morphological, physiological traits, and symbiotic) has long been used to characterise rhizobia. Useful characters have been listed by Moffett & Colwell (1968), and include growth rates, colony morphology, resistance to antibiotics, and carbon source utilisation. The techniques are very simple and do not need advanced equipment. Although more recent techniques have been developed to characterise rhizobia, such as biochemical and DNA-based techniques, the phenotypic characterisation is still regarded as an important technique and is necessary to describe novel genera and species of rhizobia (Graham *et al.*, 1991).

Other new techniques have been applied to rhizobial characterisation. For example, pyrolysis mass spectrometry (PyMS) has been shown to discriminate between closely related bacteria (Shute *et al.*, 1984; Goodacre *et al.*, 1991). The analysis is directly derived from whole cells, therefore, small changes in metabolites can be detected (Kay *et al.*, 1994; West *et al.*, 1999). The technique has been successfully used to characterise rhizobial strains (Goodacre *et al.*, 1991; Kay *et al.*, 1994) and other bacteria such as *Bacillus* (Shute *et al.*, 1984), *Escherichia coli* (Goodacre & Berkeley, 1990), and cyanobacteria (West *et al.*, 1999). Barrera *et al.* (1997) have used PyMS to study the diversity of bradyrhizobia nodulating *Lupinus* spp. They assigned the strains into 3 different groups, which were generally in agreement with their multilocus enzyme electrophoresis patterns. Standardised culturing methods, such as media and growth conditions, and bacterial behaviour may influence results, such as sporulating or non-sporulating cultures of *Bacillus* (Shute *et al.*, 1984), or the production of specialised cell types having different chemical composition as in cyanobacteria (Shute *et al.*, 1984; West *et al.*, 1999). Providing a standardised method is used, this technique has great potential to discover novel strains of bacteria (Colquhoun *et al.*, 1998).

The objective of the current study was to study the diversity of the rhizobia and non-nodulating strains of root-nodule bacteria isolated from *A. mangium* and *P. falcataria* growing in different geographical regions in Indonesia using phenotypic characteristics and biochemical properties. The term root-nodule bacteria has usually been associated with rhizobia (Jarvis *et al.*, 1982; Jordan, 1982). However, this is strictly not appropriate since other bacteria as well as rhizobia can be found in the root nodules of legumes (Sturz *et al.*, 1997). In this study the term root-nodule bacteria is used to cover both the nodulating (rhizobia) and non-nodulating bacteria isolated from the root nodules of tree legumes.

Materials and Methods

The investigation comprised of three main steps: root nodule and soil sample collection; isolation of root-nodule bacteria and plant nodulation tests; and bacterial characterisation which included phenotypic characterisation, biochemical characterisation using PyMS, and host range analysis for the rhizobial strains.

Origin of soil and root nodule samples

Root nodules and soil samples were collected from 11 different geographical areas in 8 provinces, spread across 6 different islands in Indonesia (Fig. 1). The origin of the samples where nodule or soil samples were taken are presented in Table 1. Root nodules were transported from the sampling sites to the laboratory in 25 ml vials containing 10 ml of 2.5-6.0 mm diam. sterile silica gel (Fisons, Loughborough, England) and cotton (Robinson Healthcare, Chesterfield, UK) as described by Somasegaran & Hoben (1994), or kept attached to living seedlings and put in plastic bags with soil. The latter was done for the samples taken from Garut, Mangole and Palu because seedlings were available from this area and because of the long distance of transport. Soil samples were also transported to the laboratory in plastic bags and used for trapping of rhizobia with *A. mangium* and *P. falcataria* as host plants.

For soil characterisation, samples were finely ground and N was measured using distilation and titration method after following a Kjeldahl oxidation, total organic C was determined using colorimetric method, P was analysed using molybdenum blue method, Ca, Mg, K, Na and Al were determined using an atomic absorbtion spectrometer (Anderson & Ingram, 1993). Results of the soil analyses were presented in Table 1.

Trapping of rhizobia from soil samples

Plastic pots (14 cm diam.x10 cm height) were used to grow either *A. mangium* and *P. falcataria*. Two third of the pots were filled with sterile zeolite sand (diam. 2-4 mm, pH 6.9). A 100 g sample of each soil was layered on top of the zeolite, and the pot was then filled with sterile zeolite. Two aseptically germinated seedlings (7-days-old) of each host plant were planted in the same pot to give opportunity the rhizobia to nodulate their most appropriate host (Fig. 2). The pots were then hung on racks (50 cm above the floor) in the greenhouse to avoid cross contamination from excess watering. The greenhouse conditions were: minimum/maximum temperatures 21 and 39°C respectively, and relative humidity ranged from 50 to 80%. The seedlings were watered every two days using sterile deionised water (dH_2O) and fed every 14 days with an N-free solution (Broughton & Dilworth, 1971; Appendix IA). The trap cultures were maintained in the greenhouse for three months, after which root nodules were harvested and rhizobia were isolated.

Figure 1. The map of Indonesia showing the approximate locations of soil and root-nodules sampling. The green rectangular indicates the natural habitate of both *A. mangium* and *P. falcataria*, the yellow rectangular indicates the natural habitats of *P. falcataria*. Numbers are the codes for soil origin (see Table 1).

Table 1. Origin and chemical and physical properties of soil samples taken for isolation of root nodule bacteria from Indonesia.

Soil origin	pH (H_2O)	C Org. (%)	Total N (%)	Available P- (mg kg^{-1}) Bray I	Ca (meq/ 100g)	Mg (meq/ 100g)	K (meq/ 100g)	Na (meq/ 100g)	Al (meq/ 100g)	Texture		
										Sand	Loam	Clay
1. Bogor, West Java	4.3	1.8	0.2	32.4	3.0	1.0	0.6	0.2	1.5	20.6	37.2	42.2
2. Garut, West Java	4.8	2.2	0.2	25.1	3.2	2.1	0.7	0.3	2.4	17.3	38.2	44.5
3. Parung Panjang, West Java[a]	5.2	2.7	0.2	5.4	2.0	1.5	0.2	na	nd	na	na	na
4. Kediri, East Java	6.6	1.2	0.1	27.4	4.9	2.3	1.0	0.5	nd	13.3	43.5	43.2
5. Lampung	4.8	2.7	0.2	19.5	5.1	3.8	0.2	0.8	na	48.5	25.4	25.4
6. Pasir Mayang, Jambi	4.8	4.2	0.2	7.1	1.9	0.8	0.2	0.4	na	53.4	16.3	29.2
7. Surya Dumai, Riau	5.1	51.2	1.3	32.0	17.6	3.4	0.4	0.5	na	na	na	na
8. East Kalimantan	na	na	na	na	na	na	na	na	na	na	na	na
9. Palu, Central Sulawesi	3.1	0.8	0.1	6.8	0.9	0.4	0.3	0.5	2.9	24.6	46.1	29.3
10. Mangole, Maluku	3.5	2.1	0.2	14.6	1.8	2.0	1.0	0.2	3.4	18.7	34.3	47.0
11. Irian Jaya	6.3	5.3	0.4	16.1	6.5	2.5	0.7	0.2	nd	17.5	41.7	40.8

Note: [a] (Setiadi, 1995), na= data not available, nd= value not determined

Figure 2. Trapping of rhizobia from soil. Soil samples were trapped in pot cultures for three months. Both *Acacia mangium* and *Paraserianthes falcataria* were planted in the same pot to give opportunity to the rhizobia to nodulate appropriate host.

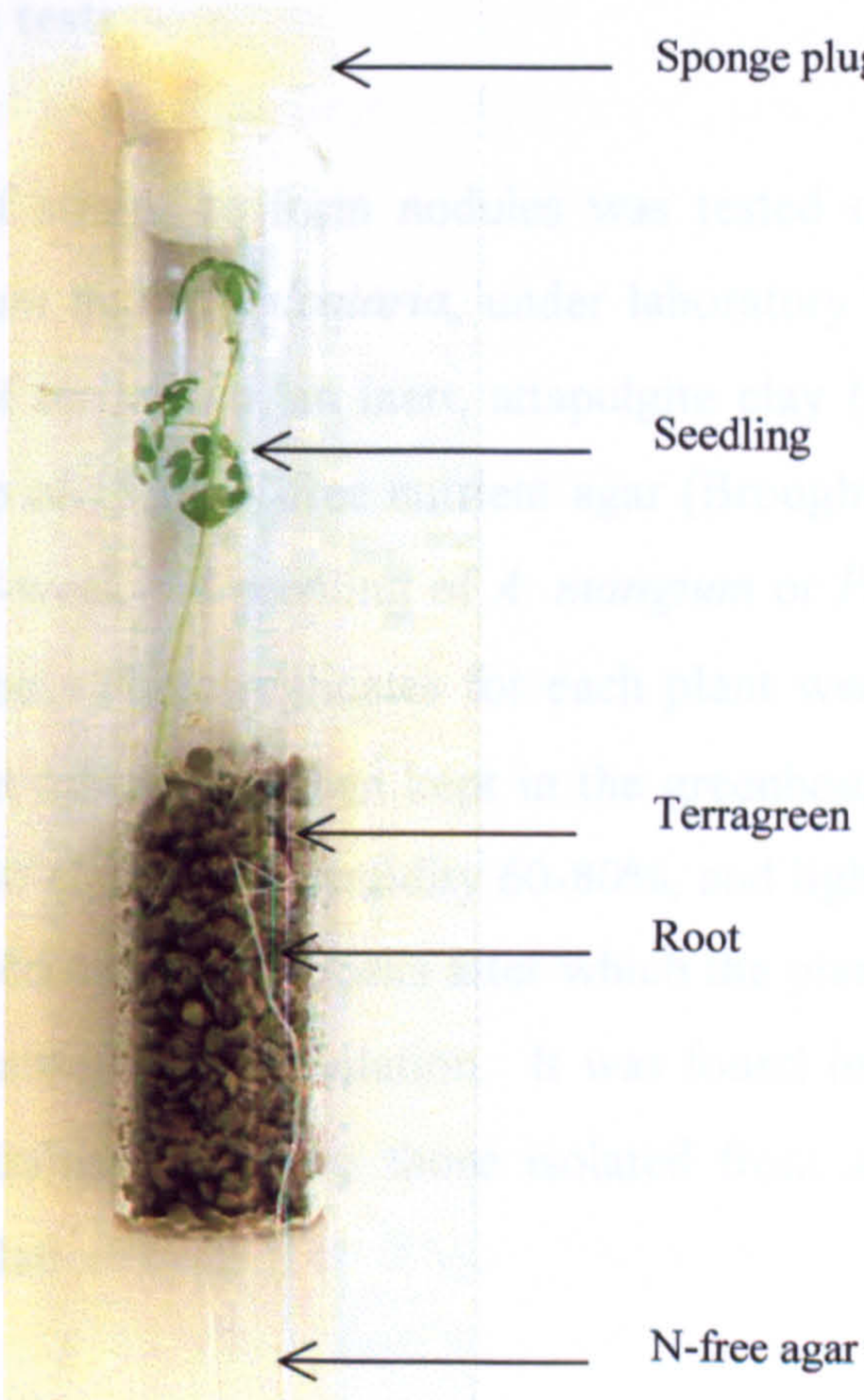


Figure 3. Test tube system to conduct the authentication test. The seedling of *Paraserianthes falcataria* was inserted next to the glass wall so that nodule formation could be observed easily.

Isolation of rhizobia

The nodules were separated from the root 3 months after sowing either *A. mangium* and *P. falcataria*, washed in distilled water, and then surface sterilised as described by Somasegaran & Hoben (1984). Nodules were immersed in 95% (v/v) ethanol for 10 sec then soaked in 3% (v/v) H_2O_2 for 5 min, and finally washed three times with sterile dH_20. Subsequently, nodules were crushed in 1.5 ml microcentrifuge tubes containing 50 µl sterile dH_20. The suspension was streaked onto yeast-extract mannitol agar (YMA) medium (Vincent, 1970; Appendix IB) amended with congo red. Colonies which did not absorb congo red, and differed from each other in morphological characteristics were selected from each plate and re-inoculated onto YMA. Pure cultures were obtained after one or more further subculturing steps.

Plant nodulation tests

The ability of strains to form nodules was tested on their respective original host plants, *A. mangium* and *P. falcataria*, under laboratory conditions in 2.5 cm diam. test tubes. A layer of terragreen, an inert, attapulgite clay (Oil Dri, Wisbech, Cambs, UK), was placed on top of 15 ml N-free nutrient agar (Broughton & Dilworth, 1971; Appendix IA) (Fig. 3). A 7-week-old seedling of *A. mangium* or *P. falcataria* was inserted into the media in each tube. Three replicates for each plant were inoculated with each strain of rhizobia. The test tubes were then kept in the greenhouse simulating tropical conditions (min. 18°C/max 40°C, relative humidity 60-80%, and light intensity 400-600 µmol $m^{-2}s^{-1}$). The test was carried out for 12 weeks after which the plants were harvested and scored for the presence and absence of nodulation. It was found in the preliminary experiment that some rhizobial strains, especially those isolated from *A. mangium*, formed nodules 12 weeks after inoculation.

Characterisation of bacterial strains

Bacterial strains were characterised by Gram staining, colony morphology, growth rates on YMA and yeast extract mannitol broth (YMB, Appendix IB), acid and alkaline reaction, carbon source utilisation tests, tolerance to NaCl, antibiotic resistances, and host

range. Pyrolysis mass spectrometry (PyMS) analyses were also conducted for some strains recovered from the root nodules of *P. falcataria*. Five type or reference strains representing 5 recognised genera of rhizobium were included in some tests: *Azorhizobium caulinodans* ATCC 43989^T, *Bradyrhizobium elkanii* ATCC 49852^T, *Bradyrhizobium japonicum* NCIMB 11477^T, *Rhizobium leguminosarum* bv *trifolii* ATCC 14479, and *Sinorhizobium meliloti* NCIMB 12075^T. All strains, including the reference and type strains, were maintained on YMA, except for *Az. caulinodans* which was maintained on TY agar media (Beringer, 1974; Appendix IC). Stock cultures of bacterial strains used for NaCl tolerance, antibiotic resistances, and carbon sources utilisation tests were prepared in YMB. All cultures were incubated at 30°C for 5 days in the dark.

The Gram stain

Cultures were scored as Gram-negative or -positive following the Gram staining procedure described by Somasegaran & Hoben (1994).

Relative growth rates on YMA and YMB

To determine the relative growth rates of the bacterial strains on YMA, the strains were streaked onto the agar medium (Lochner *et al.*, 1991). The cultures then were incubated at 30°C and observation was carried out every day for 12 days. The first appearance of visible single colonies on every plate was recorded. Two replicates were made for each bacterial strain.

To determine the growth rates (turbidity compared to a standard) in YMB, a loopful (2 mm diam.) of bacterial culture on YMA (7-days-old) was inoculated into 10 ml YMB in 25 ml universal bottles. The bottles were incubated at 30°C on a rotary shaker at 120 rpm. A set of McFarland standards (Bio-Mérieux, France) was used to compare the turbidity of the cultures 7 days after inoculation. Two replicates were made for each bacterial strain.

Acid and alkaline reaction

Root nodule solates were grown on YMA amended with bromthymol blue (25 mg l^{-1}) as a pH indicator. An acid reaction changed the colour of the medium from blue green to yellow and an alkaline reaction changed the medium to blue. The cultures were incubated at 30°C for 7 days then scored for colour changes.

Morphological characterisation

Morphological characteristics of colonies were determined on YMA 5 days after inoculation according to Moffet & Colwell (1968).

Carbon source utilisation

All carbon sources to be tested were prepared as stock solutions (sterilised by filtration) and were added to MS medium to give a final concentration of 15 mM (Jordan, 1984; Appendix ID). The carbon sources tested were L+arabinose, D-fructose, galactose, D-glucose, ß-lactose, maltose, D-mannitol, D-mannose, Na-succinate, D+raffinose, D-sorbitol, sucrose, and xylose. These carbon sources were useful to distinguish fast-growing from slow-growing rhizobia (Graham, 1964). Stock cultures of the bacterial strains were inoculated onto the test plates using a replica plater with a 8x6 array (Sigma-Aldrich Co. Ltd, Dorset, England). Each strain was replicated twice on different plates. The plates were incubated at 30°C for 7 days and the presence or absence of bacterial growth was scored.

Antibiotic resistances

The resistance of bacterial strains to antibiotics was tested on YMA (Somasegaran & Hoben, 1994). Fresh filter-sterilised antibiotics were added to melted YMA, which had been cooled to 50°C, to give final concentrations (µg ml^{-1}) of: carbenicillin, 10; erythromycin, 2.5 and 5; neomycin sulfate, 1.25 and 2.5; streptomycin sulfate, 2.5 and 10; vancomycin hydrochloride, 5. Stock solutions of antibiotic were made at 10 mg ml^{-1} in sterile dH_2O, except for erythromycin (5 mg ml^{-1} in ethanol). Inoculation was conducted

using a replica plater as described previously for the carbon source investigation. Plates were incubated at 30°C for 7 days in the dark.

NaCl tolerance

The tolerance of bacterial strains to NaCl was conducted only for strains of bacteria that were able to re-nodulate *A. mangium* or *P. falcataria* (i.e. confirmed rhizobia). Tolerance to NaCl was examined on YMA plates adjusted to the required NaCl concentration: 0, 0.5, 3.0, 5.0, 7.0 and 10.0% (w/v). Inoculation was conducted using a replica plater as described previously for carbon utilisation tests. Two replicates were prepared for each strain on different plates. Cultures were then incubated at 30°C for 7 days in the dark after which the plates were scored for the presence or absence of bacterial growth.

Host range tests

Six rhizobial strains from *A. mangium* (AMAG 3010, AMBG 2030, AMJB 1010, 1020, 3010, and AMKT 4010) and seven rhizobial strains from *P. falcataria* (PFAG 5030, 5040, 5070, 6030, 6040, PFIR 3040, PFJB 1040) representing different groups and clustered on a phenotypic basis were selected for the test. *Rhizobium leguminosarum* bv *trifolii* ATCC 14479 and *S. meliloti* NCIMB 12075^T were also included in the tests. Seven host plants representing the original host for the major recognised genera of rhizobia were used in this study: *Galega officinalis*, *Lotus corniculatus*, *Medicago sativa*, *Trifolium pratense*, *Vicia faba*, *Cicer arietinum*, *Pisum sativum*. Later the strains were also tested for their ability to nodulate *Glycine max*. In this test only the type strains of *B. elkanii* ATCC 49852^T and *B. japonicum* NCIMB 11477^T were used, because *G. max* is known to be nodulated effectively by these strains. The experiment was conducted as for the plant nodulation test described earlier.

Data Analyses for the phenotypic data

The data from Gram stain determinations, colony morphology, acid and alkaline production, carbon source utilisation tests and resistance to antibiotics were used to construct a dendrogram using an unweighted pair-group method using arithmetic average (UPGMA). The UPGMA analysis was performed using the statistical package Minitab version 11 for Windows.

PyMS analyses

A total of 35 bacterial strains were compared, including 31 rhizobial strains isolated from *P. falcataria* (PFAG 1030, 2020, 2030, 5030, 5040, 5050, 5070, 5100, 6030, 6040, 6070, 6080, PFBG 2012, 2060, PFGR 1010, PFIR 2030, 3010, 3040, 3050, 3060, 3093, 3120, PFJB 1010, 1040, PFLP 1010, PFMG 1020, PFPL 2020, PFRU 1010, 3010, 5012, 5020) and type strains of *Azorhizobium caulinodans* ATCC 43989^T, *B. japonicum* NCIMB 11477^T and *Sinorhizobium meliloti* NCIMB 12075^T, and a reference strain of *R. leguminosarum* bv *trifolii* ATCC 14479. Bacterial strains were grown on TY medium and incubated at 30°C for 3 days. To perform PyMS analysis, Ni-Fe (50:50) foils with Curie-point 530°C (Horizon Instruments, Heathfield, Sussex, England) were inserted, using forceps, into clean pyrolysis tubes (Horizon Instruments) so that 2/3 of the foil protruded from the mouth of the tubes. The bacterial colonies were picked from across the plate, avoiding the surface of the growth medium, using a 2 mm diam. platinum wire loop, and smeared on one surface of the protruding foils to give a uniform surface coating. Triplicate samples were prepared for each strain. The samples were air-dried on the bench for 30 min, then were further dried in the oven at 80°C for 10 min. Subsequently, the foils were pushed 10 mm further into the tube using a stainless steel depth gauge, to locate the samples in the tubes for pyrolysis. Viton O-rings (Horizon Instruments) were placed on the tubes. The samples were then pyrolysed in the RAPyD-400 pyrolysis mass spectrometer (Horizon Instrument) at 530°C for 3 sec. Integrated ion counts for each sample at unit mass intervals from 51-200, together with the total ion counts of each sample and its PyMS sequence number, were recorded automatically using a PC connected to the PyMS machine. The same computer was used to programme the PyMS. GENSTAT version 5.1 was employed to perform multivariate analysis of the spectral data.

The data matrix (mass spectral intensities) generated by GENSTAT was used to cluster the strains using UPGMA-analysis with Minitab version 11 for Windows.

Results

Root nodule collection and trapping

Root nodules of *A. mangium* and *P. falcataria* collected from the field exhibited indeterminate growth (Fig. 4). At this stage the root nodules of *A. mangium* could readily be distinguished from those of *P. falcataria*. Root nodules of *A. mangium* had longer branches and a darker colour (dark brown) compared with the root nodules of *P. falcataria* (light brown). Thirty-seven strains of root-nodule bacteria were recovered from *A. mangium* and seventy-one strains were recovered from *P. falcataria* (Table 2). These strains were selected because they did not absorb or weakly absorbed congo red when they were grown on YMA amended with congo red (Fig. 5).

Plant nodulation tests

All 109 strains were tested for their ability to nodulate their respective hosts, *A. mangium* and *P. falcataria*. However, only 26 nodulated *A. mangium* and 32 strains nodulated *P. falcataria* (Table 2). Two additional strains of *Bradyrhizobium* from Brazil, BR 3609 and BR 3617, originally isolated from *Acacia auriculiformis* and *A. mangium*, respectively, were also inoculated on *A. mangium* and were also able to form nodules.

Morphological characterisation

Morphological characteristics, such as colony size, shape, colour and texture, growth rates on YMA and in YMB, and acid or alkaline reaction of the rhizobia and the non-nodulating strains of the root-nodule bacteria, are presented in Table 3. Rhizobial strains were generally gummy (74%) and opaque (98%) with an off-white colour (98%). They also exhibited slow growth on YMA (91%), and produced an alkaline reaction (97%).

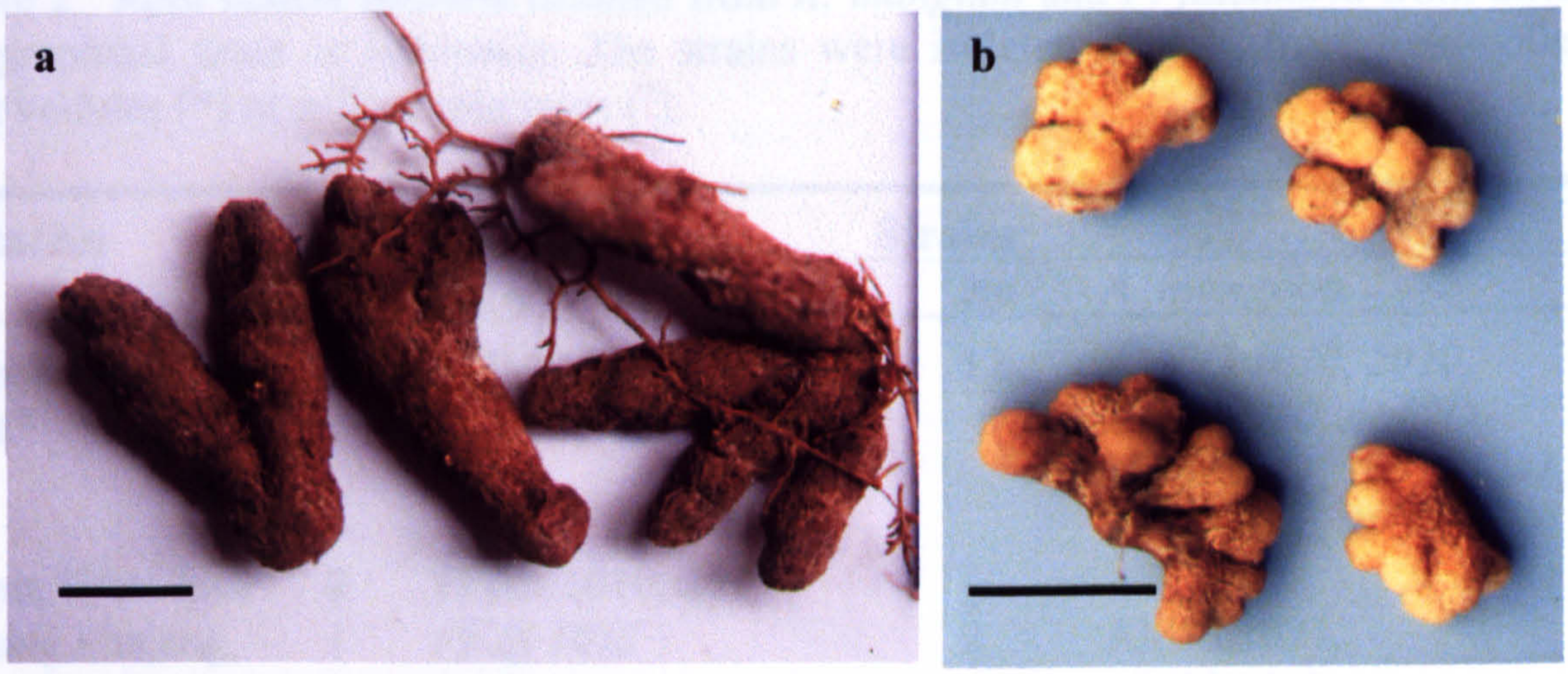


Figure 4. Root nodules of *Acacia mangium* (a) and *Paraserianthes falcataria* (b) recovered from the field. Root nodules of *A. mangium* show different morphological characteristics from those of *P. falcataria* in terms of colour and shape. Bars= 1 cm.

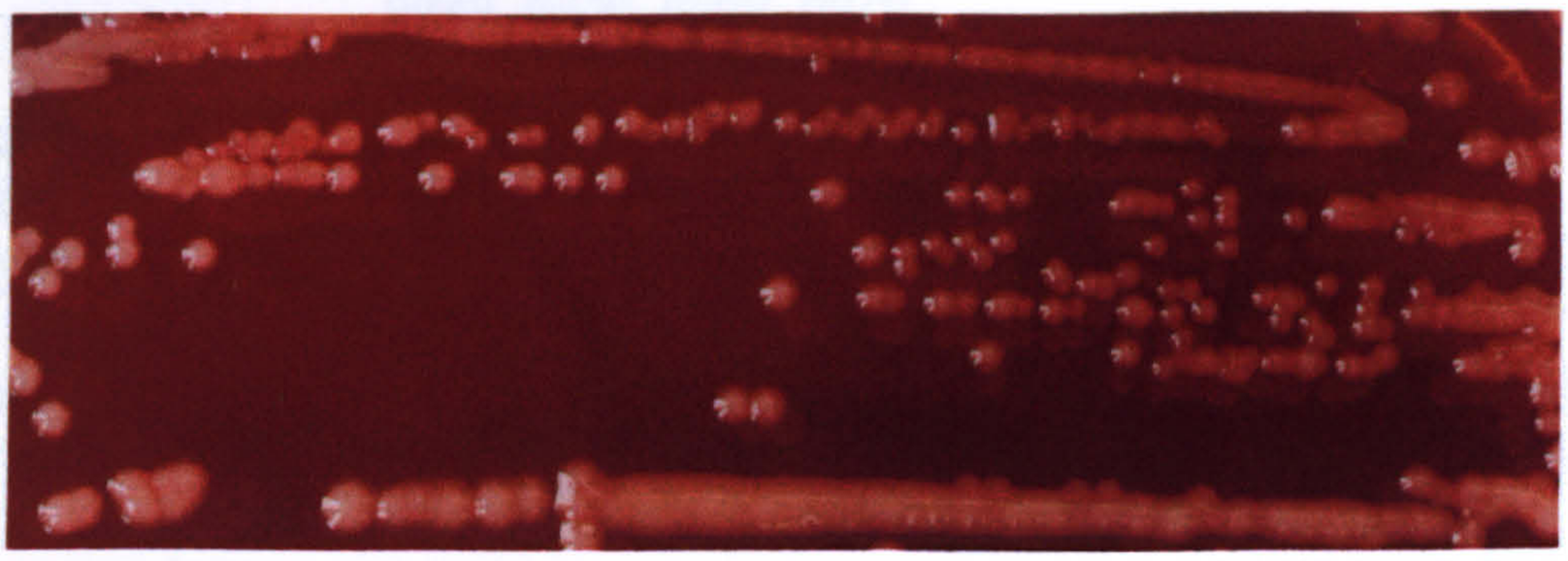


Figure 5. Colonies of root-nodule bacteria on YEMA+congo red. Those did not or weakly absorbed congo red were selected for further subculturing.

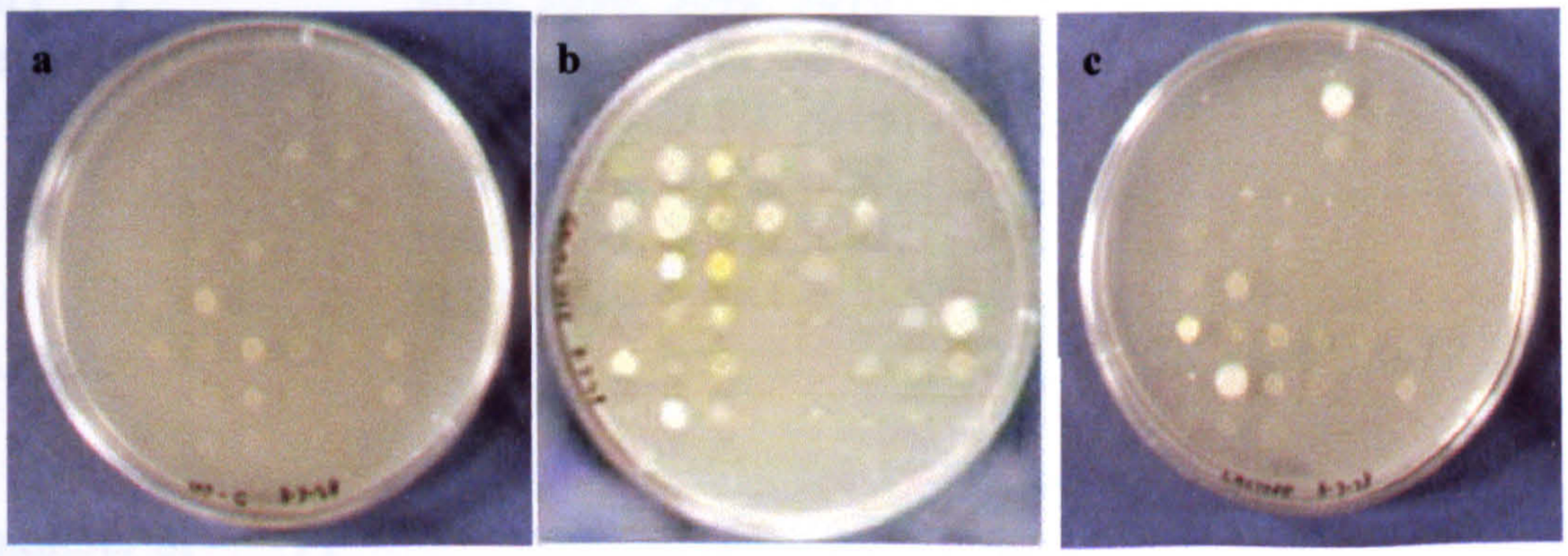


Figure 6. Growth of root-nodule bacteria isolated from the root nodules of *Acacia mangium* and *Paraserianthes falcataria* on media containing carbon sources, a) no-carbon source, b) galactose, c) lactose. Inoculation was performed using a replica plater with a 8x6 array.

Table 2. Root nodule bacteria isolated from *A. mangium* and *P. falcataria* from different geographical areas in Indonesia. The strains were isolated directly from field-collected root nodules (*) or soil sample traps (+).

Location	Strains			
	No	*P. falcataria*	No	*A. mangium*
Bogor, West Java*	2	PFBG 2012, 2060	12	*AMBG 1010, 2010, 2020, 2030, AMNO 1010, 1021, 1031, 1041, 1042, 2010, 2020, 2040*
Garut, West Java*	2	PFGR 1010, 2010	-	-
Parung Panjang, West Java*	1	*PFJS 1012*	2	AMJS 1021, AMJG1010
Kediri, East Java*	26	*PFAG 1030*, 2020, *2030, 5010, 5020, 5030, 5040, 5050, 5061, 5070, 5080, 5090, 5100, 5110, 5120, 6010, 6020, 6030, 6040, 6050, 6060, 6070, 6080, 6090, 6100, 6110*	5	*AMAG 1010, 1030, 1050, 3010, 4010*
Lampung, Lampung+	5	PFLP 1010, 1020, 1031, 1033, *1040*	2	AMLP 1010, 2010
Pasir Mayang, Jambi+	2	*PFJB 1010, 1040*	5	*AMJB 1010, 1020, 1030, 1040, 3010*
Surya Dumai, Riau+	6	*PFRU 1010*, 3010, 3020, 4010, 5012, *5020*	7	AMRU 1010, 1020, *2010*, 3020, 4010, 6010, 7010
East Kalimantan+	-	-	5	*AMKT 1010*, 2011, *2020*, 2030, *4010*
Palu, Central Sulawesi (*,+)	3	PFPL 1012, 2010, 2020	-	-
Mangole, Maluku (*,+)	7	PFMG 1020, 1030, 2020, 3010, 3030, 3040, 5010	-	-
Irian Jaya*	17	PFIR 1020, 1031, 2010, 2030, 2050, 2080, 3010, 3020, 3030, *3040*, 3050, 3060, 3091, 3092, 3093, 3110, 3120	-	-
EMBRAPA, Brazil[a]	-	-	2	*BR 3609*[b], *3617*
Total number of strains	71		40	

[a]obtained from EMBRAPA-CNPAB through Dr E.M. Ribeiro da Silva, [b] originally isolated from *Acacia auriculiformis*. Strains with italised codes were able to nodulate their respective hosts.

Table 3. Colony morphology of the nodulating and non-nodulating strains of root nodule bacteria isolated from root nodules of *A. mangium* and *P.falcataria.*

	Nodulating strains (58[a])		Non-nodulating strains (51)	
	Number[b]	(%)	Number	(%)
Size				
- small (1-2 mm)	20	34	21	41
- medium (2-5 mm)	38	66	30	59
Shape				
- convex	43	74	12	24
- rough	0	0	0	0
- entire edge	58	100	51	100
Texture				
- watery	15	26	21	41
- gum production	43	74	18	35
- mucilagenous	0	0	11	22
Colour				
- opaque	57	98	30	59
- translucent	1	2	20	39
- white	1	2	11	22
- off-white	57	98	20	39
- white precipitate	8	14	0	0
- yellow on TY	0	0	5	10
Growth rate on YMA				
- fast (3-5 days)	5	9	49	96
- slow (5-7 days)	53	91	2	4
Growth rate on YMB				
- fast (2-3 days)	0	0	37	73
- slow (3-5 days)	58	100	14	27
Acid/alkaline production				
- acid	2	3	31	61
- alkaline	56	97	4	8
- neutral	0	0	16	31

[a]number of strains tested, [b] number of the strains that showed positive results.

Table 4. Carbon source utilisation by rhizobia and non-nodulating strains of root nodule bacteria isolated from root nodules of *A. mangium* and *P. falcataria*.

Carbon source	Nodulating (58[a])		Non-nodulating (51)	
	Number [b]	(%)	Number	(%)
L+ Arabinose	29	50	40	78
D-Fructose	13	22	40	78
Galactose	13	22	36	71
D-Glucose	33	57	39	76
β-Lactose	11	19	26	51
Maltose	36	62	44	86
D-Mannitol	58	100	40	78
D-Mannose	19	33	42	82
Na-Succinate	12	21	39	76
D+ Raffinose	24	41	33	65
D- Sorbitol	15	26	29	57
Sucrose	21	36	36	71
Xylose	34	59	37	73

A small number of strains of non-nodulating bacteria had similar morphological characters as the rhizobia. However, most of the non-nodulating strains were fast growing on YMA (96%) and produced an acid reaction (61%). Substantial numbers (30%) of the strains showed a neutral reaction. The type strain of *B. japonicum* produced an alkaline reaction, while those of *R. leguminosarum* bv *trifolii* and *S. meliloti* produced an acid reaction.

Carbon source utilisation

In general, the rhizobial strains used a narrower range of carbon sources than their non-nodulating counterparts. Figure 6 shows examples of the growth of different strains of root-nodule bacteria on media containing different carbon sources. Only arabinose, glucose, maltose, mannitol and xylose were used by more than 50% of the rhizobia. In contrast, 51-86% of the non-nodulating strains were able to use all the carbon sources tested (Table 4). All strains of rhizobia were able to use mannitol as a source of carbon.

When the pattern of carbon source utilisation was used for typing of the root-nodule strains, 79 patterns were found, but only 9 patterns were shared by more than one strain (Table 5). Pattern 1 was the most common pattern being shared by 20 strains which were able to use all carbon sources tested.

Antibiotic resistance

More than 50% of the strains of root-nodule bacteria were resistant to all antibiotics and dosages tested (Table 6). Carbenicillin was the least toxic antibiotic, 59% of rhizobial and 82% of the non-nodulating strains were able to grow on the media amended with 10 $\mu g\ ml^{-1}$ of this antibiotic. Typing of the strains using the antibiotic resistance patterns generated 30 different fingerprints, and 11 patterns were shared by more than 1 strain (Table 7). Pattern 1 was shared by 43 strains, which were resistant to all antibiotics and dosages used. This pattern was not exclusive to the rhizobial strains.

Table 5. Carbon source utilisation patterns of nodulating and non-nodulating strains of root nodule bacteria. Thirteen carbon sources were used in the study. Only patterns that were shared by more than one strain were presented here.

No.	Strains	Carbon source utilisation pattern												
		ara	fru	gal	glu	lac	mal	mat	mas	suc	raf	sor	sur	xyl
1.	PFAG 5040, PFAG 5050, PFIR 3040, AMBG 1010, AMKT 1010, *S. meliloti*, PFBG 2060, PFIR 1031, PFIR 2030, PFIR 3010, PFIR 3050, PFIR 3093, PFIR 3120, PFGR 1010, PFPL 2020, AMKT 2030, AMLP 2010, AMRU 1010, AMRU 4010, AMJG 1010	+	+	+	+	+	+	+	+	+	+	+	+	+
2.	PFRU 1010, AMNO 1041, AMNO 1042, AMNO 2040, AMBG 2010, AMBG 2020, AMBG 2030, AMAG 1050	-	-	-	-	-	-	+	-	-	-	-	-	-
3.	PFAG 5030, PFAG 6110	+	-	-	+	-	+	+	-	-	+	-	-	+
4.	PFAG 6030, PFAG 6040	+	-	-	+	-	+	+	-	-	+	+	+	+
5.	AMNO 2010, AMAG 4010	-	-	-	-	-	-	+	-	-	-	+	-	-
6.	AMKT 4010, AMJS 1021	+	+	+	+	+	+	+	+	+	+	-	+	+
7.	*R. leguminosarum* bv *trifolii*, PFIR 3060	+	+	+	+	+	+	+	+	-	+	+	+	+
8.	PFIR 3091, PFMG 3030	+	-	-	+	-	+	+	+	-	-	-	+	-
9.	AMRU 1020, AMRU 6010	+	+	+	+	-	+	+	+	+	+	-	+	+

ara= L+ Arabinose, fru= D- Fructose, gal= Galactose, glu= D-Glucose, lac= β-Lactose, mal= Maltose, mat= D-Mannitol, mas= D-Mannose, suc= Na Succinate, raf= D+ raffinose, sor= D-sorbitol, sur= Sucrose, xyl= Xylose.

Table 6. Resistance to different antibiotics of nodulating and non-nodulating strains of root nodule bacteria isolated from *A. mangium* and *P. falcataria*.

Antibiotics (μg ml^{-1})	Nodulating strains (58[a])		Non-nodulating strains (51)	
	Number[b]	(%)	Number	(%)
Carbenicylin (10)	34	59	42	82
Erythromycin (2.5)	33	57	31	61
Erythromycin (5)	33	57	26	51
Neomycin sulfate (1.25)	42	72	40	78
Neomycin sulfate (2.5)	42	72	41	80
Streptomycin sulfate (2.5)	44	74	45	88
Streptomycin sulfate (10)	40	61	40	78
Vancomycin hydrochloride	30	52	27	53

[a]number of strains tested, [b] number of strain resistance to the corresponding antibiotic and dose.

Table 7. Antibiotic resistance patterns of rhizobial and non-nodulating strains of root nodule bacteria isolated from root nodules of *A. mangium* and *P. falcataria*. Only typing patterns that were shared by more than one strain were presented here.

No.	Strains	Pattern of fingerprint							
		car 10	ery 2.5	ery 5	neo 1.25	neo 2.5	str 2.5	str 10	van 5
1.	PFAG 1030, PFAG 2030, PFAG 5010, PFAG 5030, PFAG 5040, PFAG 5050, PFAG 5061, PFAG 5090, PFAG 5100, PFAG 5120, PFAG 6030, PFAG 6040, PFAG 6050, PFAG 6060, PFAG 6070, PFAG 6080, PFAG 6090, PFAG 6100, PFAG 6110, PFRU 1010, PFRU 5020, PFJB 1010, AMNO 1021, BR 3617, AMAG 1010, AMJB 1030, *S. meliloti*, *Az. caulinodans*, PFIR 2010, PFIR 2030, PFIR 3060, PFIR 3093, PFPL 2020, PFAG 2020, PFRU 3010, PFRU 3020, AMJS 1021, AMKT 2011, AMKT 2030, AMLP 2010, AMRU 3070, AMRU 4010, AMRU 6010	+	+	+	+	+	+	+	+
2.	PFAG 5080, PFAG 6020, AMBG 1010, AMJB 1020, PFIR 3092, PFMG 1030, PFMG 3040, PFMG 5010, PFPL 2010, AMJG 1010	+	-	-	+	+	+	+	-
3.	PFAG 5110, PFAG 6010, PFJS 1012, AMNO 1010, AMNO 1022, AMAG 3010, AMKT 2020, PFIR 3030, PFIR 3091, PFGR 2010, PFMG 3010, PFMG 3030, PFPL 1012	-	-	-	-	-	+	+	-
4.	AMNO 1031, AMNO 1041, AMNO 2020, AMNO 2040, AMBG 2010, AMBG 2020, *B.*	-	-	-	-	-	-	-	-
5.	AMNO 1042, AMAG 1030, AMJB 1010, *R. leguminosarum* var *trifolii*	-	+	+	+	+	-	-	-
6.	AMNO 2010, AMBG 2030	-	-	-	+	+	-	-	-
7.	PFLP 1031, PFMG 1020, AMRU 7010	+	+	-	+	+	+	+	-
8.	AMKT 1010, AMJB 1040, AMJB 3010	-	-	-	-	-	+	-	-
9.	PFIR 3020, PFIR 3110	+	+	+	+	+	+	+	-
10.	PFBG 2060, PFIR 3120, PFLP 1010, AMLP 1010, AMRU 1010	+	+	+	+	+	-	-	+
11.	PFGR 1010, PFRU 5012	+	+	+	+	+	+	-	+

car= carbenicillin, ery= erythromycin, neo= neomycin, str= streptomycin, van= vancomycin, number under each antibiotic was the dose of the respective antibiotics

NaCl tolerance

More than 50% of the rhizobial strains were tolerant to NaCl up to the concentration of 7% (w/v), but all strains failed to grow on the media amended with 10% (w/v) NaCl (Table 8).

Host range

The results of the host range test are presented in Table 9. From 13 strains tested, none of them produced root nodules on *C. arietinum*, *Ga. officinalis*, and *V. faba*. Two strains (AMJB 1020 and PFIR 3040) were able to nodulate *L. corniculatus*, and four strains produced root nodules when associated with *G. max*. Strains AMAG 3010, AMBG 2030, and PFAG 5070 produced galls (or pseudonodules *sensu* Trinick, 1980), abnormal nodules and did not fix N, when associated with *T. pratense* (Fig. 7). There was no indication of nitrogen fixation associated with gall production since the plant leaves were yellowish.

Cluster analysis

Cluster analysis for all of the root-nodule strains and type strains separated the strains into two main groups with 25% average similarity (Fig. 8a, b). The first group consisted of 6 clusters which contained most of the rhizobial strains and the type strains of *B. japonicum*, while the second group comprised 5 clusters which contained mostly the non-nodulating strains and fast-growing type strains of rhizobia. *Sinorhizobium meliloti* and *R. leguminosarum* bv *trifolii* were placed in the same sub-cluster with only approximately 50% similarity, while *Az. caulinodans* was placed in a different cluster from the latter two type strains.

Cluster 1 contained mainly strains of rhizobia isolated from *P. falcataria* growing in Kediri, East Java. These strains were able to use arabinose, maltose, mannitol and xylose as carbon sources and were resistant to all the antibiotics tested. Cluster 2 comprised of strains that were able to use all the carbon sources and were resistant to all the antibiotics tested. These strains were isolated from both *A. mangium* and *P. falcataria*. Strains in cluster 3 were able to use arabinose, glucose, maltose, mannitol and xylose as carbon

Table 8. Tolerance of the 58 nodulating strains of rhizobia isolated from *A. mangium* and *P. falcataria* to NaCl.

NaCl concentration (%)	Number[a]	(%)
0	42	72
0.5	49	84
3	39	67
5	39	67
7	33	57
10	0	0

[a]number of strains tolerant to the corresponding NaCl concentration.

Table 9. Ability of different rhizobial strains obtained from the root nodules of *A. mangium* and *P. falcataria* to form nodules with other leguminous plant species.

Strain	Plant species							
	Cicer	*Galega*	*Glycine*	*Lotus*	*Medicago*	*Pisum*	*Trifolium*	*Vicia*
AMAG 3010	-	-	-	-	-	-	+	-
AMBG 2030	-	-	+	-	±	-	±	-
AMJB 1010	-	-	+	-	-	-	-	-
AMJB 1020	-	-	-	+	-	-	-	-
AMJB 3010	-	-	-	-	-	-	-	-
AMKT 4010	-	-	-	-	-	-	-	-
PFAG 5030	-	-	-	-	-	-	-	-
PFAG 5040	-	-	+	-	-	-	-	-
PFAG 5070	-	-	+	-	-	-	±	-
PFAG 6030	-	-	-	-	-	-	-	-
PFAG 6040	-	-	-	-	-	-	-	-
PFIR 3040	-	-	+	+	-	-	-	-
PFJB 1040	-	-	-	-	-	-	-	-
B. elkanii	nt	nt	+	nt	nt	nt	nt	nt
B. japonicum	nt	nt	+	nt	nt	nt	nt	nt
R. leguminosarum. bv *trifolii*	-	-	-	-	-	-	+	-
S. meliloti	-	-	-	-	+	-	-	-

- did not form root nodules on the respective host, + formed root nodules on the respective host, ± produced gall, nt= not tested

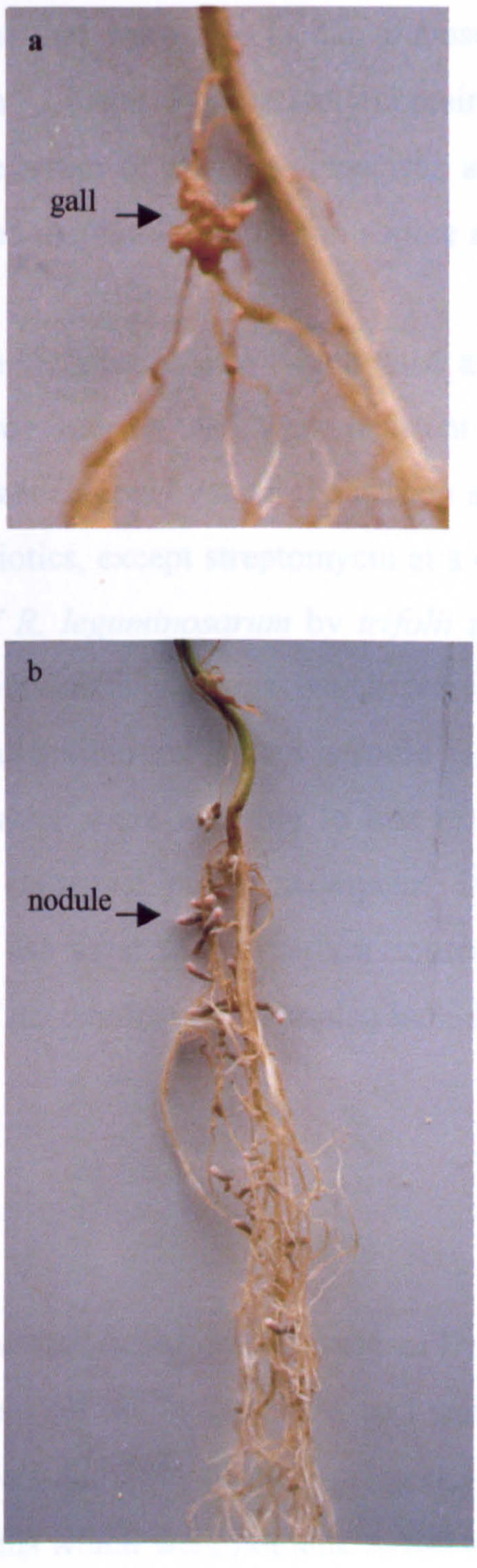


Figure 7. A gall (pseudonodule *sensu* Trinick, 1980), formed by isolate AMBG 2030 on *Trifolium pratense* (a), the shape and colour were distinct compared with normal nodules formed by this plant species (b).

sources and were not tolerant to erythromycin and streptomycin. Cluster 4 contained strains that could not use carbon sources other than mannitol and were resistant only to neomycin. This cluster was dominated by rhizobial strains isolated from *A. mangium*. Cluster 5 comprised of strains that were able to use glucose, mannitol and xylose, and were resistant to streptomycin. Cluster 6 exclusively contained rhizobial strains isolated from *A. mangium*. The type strain of *B. japonicum* was also included in this cluster. Strains in this cluster only used mannitol as a carbon source and were not resistant to any antibiotic tested.

Cluster 7 contained non-nodulating strains which used arabinose, maltose, mannitol, mannose and sucrose as carbon sources, and were resistant to streptomycin. Cluster 8 comprised of one strain only which could use all the carbon sources tested. These strains were not resistant to any antibiotics, except streptomycin at a dose of 2.5 $\mu g\ ml^{-1}$. Cluster 9 included the type strains of *R. leguminosarum* bv *trifolii* and *S. meliloti*. This cluster consisted mostly of the non-nodulating strains which were able to use all the carbon sources and were resistant to all antibiotics, except in some cases, vancomycin. Cluster 10 was represented by strains which were not able to use lactose and sorbitol as carbon sources and were resistant to carbenycillin and neomycin. In the last cluster, cluster 11, the strains were not able to use most of the carbon sources and were resistant to all antibiotics. The type strain of *Az. caulinodans* was also included in this cluster.

PyMS analysis

Four clusters were generated based on the result of PyMS analysis (Fig 9). Cluster 1 comprised strains which could not utilise succinate, and were sensitive to streptomycin. These strains were fast- growers and 50% of them produced a neutral reaction on YMA. Cluster 2 comprised three strains which were not able to use sorbitol, and were resistant to all antibiotics. These strains were fast-growers and alkali-producers. Cluster 3 comprised strains which were able to use arabinose, glucose, maltose and raffinose for their source of carbon, and were resistant to carbenicylin and vancomycin. These strains were fast growers and acid producers. The type strain of *Az. caulinodans* and the reference strain of *R. leguminosarum* bv *trifolii* were grouped in this cluster. Cluster 4 was dominated by rhizobial strains isolated from the root nodules of *P. falcataria* and the type strains of *B.*

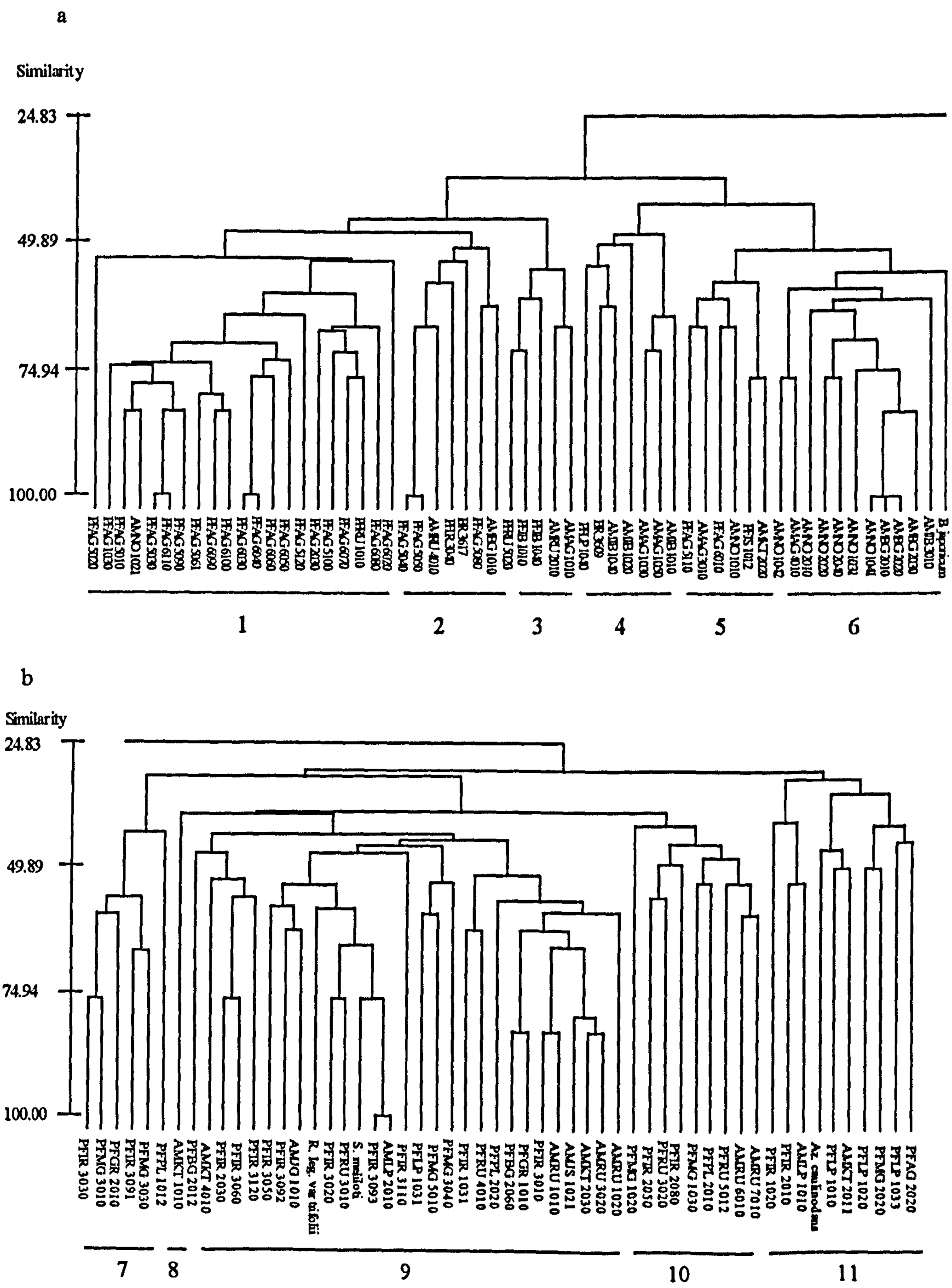


Figure 8. Similarity dendrograms (ordered by UPGMA) derived from phenotypic data of the root nodule bacteria isolated from *A. mangium* and *P. falcataria* growing in Indonesia. a) Group 1 consisted of the rhizobial isolates and the type strain of *B. japonicum*, b) Group 2 mainly consisted of non-nodulating isolates of the root nodule bacteria, and type strains of *Az. caulinodans* and *S. meliloti*, and a reference strain of *R. leguminosarum* var *trifolii*.

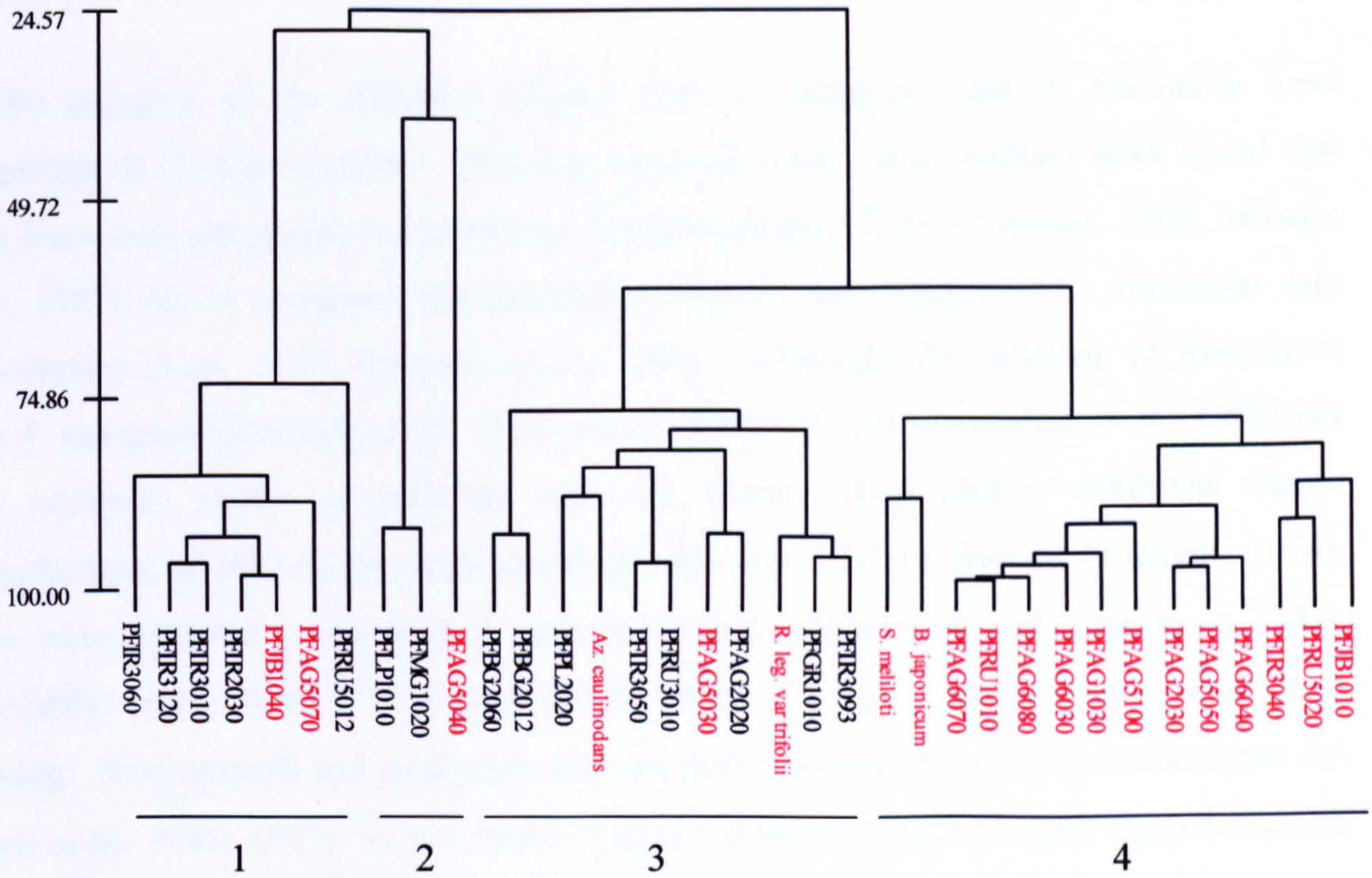


Figure 9. Similarity dendrogram (ordered by UPGMA) derived from PyMS spectral data on 31 strains of rhizobia (red) and non-nodulating root-nodule bacteria (black) isolated from *P. falcataria* from Indonesia and type strains of *Az. caulinodans* and *B. japonicum*, and *S. meliloti*, and a reference strain of *R. leguminosarum* bv *trifolii*. The bacterial strains were grouped into 4 different clusters.

japonicum and *S. meliloti*. All strains used mannitol as a source of carbon, and they were resistant to all antibiotics. Apart from *S. meliloti*, all strains were slow-growers and alkali-producers.

Discussion

The majority of the rhizobia isolated from *A. mangium* and *P. falcataria* were categorised as *Bradyrhizobium*. This was expected, since other workers have found that these trees were principally nodulated by *Bradyrhizobium* (Turk & Keyser, 1992; Moreira *et al.*, 1993). For *A. mangium*, this has also previously been supported by molecular data (Nuswantara *et al.*, 1997; Fremont *et al.*, 1999). Although, the isolation of *Rhizobium* from *A. mangium* (Fremont *et al.*, 1999) and *P. falcataria* (Padmanabhan *et al.*, 1990) has been reported, strains of rhizobia were not found which clearly exhibited classic characteristics of *Rhizobium*, such as fast growth and acid production (Vincent, 1974). There were rhizobial strains from *A. mangium* which produced an acid reaction on YMA, i.e. AMBG 1010, AMKT 1010, and AMKT 4010. However, these strains were slow-growing. Slow growth and acid production are also characteristics of *Mesorhizobium loti* (Jarvis *et al.*, 1982, 1997). In the cluster analysis based on the phenotypic characterisation data, the strains AMKT1010 and AMKT4010 were placed in the same cluster as the non-nodulating strains and were more closely related to *R. leguminosarum* bv *trifolii* and *S. meliloti* than *B. japonicum*.

The recovery of rhizobia from root nodules of *A. mangium* and *P. falcataria* was low, only 53% of the total bacterial strains obtained from the root nodules. This result was lower than the 95% obtained by Mpepereki & Wollum (1991) for *B. japonicum* from nodules on *G. max*. In our study, colonies were selected from the mixed cultures for further sub-culture based on the inability of the colony to absorb congo red. Rhizobial strains commonly absorb congo red weakly (Vincent, 1970). Colony morphology such as circular, convex, mucous, glistening, opaque and white to beige-coloured colonies are typical of rhizobia, but are also shared by other bacteria, such as *Agrobacterium* spp. (Kersters & De Ley, 1984). Some *Pseudomonas* spp. may also share the same colony morphology and other traits with rhizobia (Elkan, 1971; Vincent, 1974). Sturz *et al.* (1997) have reported that root nodules of red clover were occupied by 13 different species of bacteria, including *Rhizobium leguminosarum* bv *phaseoli*, *R. leguminosarum* bv

trifolii, *M. loti*, *Pseudomonas corrugata*, *Pseudomonas fragi* and *Agrobacterium tumefaciens*. Therefore, these bacterial species could have mistakenly been picked up during the isolation and purification processes. The non-nodulating strains might also be rhizobia that have lost their infectivity during isolation and subsequent subculturing (Vincent, 1970). Results of authentication tests are rarely reported, giving the impression that all bacteria isolated from root nodules are rhizobia. The results of this study have emphasised the need for authentication tests (Vincent, 1970) to verify that strains of bacteria from root nodules are true rhizobia (i.e. capable of nodulating the host plant).

Carbon source utilisation patterns are sensitive tools to study the diversity of rhizobia. Although, in general, rhizobial strains from *A. mangium* and *P. falcataria* used a narrow range of carbon sources, many individual strains had a unique pattern. The data show that 79 patterns of carbon source utilisation were obtained. Some strains of the rhizobia (e.g. PFAG 5040, PFAG 5050, PFIR 3040, AMBG 1010, and AMKT 1010) were able to use all the carbon sources tested. This characteristic is typical of fast-growing rhizobia (Graham, 1964; Moffett & Colwell, 1968; Martinez-de Drets *et al.*, 1974). In many studies involving carbon source utilisation (Graham, 1964; Moffett & Colwell, 1968; Martinez-de Drets *et al.*, 1974), the authors tended to ignore the patterns produced by individual strains, which varied greatly (Padmanabhan *et al.*, 1990). Padmanabhan *et al.* (1990) found that *Bradyrhizobium* strains showed greater variation in carbon source utilisation than the *Rhizobium* strains. Despite the value of this approach to assess differences among strains, characterisation using carbon source utilisation patterns does not give consistent results among published studies (Graham, 1964; Padmanabhan *et al.*, 1990; Irisarri *et al.*, 1996). In our study, and the study carried out by Irisarri *et al.* (1996) using slow-growing rhizobial strains isolated from *Lotus subbiflorus*, substantial numbers of the strains utilised maltose as a sole source of carbon. In contrast, Graham (1964) and Padmanabhan *et al.* (1990) found that only very small numbers of the slow-growing strains from *Glycine max*, *Alysicarpus vaginalis*, *Mimosa invisa*, *Desmodium ovalifolium* used maltose.

Antibiotic resistant patterns varied among strains as for carbon source utilisation profiles. Thirty unique patterns were obtained in our study. Forty three percent of the strains were resistant to all antibiotics tested. The rest of the strains were either resistant to one or more antibiotics, or sensitive to all antibiotics. Therefore, antibiotic resistance profiles are also a good technique to evaluate inter-strain variation. The sensitivity of this technique to reveal the diversity of strains within species of rhizobia has been successfully

demonstrated by other workers (Mahler & Bezdicek, 1978; Josey *et al.*, 1979; Beynon & Josey, 1980). Resistance patterns to certain antibiotics have long been used to mark rhizobial strains for ecological studies, such as to monitor the survival and the competitiveness of rhizobial strains (Sishido & Pepper, 1990; Lochner *et al.*, 1991; Tas *et al.*, 1996). A comparison of our data and those from other studies is difficult, since there is no standard method for this kind of test. Some workers might incorporate the antibiotic solution into the media (Josey *et al.*, 1979; Batzli *et al.*, 1992) whilst others used antibiotic discs (Nour *et al.*, 1994; Frémont *et al.*, 1999). Types of antibiotics and dosages used also varied among workers.

Morphological characteristics, patterns of carbon source utilisation and antibiotic resistance which have been used for characterisation of bacteria, including rhizobia (Graham, 1964; Norris, 1965; Moffett & Colwell, 1968) can separate rhizobia from the non-nodulating strains of the root nodule bacteria isolated from *A. mangium* and *P. falcataria*. The result of the cluster analysis (Fig 6) showed that rhizobial strains from *A. mangium* and *P. falcataria* were separated from the non-nodulating strains and placed together with the slow-growing type of rhizobium, *B. japonicum*. However, the fast-growing rhizobia *R. leguminosarum* bv *trifolii*, *S. meliloti* and *Az. caulinodans* were placed in clusters containing the non-nodulating bacteria. *Azorhizobium caulinodans* was only distantly related to *R. leguminosarum* bv *trifolii* and *S. meliloti* on this basis. With only a limited number (44) of characters used, this characterisation technique could help to reduce the number of root-nodule bacteria to be included in the authentication tests if it was known that the particular host plant species were principally nodulated by *Bradyrhizobium*. This screening technique, nevertheless, is not suitable for fast-growing species of rhizobia, since the rhizobial strains may be intermixed with the non-nodulating strains as demonstrated here with the type strains of *R. leguminosarum* bv *trifolii*, *S. meliloti* and *Az. caulinodans*.

Rhizobial strains of *A. mangium* and *P. falcataria* did not, in general, form nodules on host plants known to be nodulated solely by fast-growing rhizobia. Two strains were able to nodulate *L. corniculatus*, and six strains were able to nodulate *G. max*. These two plant species are nodulated by both fast-growing and slow-growing rhizobia (Jarvis *et al.*, 1982, 1997; Jordan, 1982; Scholla & Elkan, 1984; Chen *et al.*, 1988). The host range data show that majority of the rhizobial strains were distinct from the *B. japonicum* strains nodulating *G. max*, which was indicated by their inability to form nodules with this plant species. Strains PFIR 3040 and AMJB 1020 seem to have a broader host range than the others, as

exhibited by their ability to form nodules with *G. max*, and *L. corniculatus*. This shows evidence for the weakness of the cross-inoculation group concept for rhizobia (Baldwin & Fred, 1929; see Giller & Wilson, 1993). *Glycine max* and *L. corniculatus* are not from the same cross-inoculation group (Somasegaran & Hoben, 1994), yet could be nodulated by the same rhizobial strains, in this case AMJB1020 and PFIR3040.

PyMS analysis grouped almost all strains of rhizobia nodulating *P. falcataria* in the same cluster (cluster 4) with 75% similarity, together with the type strains of *B. japonicum*. The clusters produced with PyMS data paralleled the differences between groups previously clustered using phenotypic characters. However, the clustering of the strains did not express the current rhizobial phylogeny (Young & Haukka 1996), and it is interesting that *B. japonicum* was grouped together with *S. meliloti*. Some rhizobial strains were also placed in the same cluster together with non-nodulating strains of root-nodule bacteria. The sensitivity of PyMS analysis, which could distinguish closely related strains of bacteria (Goodacre & Berkeley, 1990), failed to distinguish some rhizobial strains, including the type strain of *Az. caulinodans* and the reference strain of *R. leguminosarum* bv. *trifolii*, from the non-nodulating strains. In previous studies with rhizobia, PyMS was employed to distinguish closely related strains of the bacteria, eg. *Bradyrhizobium* spp. [*Lupinus*] (Barrera *et al.*, 1998), *B. japonicum* (Kay *et al.* 1994), and *S. meliloti* (Goodacre *et al.*, 1991). Therefore, these strains generally have similar phenotypic characteristics, especially growth rates and the preference for growth media. This technique is not commonly used to analyse bacteria with such diverse phenotypic characteristics as in this current study, and no outgroups are usually included in the analysis. The current study showed that care needs to be taken to use PyMS analysis to identify newly isolated rhizobia from root nodules even in the presence of type strains of these bacteria. Plant nodulation test should be carried out prior to PyMS analysis to ensure that the strains are rhizobia.

In the current study, the medium used to grow the strains of the root nodule bacteria was TY medium. This is different from the media used by other workers to grow rhizobial strains for PyMS analysis, such as YMA (Barrera *et al.*, 1997), peptone salt yeast extract agar (Kay *et al.*, 1994), or lab M nutrient agar (Goodacre *et al.*, 1991). This was because in preliminary studies using YMA, peptone salt yeast extract agar, and lab M nutrient agar, and TY medium, TY medium was the best for all strains. Other media resulted in variable growth of the bacterial strains; e.g. *Az. caulinodans* did not grow well on YMA.

On peptone-based media, the slow-growing strains had not grown after 7 days, whilst the fast-growing strains had produced substantial colonies.

This is the first use of PyMS analysis to separate rhizobia from the non-nodulating strains of the root nodule bacteria isolated from *A. mangium* and *P. falcataria*. The fact that most rhizobial strains were placed in one cluster together with the type strain of *B. japonicum* shows the potential use of PyMS to screen rhizobial strains from newly-isolated root nodule bacteria from these tree species. The use of PyMS for screening would save time and effort for subsequent authentication tests of newly-isolated root nodule bacteria by identifying those bacteria that are likely to be rhizobia. Further study need to be done, involving root-nodule bacteria isolated from different leguminouse plant species, which primarily nodulated by *Bradyrhizobium* sp., to verify the capacity of PyMS analysis to distinguish *Bradyrhizobium* sp. from other root nodule bacteria as demonstrated in the current study using *A. mangium* and *P. falcataria*.

Finally, results from this current study showed that phenotypic characterisation and PyMS were useful techniques to study the diversity of rhizobia nodulating *A. mangium* and *P. falcataria* from Indonesia. However, despite the huge workload in phenotypic characterisation coupled with no standard procedures to carry out the test (e.g. no standardisation for antibiotic tests), this technique lacks of comparability between studies. The PyMS analysis, on the other hand, is a rapid and sensitive method to characterise bacteria, including rhizobia; nevertheless, this technique is sensitive to cultural changes, such as type of medium used and age of cultures. This can be a problem when a wide range of bacterial species (such as the root-nodule bacteria used in the current study) having different requirements to grow (e.g. growth medium), and growing in different rates, need to be tested.

References

Anderson JM, Ingram JSI. 1993. Tropical soil biology and fertility. 2ed. C.A.B. International, Wallingford.

Baldwin IL, Fred EB. 1929. Nomenclature of the root-nodule bacteria of the leguminosae. *Journal of Bacteriology* 17: 141-50.

Barrera LL, Trujillo ME, Goodfellow M, Garcia FJ, Hernandez-Lucas I, Davila G, Van Berkum P, Martinez-Romero E. 1997. Biodiversity of bradyrhizobia nodulating *Lupinus* spp. *International Journal of Systematic Bacteriology* 47: 1086-1091.

Batzli JM, Graves WR, VanBerkum. 1992. Diversity among rhizobia effective with *Robinia pseudoacacia* L. *Applied and Environmental Microbiology* **58**: 2137-2143.

Beringer JE. 1974. R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**: 188-198.

Beynon JL, Josey DP. 1980. Demonstration of heterogeneity in natural populations of *Rhizobium phaseoli* using variation in intrinsic antibiotic resistance. *Journal of General Microbiology* **118**: 437-442.

Broughton WJ, Dilworth MJ. 1971. Control of leg-haemoglobin synthesis in snake beans. *Biochemistry Journal* **125**: 1075-1080.

Chen WX, Yan GH, Li JL. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen.nov. *International Journal of Systematic Bacteriology* **38**: 392-397.

Colquhoun JA, Mexson J, Goodfellow M, Ward AC, Horikoshi K, Bull AT. 1998. Novel rhodococci and other mycolate actinomycetes from the deep sea. *Antonie van Leuwenhoek* **74**: 27-40.

Elkan GH. 1971. Biochemical and genetical aspects of the taxonomy of *Rhizobium japonicum*. In Lie TA, Mulder EG. eds. *Biological nitrogen fixation in natural and agricultural habitats*. *Plant and Soil*, special volume, The Hague, Martinus Nijhoff. The Netherland. 85-104.

Fremont M, Prin Y, Chauviere M, Diem HG, Pwee KH, Tan TK. 1999. A comparison of *Bradyrhizobium* strains using molecular, cultural and field studies. *Plant Science* **141**: 81-91.

Galiana A, Chaumont J, Diem HG, Dommergues YR. 1990. Nitrogen-fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp. *Biology and Fertility of Soils* **9**: 261-267.

Galiana A, Prin Y, Mallet B, Gnahoua GM, Poitel M, Diem HG. 1994. Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium* strains under field conditions: Long-term effect on plant growth and persistence of the introduced strains in soil. *Applied and Environmental Microbiology* **60**: 3974-3980.

Giller KE, Wilson KJ. 1993. *Nitrogen fixation in tropical cropping systems*. C.A.B. International, Wallingford.

Goodacre R, Berkeley RCW. 1990. Detection of small genotypic changes in *Escherichia coli* by pyrolysis mass spectrometry. *FEMS Microbiology Letters* **71**: 133-138.

Goodacre R, Hartmann A, Beringer JE, Berkeley RCW. 1991. The use of pyrolysis mass spectrometry in the characterisation of *Rhizobium meliloti*. *Letters in Applied Microbiology* **13**: 157-160.

Graham PH. 1964. Studies on the utilisation of carbohydrates and Krebs cycle intermediates by *Rhizobia*, using an agar plate method. *Antonie van Leuwenhoek* **30**: 68-72.

Graham PH, Sadowsky MJ, Keyser HH, Barnet YM, Bradley RS, Cooper JE, De Ley DJ, Jarvis BDW, Roslycky EB, Strijdom BW, Young JPW. 1991. Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *International Journal of Systematic Bacteriology* **41**: 582-587.

Irisarri P, Milnitsky F, Monza J, Bedmar EJ. 1996. Characterisation of rhizobia nodulating *Lotus subbiflorus* from Uruguayan soils. *Plant and Soil* **180**: 39-47.

Jarvis BDW, Pankhurst CE, Patel JJ. 1982. *Rhizobium loti*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* **32**: 378-380.

Jarvis BDW, Van Berkum P, Chen WX, Nour SM, Fernandez MP, Cleyet-Marel JC, Gillis M. 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen.nov. *International Journal of Systematic Bacteriology* **47**: 895-898.

Jordan DC. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen.nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology* **32**: 136-139.

Jordan DC. 1984. Family III. Rhizobiaceae Conn 1938, 321AL. In Krieg NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology, vol. 1.* Williams & Wilkins, Baltimore, London, England. 234-254.

Josey DP, Beynon JL, Johnston AWB, Beringer JE. 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *Journal of Applied Bacteriology* **46**: 343-350.

Kay HE, Coutinho HLC, Fattori M, Manfio GP, Goodacre R, Nuti MP, Basaglia M, Beringer JE. 1994. The identification of *Bradyrhizobium japonicum* strains isolated from Italian soils. *Microbiology* **140**: 2333-2339.

Kersters K, DeLey J. 1984. Genus III. Agrobacterium Conn 1942, 359AL. In Krieg NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology, vol. 1.* Williams & Wilkins, Baltimore, London, England. 244-254.

Lafay B, Burdon JJ. 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in South-Eastern Australia. *Applied and Environmental Microbiology* **64**: 3989-3997.

Lochner HH, Strijdom BW, Steyn PL. 1991. Limitation of colony morphology and antibiotic resistance in the identification of a *Bradyrhizobium* sp. (*Lotus*) strain in soil. *Biology and Fertility of Soils* **11**: 128-134.

Mahler RL, Bezdicek DF. 1978. Diversity of *Rhizobium leguminosarum* in the Palous Eastern Washington. *Applied and Environmental Microbiology* **36**: 780-782.

Martinez-de Drets G, Arias A, Rovira de Cutinella M. 1974. Fast- and slow-growing rhizobia: differences in sucrose utilisation and invertase activity. *Canadian Journal of Microbiology* **20**: 605-609.

Moffett ML, Colwell RR. 1968. Adansonian analysis of the Rhizobiaceae. *Journal of Geneneral Microbiology* **51**: 245-266.

Moreira FMS, Haukka K, Young JPW. 1998. Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. *Molecular Ecology* 7: 889-895.

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. 1993. Characterisation of rhizobia isolated from different divergence groups of tropical leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology* **16**: 135-146.

Mpepereki S, Wollum AG. 1991. Diversity of indigenous *Bradyrhizobium japonicum* in North Carolina Soils. *Biology and Fertility of Soils* **11**: 121-127.

Mpepereki S, Makonese F, Wollum AG. 1997. Physiological characterisation of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* **22**: 275-292.

Norris DO. 1965. Acid production by *Rhizobium.* A unifying concept. *Plant and Soil* **22**: 143-166.

Nour SM, Cleyet-Marel J-C, Beck D, Effosse A, Fernandez MP. 1994. Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.). *Canadian Journal of Microbiology* **40**: 345-354.

Nuswantara S, Fujie M, Sukiman HI, Yamashita M, Yamada T. 1997. Phylogeny of bacterial symbionts of the leguminous tree *Acacia mangium*. *Journal of Fermentation and Bioengineering* **84**: 511-518.

Padmanabhan S, Hirtz R-D, Broughton WJ. 1990. Rhizobia in tropical legumes: cultural characteristics of *Bradyrhizobium* and *Rhizobium* sp. *Soil Biology & biochemistry* **22**: 23-28.

Scholla MH, Elkan GH. 1984. *Rhizobium fredii* sp.nov., a fast-growing species that effectively nodulates soybeans. *International Journal of Systematic Bacteriology* **34**: 484-486.

Setiadi Y. 1995. The practical application of arbuscular mycorrhizal fungi for reforestation in Indonesia. PhD thesis. University of Kent at Canterbury. England.

Shute LA, Gutteridge CS, Norris JR, Berkeley RCW. 1984. Curie-point pyrolysis mass spectrometry applied to characterisation and identification of selected *Bacillus* species. *Journal of General Microbiology* **130**: 343-355.

Sishido M, Pepper IL. 1990. Identification of dominant indigenous *Rhizobium meliloti* by plasmid profiles and intrinsic antibiotic resistance. *Soil Biology & biochemistry* **22**: 11-16.

Somasegaran P, Hoben HJ. 1994. Handbook for rhizobia. Springer-Verlag, New York, USA.

Sturz AV, Christie BR, Matheson BG, Nowak J. 1997. Biodiversity of endophytic bacteria which colonise red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils* **25**: 13-19.

Tas E, Leinonen P, Saano A, Räsänen LA, Kaijalainen S, Piippola S, Hakola S, Lindström K. 1996. Assessment of competitiveness of rhizobia infecting *Galega orientalis* on the basis of plant yield, nodulation, and strain identification by antibiotic resistance and PCR. *Applied and Environmental Microbiology* **62**: 529-535.

Trinick MJ. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus, Leuchaena leucocephala, Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial group. *Journal of Applied Bacteriology* **49**: 39-53.

Turk D, Keyser HH. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Canadian Journal of Microbiology* **38**: 451-460.

Umali-Garcia M, Libuit JS, Baggayan RL. 1988. Effects of rhizobium inoculation on growth and nodulation of *Albizia falcataria* (L.) Fosh. and *Acacia mangium* Willd. in the nursery. *Plant and Soil* **108**:71-78.

Vincent JM. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publication, Oxford.

Vincent JM. 1974. Root nodule symbiosis with *Rhizobium.* In Quispel A., ed. *The Biology of Nitrogen Fixation.* North-Holland Publishing Company, Amsterdam, The Netherlands. 265-341.

West NJ, Adams DG, Sisson PR, Freeman R, Hawkey PM. 1999. Pyrolysis mass spectrometry analysis of free-living and symbiotic cyanobacteria. *Antonie van Leuwenhoek* **75**: 201-206.

Young JPW, Haukka KE. 1996. Diversity and phylogeny of rhizobia. *New Phytologist* **133**: 87-94.

III. Ribotyping of rhizobia nodulating *Acacia mangium* and *Paraserianthes falcataria* from different geographical areas in Indonesia using PCR-RFLP-SSCP analyses

Introduction

Acacia mangium and *Paraserianthes falcataria* are two nitrogen-fixing leguminous tree species which are widely grown in Indonesia and many other tropical countries. These species are chosen mainly because of their fast growth and ability to fix nitrogen (N) from the atmosphere. *Acacia mangium* and *P. falcataria* have a limited natural distribution in Maluku and Irian Jaya in the eastern part of Indonesia (see Chapter 2, Fig. 1). *Acacia mangium* can also be found growing naturally in Papua New Guinea and Northern Australia (Pinyopusarerk *et al.* 1993), while *P. falcataria* also occurs naturally in the Solomon Islands and the Bismarck Archipelago (Nielsen *et al.* 1983; Martawijaya *et al.* 1989).

Despite the extensive planting of *A. mangium* and *P. falcataria,* studies of the characteristics of rhizobia nodulating these tree species are limited, and are mainly concerned with the nitrogen-fixation potential of the microsymbionts (Umali-Garcia *et al.* 1988; Galiana *et al.* 1990, 1994). The rhizobia that nodulate these trees have not been assigned to any particular species, but at a generic level, these rhizobia belong to *Bradyrhizobium* (Souvanavong and Galiana 1991; Moriera *et al.* 1993). Interactions between rhizobia and *A. mangium* has received more attention than *P. falcataria,* because of the importance of the species for plantation forests and the other uses. Souvanavong and Galiana (1991) characterised 43 isolates of rhizobia from the root nodules of *A. mangium* growing in its natural range in Australia, and outside its natural habitat, in the Congo, French Guyana, Ivory Coast, and Senegal. Based on their growth rates, the authors classified them as *Bradyrhizobium* species. Moriera *et al.* (1993) included a limited number of rhizobia isolated from *A. mangium* and *P. falcataria* as part of their study of rhizobial diversity isolated from divergent groups of tropical legumes grown in Brazil. Isolates of rhizobia nodulating *A. mangium* and *P. falcataria* were found to group with the genus *Bradyrhizobium* based on their growth rates and electrophoretic protein profiles. The slow-growing, alkali-producing genus *Bradyrhizobium* contains three species, *B. japonicum* (Jordan 1982), *B. elkanii* (Kuykendall *et al.* 1992), and *B. liaoningensis* (Xu *et al.* 1995). No formal description has been given to *B. elkanii* (Young 1996). The

KENT
TEMPLEMAN
LIBRARY

separation of this species from *B. japonicum* was based on DNA-DNA hybridisation (Kuykendall *et al.* 1992). Meanwhile, *B. liaoningensis* was proposed after a polyphasic study (combination of phenotypic, biochemical, and genetic characterisations) was carried out for *Bradyrhizobium* spp. isolated from *Glycine* spp. in China (Xu *et al.* 1995), and this species found to be different from the other two species of *Bradyrhizobium*. More detailed characterisation of rhizobia nodulating *A. mangium* and *P. falcataria* is needed to reveal their relationships to named *Bradyrhizobium* species.

The development of DNA fingerprinting, especially involving the polymerase chain reaction (PCR), has made characterisation and identification of bacteria more rapid and accurate. There are several PCR-based DNA techniques that have been used to characterise rhizobia; namely random amplified polymorphic DNA (RAPD)-PCR (Dooley and Harrison 1993; Selenska-Pobell *et al.* 1995; Kishinevsky *et al.* 1996; Mathan *et al.* 1996), restriction fragment length polymorphisms (PCR-RFLP) of the 16S rRNA gene and the inter-genic spacer (IGS) regions (Laguerre *et al.* 1994, 1996; Khbaya *et al.* 1998; Lafay and Burdon 1998), repetitive extragenic palindromic (REP)-PCR (De Bruijn 1992; Teaumroong and Boonkerd 1998), and most recently denaturing gradient gel electrophoresis (DGGE) (De Oliveira *et al.* 1999). A combination of these techniques (Selenska-Pobell *et al.* 1995; Laguerre *et al.* 1997; Vinuesa *et al.* 1998), has also been used. Several of these techniques proved useful for studying the diversity of rhizobia below the species level. However, to assign individual isolates to genus or species, sequence analysis of the 16S rRNA gene has been regarded as the best phylogenetic tool. This is partly because of its reproducibility and the availability of sequence databases to compare the sequence data. The use of the 16S rRNA gene to characterise, identify and construct phylogenies of bacterial isolates, including rhizobia has been widely accepted. The 16S rRNA gene consists of both conserved and variable regions that makes it ideal for typing (Woese 1987). However, sequencing is a time-consuming and expensive technique if large sample sizes are required.

Single-strand conformational polymorphism (SSCP) is a simple and sensitive technique that can detect minor DNA sequence differences (Hayashi and Yandell 1993; Sheffield *et al.* 1993). In PCR-SSCP analysis double stranded PCR products are denatured. Each strand forms a specific folded conformation (conformer) based on internal inverted base-pairing, which depends on its sequence. These conformers are separated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. The migration of the conformer, and hence the banding patterns on the stained gel,

depends on its unique conformation, length, and molecular weight (Yap and McGee 1994). The sensitivity of PCR-SSCP to detect mutation could be affected by the length of the DNA fragment being analysed. The shorter the fragment will improve the sensitivity of the PCR-SSCP (Hayashi and Yandell 1993; Sheffield *et al.* 1993). Therefore, Hayashi and Yandell (1993) suggested the restriction enzyme digestion of the PCR products to below 600 bp prior to loading on MDE gel. Unlike PCR-RFLP, which usually used several restriction enzymes (Laguerre *et al.* 1994, 1996), a single enzyme is sufficient to perform PCR-RFLP-SSCP. Therefore, the latter technique is much simpler and quicker than the former one. The PCR-SSCP has been widely applied in mutation analysis (Hayashi and Yandell 1993; Schwieger and Tebbe 1998); however, there is a growing interest in the use of this technique for typing of microorganisms, such as fungi (Kumeda and Asao 1996) and bacteria (Widjojoatmodjo *et al.* 1994; Daffonchio *et al.* 1998). Results of identification using SSCP analysis of PCR-amplified 16S rRNA gene have been reported to be identical to those obtained by 16S rRNA gene sequence analysis (Tokue *et al.* 1995). However, to date, SSCP analysis has not been applied to the identification of rhizobia, and specifically to those isolated from root nodules of the leguminous tree species such as *A. mangium* and *P. falcataria.*

The objectives of the current study were to confirm that SSCP could be used to identify rhizobia isolated from *A. mangium* and *P. falcataria*, and secondly, to study the diversity of rhizobia nodulating *A. mangium* and *P. falcataria* isolated from different geographical areas in Indonesia. These trees can adapt well to new environments without any need for rhizobial inoculation, important questions are whether these trees are able to form effective symbiotic relationships with a broad range of rhizobial taxa, and whether the rhizobia that are specific for these trees are ubiquitous?

Materials and Methods

Origin of the Rhizobial Isolates

A total of 57 isolates of rhizobia nodulating *A. mangium* and *P. falcataria*, from different geographical areas in Indonesia, as well as two isolates from Brazil, were used in this study together with three type strains *B. elkanii* ATCC 49852^T, *B. japonicum* NCIMB 11477^T, and *M. loti* NCIMB 12074^T (Table 1). Two isolates of bacteria, AMKT1010 and

PFJB1050, isolated from root nodules of *A. mangium* and *P. falcataria* respectively, were also included in the study, as they had been shown (see Chapter 2) not to nodulate either tree.

Growing of Rhizobial Isolates and DNA extraction

Rhizobial isolates were grown in 5 ml tryptone yeast (TY) broth (Appendix IC) for 2 days at 30°C in the dark on a rotary shaker until the turbidity of the culture was equal to the McFarland (Bio-Mérieux, France) standard no 5. Two hundred microlitres of each culture were then transferred to a sterile universal bottle containing 5 ml peptone broth (PB; 4 g peptone, 0.48 g $MgCl_2.7H_2O$, 1 litre dH_2O), and incubated at 30°C on a rotary shaker for 3 days in the dark until the turbidity of the cultures was equal to the McFarland standard no 3-4.

The DNA extraction was conducted as follows: 3 ml (2 x 1.5 ml) of the PB culture was pelleted in a 1.5 ml microcentrifuge tube by centrifugation at 14 000 g (Model 1-15, Sigma, Germany) for 5 min. The supernatant was removed and the pellets resuspended in 1 ml TE_{10} buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), then re-pelleted cells were resuspended once more in 540 µl TE_{50} buffer (50 mM Tris-HCl, 1 mM EDTA, in dH_2O, pH 8) then incubated at 70°C for 15 min in a waterbath. Two microlitres of Proteinase-K (20 mg ml^{-1}) were added to the solution and incubated at the same temperature for another 15 min. Subsequently 30 µl of 10% SDS (sodium dodecyl sulfate) was added to the mixture and incubated for a further 15 min. Protein was extracted by adding 500 µl of phenol mixed by gentle inversion for 5 min, followed by centrifugation for 10 min, after which the aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. Five hundred microlitres of chloroform/isoamyl alcohol (24:1) was added to the solution and mixed again by inversion for 5 min, then centrifuged for 5 min. The aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. The DNA was precipitated by adding 0.8 volumes of isopropanol and gently mixing by inversion. The mixtures were incubated at -20°C for 15 min, then centrifuged for 20 min. The supernatant was removed from the DNA pellets which were washed with 70% ethanol, dried and dissolved in 100 µl of PCR-grade water (Sigma-Aldrich Chemic Gmbh, Steinheim, Germany).

Table 1. Isolates of rhizobia used and their original host and geographical origin.

Isolates and Origin	Original host	DNA code+	Isolates and Origin	Original host	DNA code
Bogor			Kediri (continued)		
AMBG 1010	Am	43	PFAG 1030	Pf	47
AMBG 2010	Am	36	PFAG 2030	Pf	35
AMBG 2020	Am	37	PFAG 5010	Pf	53
AMBG 2030	Am	13	PFAG 5020	Pf	34
AMNO 1010	Am	56	PFAG 5030	Pf	5
AMNO 1021	Am	57	PFAG 5040	Pf	9
AMNO 1022	Am	58	PFAG 5050	Pf	45
AMNO 1031	Am	41	PFAG 5061	Pf	23
AMNO 1041	Am	39	PFAG 5070	Pf	10
AMNO 1042	Am	40	PFAG 5080	Pf	51
AMNO 2010	Am	60	PFAG 5090	Pf	46
AMNO 2020	Am	59	PFAG 5100	Pf	52
AMNO 2040	Am	20	PFAG 5110	Pf	31
East Kalimantan			PFAG 5120	Pf	48
AMKT 1010*	Am	54	PFAG 6010	Pf	22
AMKT 2020	Am	38	PFAG 6020	Pf	49
AMKT 4010	Am	12	PFAG 6030	Pf	6
EMBRAPA-Brazil			PFAG 6040	Pf	7
BR 3609	Aa	18	PFAG 6050	Pf	24
BR 3617	Am	17	PFAG 6060	Pf	27
Irian Jaya			PFAG 6070	Pf	33
PFIR 3040	Pf	4	PFAG 6080	Pf	28
Jambi			PFAG 6090	Pf	26
AMJB 1010	Am	14	PFAG 6100	Pf	25
AMJB 1020	Am	15	PFAG 6110	Pf	29
AMJB 1030	Am	64	Lampung		
AMJB 1040	Am	42	PFLP 1040	Pf	21
AMJB 3010	Am	16	Riau		
PFJB 1010	Pf	50	AMRU 2010	Am	19
PFJB 1040	Pf	8	PFRU 1010	Pf	32
PFJB 1050*	Pf	55	PFRU 5020	Pf	30
Kediri			Type strains		
AMAG 1010	Am	44	*Bradyrhizobium elkanii* ATCC 49852^T		1
AMAG 1030	Am	62	*Bradyrhizobium japonicum* NCIMB 11477^T		2
AMAG 1050	Am	61	*Mesorhizobium loti* NCIMB 12074^T		3
AMAG 3010	Am	11			
AMAG 4010	Am	63			

+Isolates were selected and numbered randomly for DNA extraction and these numbers were used throughout the text to refer to the isolate. * non-nodulating root-nodule bacterium, ^{T} type strain, Aa= *Acacia auriculiformis*, Am= *Acacia mangium*, Pf= *Paraserianthes falcataria*

PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified using a pair of universal primers, Eubac27F (5'-AGA GTT TGA TCC TGG CTC AG) and Eubac1492R (5'-GGT TAC CTT GTT ACG ACT T) (De Long *et al.* 1993). The PCR reaction mixtures contained 1 µl DNA template (due to variation between extractions, template titrations were used to optimise the PCR), 2 µl 10x Super *Tth* buffer (HT Biotechnology Ltd., Cambridge, England), 1.2 µl $MgCl_2$ (25 mM stock solution), 2 µl dNTP (4 mM stock solution), 1 µl of each primer (5 µM stock solution), 1.5 units of Super *Tth* (HT Biotechnology Ltd., Cambridge, England), and 12.5 µl PCR-grade water (Sigma-Aldrich Chemic Gmbh, Steinheim, Germany). The amplification was carried out using a PTC-200 thermocycler (MJ Research, Watertown, Massachusetts, USA) with the following programme: 95°C for 3 min; 60 °C for 45 s; 72 °C for 2 min; 95 °C for 1 min 30 s; 57 °C 45 s; 72 °C 2 min; 4x (95 °C for 1 min 30 s; 57 °C 45 s; 72 °C 2 min), 95 °C for 1 min 30 s; 55 °C 45 s; 72 °C 2 min, 14x (95 °C for 1 min 30 s; 55 °C 45 s; 72 °C 2 min), 95 °C for 1 min 30 s; 55 °C 45 s; 72 °C 3 min, 14x (95 °C for 1 min 30 s; 55 °C 45 s; 72 °C 3 min), 95 °C for 1 min 30 s; 55 °C 45 s; 72 °C 10 min. The molecular weight of PCR products were determined by agarose gel electrophoresis and staining with ethidium bromide.

PCR-RFLP of 16S rRNA gene

The PCR products were digested with two restriction enzymes *Msp*I and *Rsa*I (Promega Co., Madison, USA) separately. The digestion reaction was as follows: 4 µl PCR product, 2 µl 10x respective restriction enzyme (RE) buffer (Promega Co., Madison, USA), 0.2 µl bovine serum albumine (BSA; 10 µg μl^{-1} stock solution), 0.5 µl restriction enzyme (10 unit μl^{-1}), and 13.3 µl PCR water. The mixture was incubated at 37°C for 3 hrs in the thermocycler. The digestions were electrophoresed on 2% agarose gels or stored in microcentrifuge tubes in a domestic freezer (-20°C) until required.

PCR-RFLP-SSCP of 16S rRNA gene

Preparation of single-stranded DNA

To run the restriction digest products on an MDE (Mutation Determination Enhancement) gel, 5 µl of the *Msp*I- or *Rsa*I-digestion product was added to 15 µl denaturing buffer solution containing 95% formamide in 0.2 µl PCR tubes, 10 mM NaOH, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol. The mixtures were incubated at 95°C for 2 min in the thermocycler then put on wet ice and loaded as quickly as possible to avoid re-annealing of the denatured DNA.

Gel electrophoresis and staining

Each gel for SSCP was prepared by mixing 12.5 ml MDE gel solution (Flowgen, Staffordshire, UK), 31.28 ml milli-Q water, 6 ml 5x TBE, 20 µl Temed (N,N,N',N'-tetramethylethylenediamine), and 200 µl ammonium persulfate 10% (w/v) in a 100 ml beaker. The solution then was injected between a pair of sequencing gel plates, the thermal (33 x 41.7 cm^2) and the binding silane plates (33 x 39 cm^2), according to the manufacturer's instructions (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA) and left to polymerise for one hour. The glass sandwich then was assembled in the gel tank (Hoefer SQ3 Sequencer, Hoefer Pharmacia Biotech Inc.) and 4.5 µl denatured products were loaded into each well. The gel was electrophoresed at 4 Watts for 8.17 volt hours.

The single strand DNA bands were stained with silver. The thermal plate was removed from the sandwich and the gel sticking on the binding silane plate was soaked in 10% acetic acid for 20 min with constant shaking. The acetic acid was removed and the gel was washed with dH_2O three times, 2 min for each wash. Silver nitrate solution (2 l milli-Q water, 2 g $AgNO_3$, and 3 ml formaldehyde 37%) was then poured onto the gel for 30 min with constant shaking. The silver nitrate solution was removed and the gel was quickly washed with dH_2O. Subsequently, sodium carbonate solution (2 L milli-Q water, 60 g $NaCO_3$, 3 ml formaldehyde 37%, and 400 µl sodium thiosulfate 1 mg ml^{-1}) was poured onto the gel until bands developed. The staining reaction was stopped by replacing the sodium carbonate solution with 10% acetic acid for 15 min and subsequently with dH_2O for 30 min with constant shaking. Finally, the gel was dried in the oven at 70°C for

2-3 hrs. The gel was then scanned using a flat bed scanner (EPSON GT-9600, EPSON PTE Ltd, Singapore) and the image saved for further analyses. Profiles were then compared by eye and grouped according to similarity of band migration.

16S rRNA gene sequence analysis

The PCR-RFLP-SSCP profiles grouped into seven patterns over the 64 band varieties. Seven representative isolates were sequenced, corresponding to *B. elkanii* ATCC 49852^T, *B. japonicum* NCIMB 11477^T, *M. loti* NCIMB 12074^T, AMBG 1010, AMKT 2020, PFAG5100, PFRU 5020. At least 1000 bp within the 16S rRNA gene were sequenced for these isolates. The products of 16S rRNA gene amplification were sent to the Advanced Biotechnology Centre (ABC, Imperial College School of Medicine, London, UK).

Results

PCR amplification of 16S rRNA gene

The 16S rRNA gene was successfully amplified from all of the bacterial isolates and produced a single band of about 1,450 bp (Fig. 1). All rhizobial isolates and two non-nodulating root nodule bacteria produced approximately the same size amplification product.

PCR-RFLP analyses of 16S rRNA gene

***Msp*I restriction enzyme**

Restriction digests using the restriction enzyme *Msp*I resulted in 4 strong bands for the rhizobial isolates of *A. mangium* and *P. falcataria* and the type strains of *B. elkanii* and *B. japonicum* (Fig. 2) visualised on agarose gels stained with ethidium bromide. Both type strains of *Bradyrhizobium* had identical banding patterns, which was also shared with 58 isolates of rhizobia from *A. mangium* and *P. falcataria.* The rhizobial isolate AMBG1010 and the non-nodulating isolates, AMKT 1010 and PFJB 1050 had different banding patterns from the rest of the rhizobial isolates of *A. mangium* and *P. falcataria.*

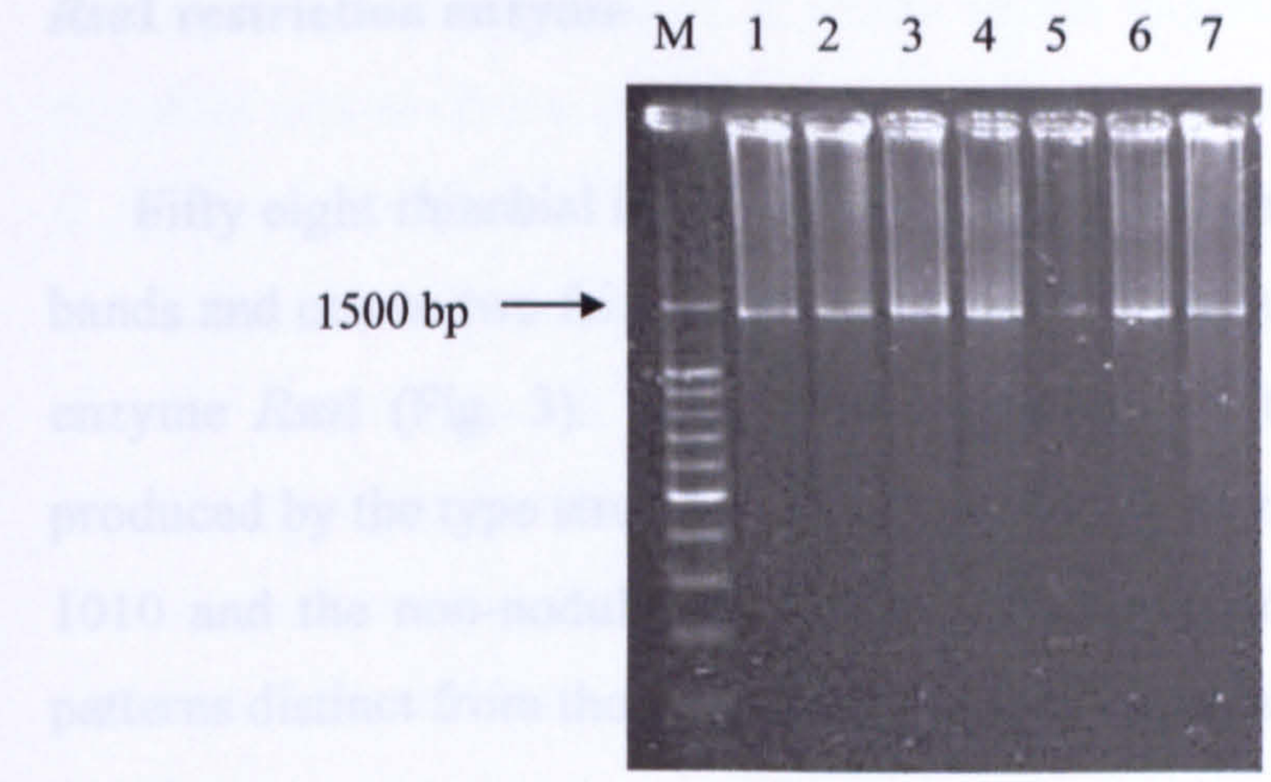


Figure 1. Examples of the 16S rRNA gene PCR products amplified from rhizobial isolates from *A. mangium* and *P. falcataria*. Single identical bands were successfully amplified from all bacterial isolates. Numbers at the top of the gel represent the DNA codes (see Table 1).

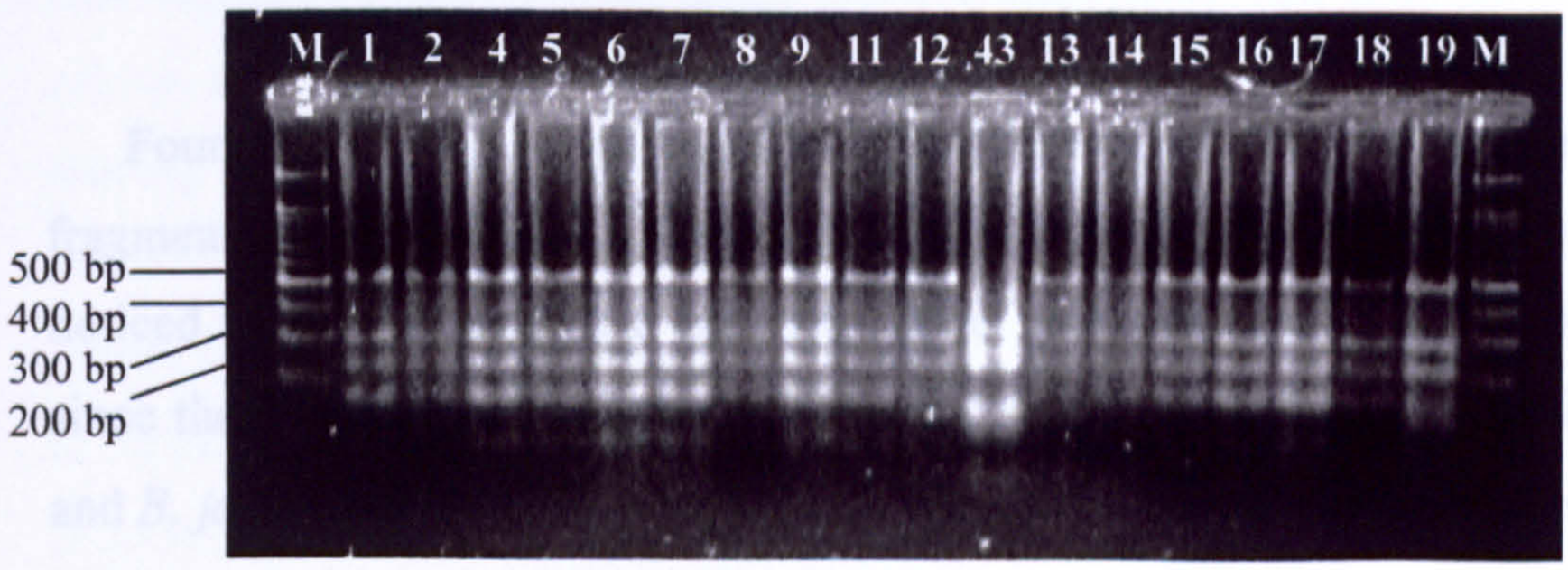


Figure 2. Products of the restriction digest of the 16S rRNA gene of some rhizobial isolates using the restriction enzyme *Msp*I. Most of the rhizobial isolates of *A. mangium* and *P. falcataria* produced the same banding patterns as *B. elkanii* (1) and *B. japonicum* (2) type strains, except isolate AMBG1010 (43). M= DNA marker weight. Numbers on top of the gel represent the DNA codes (see Table 1).

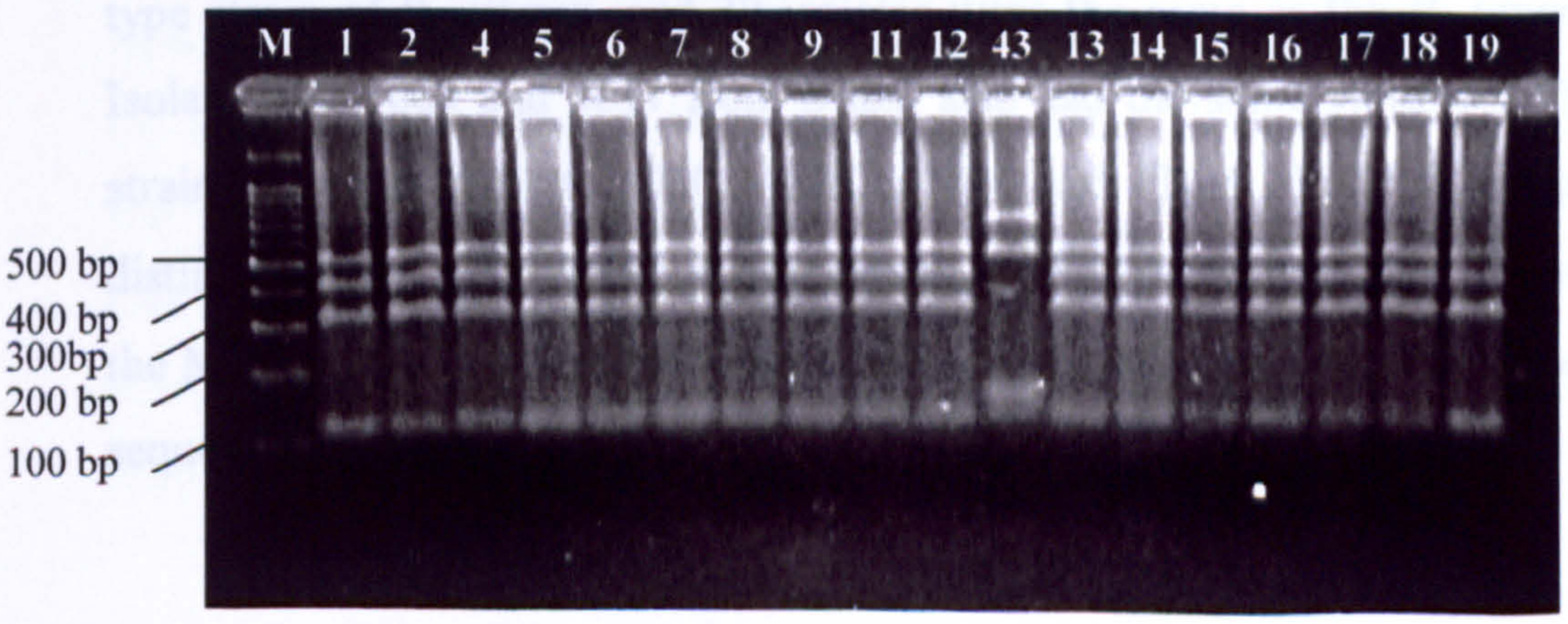


Figure 3. Products of the restriction digest of of the 16S rRNA gene of some rhizobial isolates using the restriction enzyme *Rsa*I. Most of the rhizobial isolates of *A. mangium* and *P. falcataria* produced the same banding patterns as *B. elkanii* (1) and *B. japonicum* (2) type strains, except isolate AMBG1010 (43). M= DNA marker weight. Numbers on top of the gel represent the DNA codes (see Table 1).

_Rsa_I restriction enzyme

Fifty eight rhizobial isolates from *A. mangium* and *P. falcataria* produced three strong bands and one to two faint bands after the PCR products were digested with the restriction enzyme *Rsa*I (Fig. 3). The banding patterns of these isolates were similar to those produced by the type strains of *B. elkanii* and *B. japonicum*. The rhizobial isolate AMBG 1010 and the non-nodulating isolates, AMKT 1010 and PFJB 1050 produced banding patterns distinct from those of other rhizobial isolates.

PCR-RFLP-SSCP analyses of 16S rRNA gene

_Msp_I restriction enzyme

Four to 6 strong bands could be clearly observed for each isolate after the RFLP fragments of the 16S rRNA gene were run on the MDE gel. Some faint bands were also noticed at the bottom of the gel. No further attempts were made to clarify these bands since the 4 to 6 clear bands had sufficient discriminatory power to distinguish *B. elkanii* and *B. japonicum*. The 16S rRNA genes digested with restriction enzyme *Msp*I produced 5 different banding patterns on the MDE gel for the rhizobial isolates and two non-nodulating root-nodule bacteria from *A. mangium* and *P. falcataria*. The type strain of *B. elkanii* could be distinguished from the type strain of *B. japonicum* (Fig. 4). Sixteen rhizobial isolates of *A. mangium* and *P. falcataria* had the same banding pattern as the type strain *of B. elkanii*, and 39 isolates were the same as the *B. japonicum* type strain. Isolates BR 3609 and 3617 from Brazil also had the same pattern as the *B. elkanii* type strain. Isolates AMBG 1010, AMKT 1010 and PFJB 1050 had unique SSCP profiles distinct from *B. elkanii* and *B. japonicum*. The size of the DNA fragments appeared in the MDE gel with the restriction enzyme *Msp*I, and their position in the 16S rRNA gene sequence were presented in Fig. 7.

_Rsa_I restriction enzyme

When the *Rsa*I restriction enzyme was used, 3 to 6 bands could be clearly seen for each isolate on the MDE gel. The type strains of *B. elkanii* and *B. japonicum* could be

distinguished on the basis of 6 bands which migrated to different positions. Seventeen rhizobial isolates from *A. mangium* and *P. falcataria* had identical banding patterns to the *B. elkanii* type strain. This included two isolates, AMAG 1030, and AMNO 2010, which had not produced strong bands on the MDE gel generated using the *Msp*I restriction enzyme due to low PCR-RFLP products for SSCP. Thirty-nine isolates had banding patterns that closely resembled the *B. japonicum* type strains, but differed by the top band. The uppermost band of the rhizobial isolates from *A. mangium* and *P. falcataria* migrated a shorter distance compared with that of the *B. japonicum*. In the case of isolate AMKT 2020, the top two bands were similar to the *B. japonicum*-type isolates from *A. mangium* and *P. falcataria* with a slight shift, but the rest of the bands were similar to the latter isolates and to the type strain of *B. japonicum* (Fig. 5). Isolate AMRU2010, which had the banding pattern of *B. elkanii* when digested with *Msp*I, had both banding patterns of *B. elkanii* and *B. japonicum* when digested with *Rsa*I indicating contamination due to a gel loading error. Isolate PFAG5070, which was identified as *B. japonicum* using the *Msp*I restriction enzyme, produced a unique banding pattern which was different from both the *Bradyrhizobium* type strains. For *Rsa*I digestion, DNA from this isolate was re-extracted to improve the quality of the PCR amplification product (a poor PCR amplification product resulted in a poor quality PCR-RFLP-SSCP using the restriction digest *Msp*I, see Fig. 4). Probably this isolate was contaminated during this process. Morphological changes were found in the isolate PFAG5070 on the plate, where the colony colour changed from white opaque to yellowish opaque. Isolates AMBG1010, AMKT1010, and FJB1050 had different banding patterns from the *Bradyrhizobium* type strains. When the type strain of *M. loti* was included in the analysis, it was clear that isolate AMBG1010 had the same banding pattern (Fig.5). Results of the PCR-RFLP-SSCP analyses with the restriction enzyme *Rsa*I and the size of the DNA fragments appeared in the MDE gel, and their positions in the 16S rRNA gene sequence were presented in Fig. 8. The results of the characterisation of the rhizobial isolates and the non-nodulating root nodule bacteria using both restriction enzymes is summarised in Table 2.

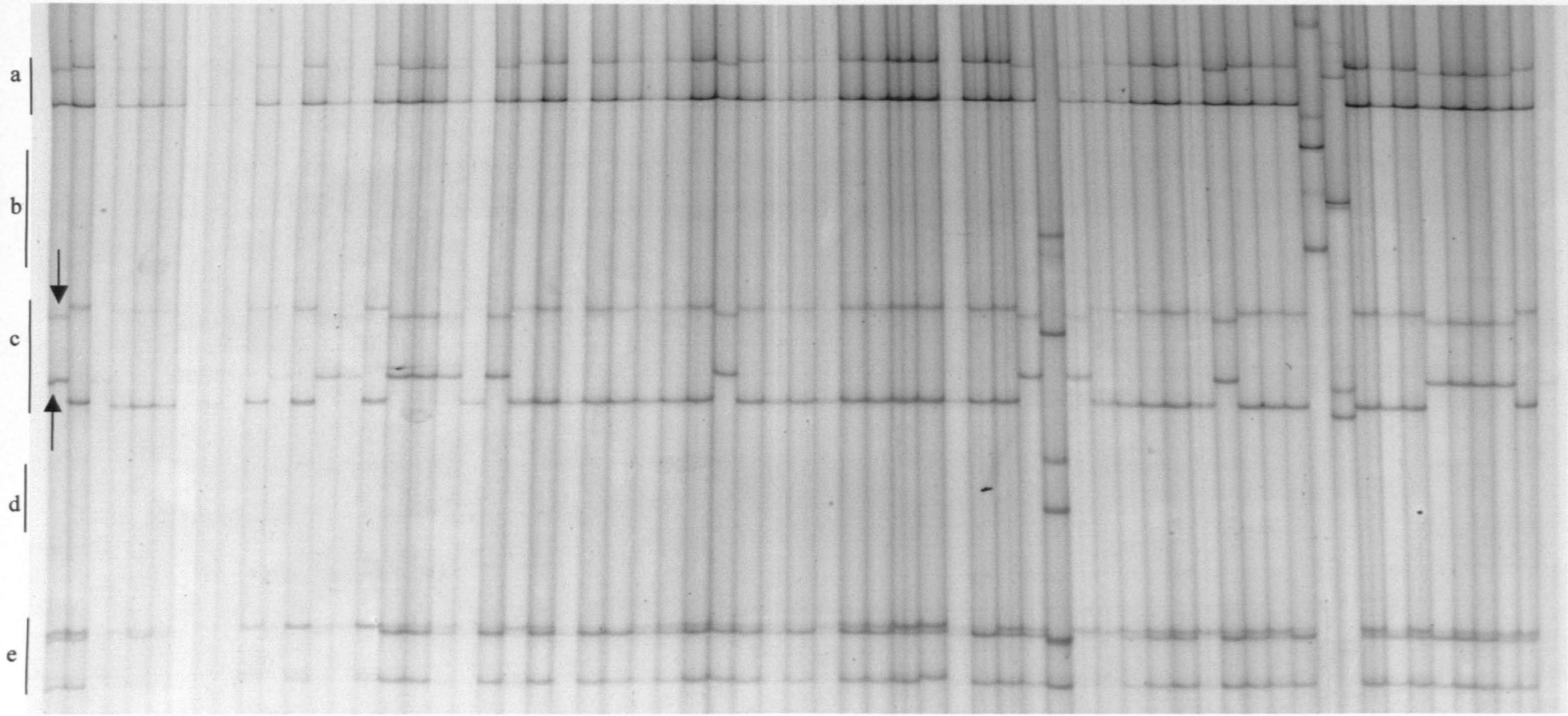


Figure 4. SSCP analysis of PCR-amplified 16S rRNA genes digested with a restriction enzyme *Msp*I. The picture shows the top part of the gel contained at least 6 strong and informative bands. Lanes 1 and 2 were the DNA of *B. elkanii* ATCC 49852 and *B. japonicum* NCIMB 11477, respectively. The bands in zone a and c areas could separate these *Bradyrhizobium* species. In zone c, the top band of *B. elkanii* migrated further than that of *B. japonicum*, and conversely its bottom band migrated less (arrows). Most of the rhizobial isolates of *A. mangium* and *P. falcataria* (4-64) showed similarities of banding patterns to either *B. elkanii* or *B. japonicum*, except three isolates in that AMBG 1010 (43), AMKT 1010 (54) and PFJB 1050 (55). Numbers at the top of the gel are the DNA codes for each rhizobial isolates (see Table 1 for details).

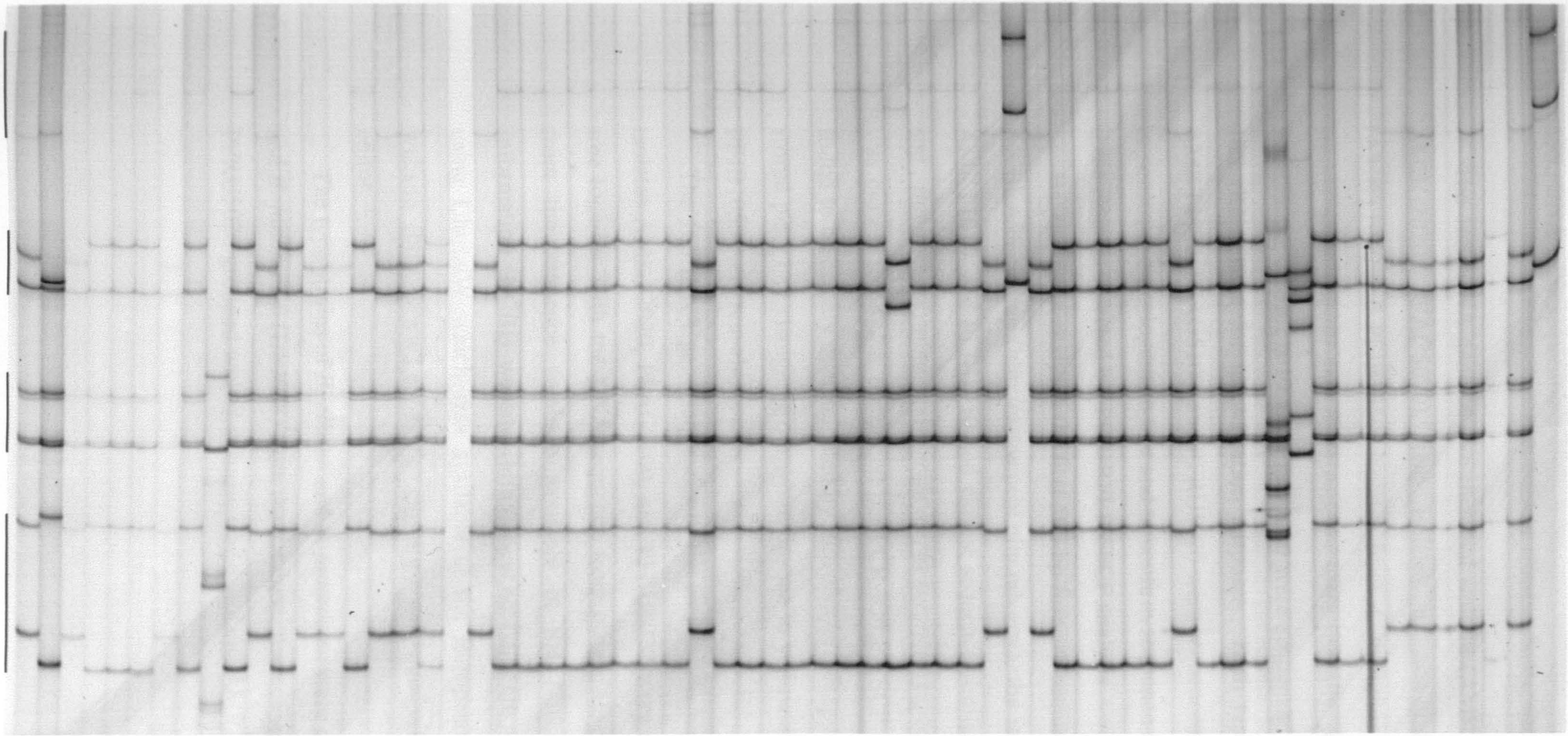


Figure 5. SSCP analysis of amplified 16S rRNA genes digested with a restriction enzyme *Rsa*I. The picture shows the top part of the gel which contained at least three strong and informative bands. Lanes 1 and 2 were the type strains of *B. elkanii* ATCC 49852 and *B. japonicum* NCIMB 11477, respectively. The bands in zone b and d discriminated these *Bradyrhizobium* species. In zone b the top band of *B. elkanii* migrated less than that of *B. japonicum*, and in zone d the distance between two bands of the former species was shorter than that of the latter one. In zone b some of rhizobial isolates of *A. mangium* and *P. falcataria* (4-64) showed similar banding patterns with *B. elkanii* (e.g. no. 12), but most of them (e.g. no. 5) did not share similar banding patterns with both *Bradyrhizobium* type strains. In this zone the banding pattern of isolate AMKT2020 (38) from *A. mangium* was different from other *Bradyrhizobium* isolates. However, generally these isolates had similar banding patterns with either *B. elkanii* or *B. japonicum* in zone c and d. Isolates PFAG 5070 (10), AMBG 1010 (43), AMKT 1010 (54), and PFJB 1050 (55) did not identical to any of the *Bradyrhizobium* type strains. Isolate AMBG 1010 (43) was identical to the type strain of *M. loti* NCIMB 12074 (3) sharing similar banding patterns at the a and b areas. Numbers at the top of the gel are the DNA codes for each of rhizobial isolate (see Table 1 for details).

16S rRNA gene sequencing

The 16S rRNA gene of five rhizobial isolates (AMKT2020, PFAG5100, PFRU5020, AMBG2020, PFAG5070), two isolates of non-nodulating root nodule bacteria (AMKT1010 and PFJB1050) isolated from *A. mangium* and *P. falcataria*, and the type strains of *B. elkanii* and *B. japonicum* were partially sequenced. Isolates AMBG1010, PFAG5070, AMKT1010 and PFJB1050 were sequenced only for the first 500 bp of the 16S rRNA gene for identification purposes. Both type strains and rhizobial isolates AMKT2020, PFAG5100, and PFRU5020 were sequenced for the first 1000 bp of the gene to construct a phylogenetic tree and to confirm sequence similarities or differences among these isolates detected by PCR-RFLP-SSCP analysis.

Based on the analyses the first 500 bp forward sequence of the 16S rRNA gene using the BLAST search (http://www.ncbi.nlm.nih.gov), the isolates AMBG1010, PFAG5070, AMKT1010, and PFJB1050 had closest sequence similarities to *M. loti*, *Flavobacterium* spp., *Bacillus polymixa*, and *Burkholderia kuranei*, respectively. That isolate AMBG1010 was closest to *M. loti* confirmed the PCR-RFLP-SSCP result. Isolate PFAG5070 was found to be heavily contaminated by *Flavobacterium* spp. (the colour of the colony in the stored plate had changed from white opaque to yellowish), and isolates AMKT1010 and PFJB1050 were non-rhizobia.

The 16S rRNA gene sequences of the two *Bradyrhizobium* type strains and rhizobial isolates AMKT2020, PFAG5100, and PFRU5020 were used to construct a phylogenetic tree together with the DNA sequence from other recognised rhizobial species obtained from the sequence database available in the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The alignment was made using ClustalW which was available at the European Bioinformatics Institute (EBI) website (http://www2.ebi.ac.uk/ clustalw) and manipulated in the JalView alignment editor.

The type strains of *B. elkanii* and *B. japonicum* were grouped with *B. elkanii* (U35000) and *B. japonicum* (U69638) from the database, respectively (Fig. 6). As expected, isolate PFRU5020 grouped with *B. elkanii*, whilst isolates AMKT2020 and PFAG5100 clustered

Table 2. Summary of results of PCR-RFLP-SSCP analysis of rhizobial isolates and non-nodulating root nodule bacteria isolated from *A. mangium* and *P. falcataria* from Indonesia.

DNA code	Isolate	Restriction enzymes		DNA code	Isolate	Restriction enzymes	
		*Msp*I	*Rsa*I			*Msp*I	*Rsa*I
				continued			
1	*B. elkanii* ATCC 49852^T	Be	Be	33	PFAG6070	Bj	Bj
2	*B. japonicum* NCIMB	Bj	Bj	34	PFAG5020	Bj	Bj
3	*M. loti*^{T} NCIMB 12074^T	nd	Ml	35	PFAG2030	Bj	Bj
4	PFIR3040	Be	Be	36	AMBG2010	Bj	Bj
5	PFAG5030	Bj	Bj	37	AMBG2020	Bj	Bj
6	PFAG6030	Bj	Bj	38	AMKT2020	Bj	Bj
7	PFAG6040	Bj	Bj	39	AMNO1041	Bj	Bj
8	PFJB1040	Be	Be	40	AMNO1042	Bj	Bj
9	PFAG5040	Bj	Bj	41	AMNO1031	Bj	Bj
10	PFAG5070	Bj	u	42	AMJB1040	Be	Be
11	AMAG3010	Bj	Bj	43	AMBG1010	u	Ml
12	AMKT4010	Be	Be	44	AMAG1010	Be	Be
13	AMBG2030	Bj	Bj	45	PFAG5050	Bj	Bj
14	AMJB1010	Be	Be	46	PFAG5090	Bj	Bj
15	AMJB1020	Be	Be	47	PFAG1030	Bj	Bj
16	AMJB3010	Bj	Bj	48	PFAG5120	Bj	Bj
17	BR3617	Be	Be	49	PFAG6020	Bj	Bj
18	BR3609	Be	Be	50	PFJB1010	Be	Be
19	AMRU2010	Be	Be	51	PFAG5080	Bj	Bj
20	AMNO2040	Bj	Bj	52	PFAG5100	Bj	Bj
21	PFLP1040	Be	Be	53	PFAG5010	Bj	Bj
22	PFAG6010	Bj	Bj	54	AMKT1010	u	u
23	PFAG5061	Bj	Bj	55	PFJB1050*	u	u
24	PFAG6050	Bj	Bj	56	AMNO1010	Bj	Bj
25	PFAG6100	Bj	Bj	57	AMNO1021	Bj	Bj
26	PFAG6090	Bj	Bj	58	AMNO1022	Bj	Bj
27	PFAG6060	Bj	Bj	59	AMNO2020	Be	Be
28	PFAG6080	Bj	Bj	60	AMNO2010	nd	Be
29	PFAG6110	Bj	Bj	61	AMAG1050	Be	Be
30	PFRU5020	Be	Be	62	AMAG1030	nd	Be
31	PFAG5110	Bj	Bj	63	AMAG4010	nd	Be
32	PFRU1010	Bj	Bj	64	AMJB1030	Be	Be

^{T} type strain, Be= the banding pattern was similar to that of *B. elkanii* ATCC 49852^T , Bj= the banding pattern was similar to that of *B. japonicum* NCIMB 11477^T , Ml= the banding pattern was similar to that of *M. loti* NCIMB 12074^T , nd= no data, u= the banding pattern was unique, which was different from that of both *B. elkanii* ATCC 49852^T and *B. japonicum* NCIMB 11477^T, * non-nodulating root-nodule bacterium.

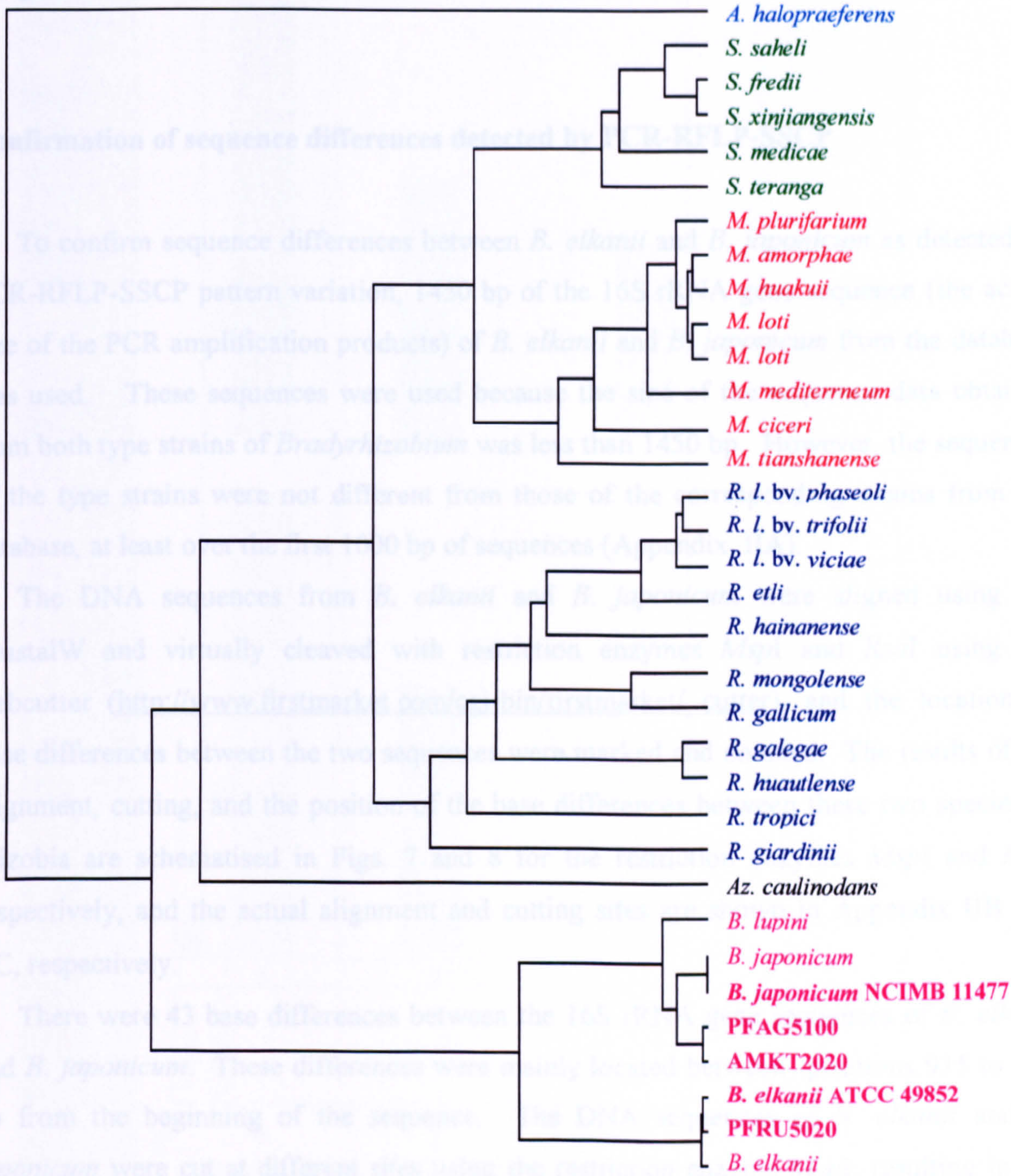


Figure 6. Phylogenetic position of the 16S rRNA gene sequences obtained from isolates used in this study (AMKT2020, PFAG5100, and PFRU5020), and type strains of *B. elkanii* ATCC 49852 and *B. japonicum* NCIMB 11477. The accession numbers for other isolates from top to bottom are: D13943, X68390, X67231, D12796, L39882, X68387, Y14158, AF041442, D12797, Y14159, Y14160, L38825, U07934, U71079, U29388, U31074, U89831, U28939, U71078, U89822, AF008130, AF025853, AF025852, U89832, U86344, X94200, U69636, U69638, U35000. Note: A.= *Azospirillum*, Az.= *Azorhizobium*, B.= *Bradyrhizobium*, M.= *Mesorhizobium*, R.= *Rhizobium*, R.l.= *Rhizobium leguminosarum*, S. = *Sinorhizobium*.

with *B. japonicum*. These grouping confirmed the results obtained by PCR-RFLP-SSCP analysis.

Confirmation of sequence differences detected by PCR-RFLP-SSCP

To confirm sequence differences between *B. elkanii* and *B. japonicum* as detected by PCR-RFLP-SSCP pattern variation, 1450 bp of the 16S rRNA gene sequence (the actual size of the PCR amplification products) of *B. elkanii* and *B. japonicum* from the database was used. These sequences were used because the size of the sequence data obtained from both type strains of *Bradyrhizobium* was less than 1450 bp. However, the sequences of the type strains were not different from those of the corresponding strains from the database, at least over the first 1000 bp of sequences (Appendix IIA).

The DNA sequences from *B. elkanii* and *B. japonicum* were aligned using the ClustalW and virtually cleaved with restriction enzymes *Msp*I and *Rsa*I using the webcutter (http://www.firstmarket.com/cgi-bin/firstmarket/_cutter), and the location of base differences between the two sequences were marked and counted. The results of the alignment, cutting, and the position of the base differences between these two species of rhizobia are schematised in Figs. 7 and 8 for the restriction enzymes *Msp*I and *Rsa*I respectively, and the actual alignment and cutting sites are shown in Appendix IIB and IIC, respectively.

There were 43 base differences between the 16S rRNA gene sequences of *B. elkanii* and *B. japonicum*. These differences were mainly located between positions 935 to 986 bp from the beginning of the sequence. The DNA sequences of *B. elkanii* and *B. japonicum* were cut at different sites using the restriction enzyme *Msp*I; resulting in the different numbers of fragments produced: 8 for *B. elkanii* and 7 for *B. japonicum* (Fig. 7b). However, on the agarose gel, only 4 fragments longer than 150 bp could be detected (Fig. 7c). On the MDE gel, single strand DNA fragments corresponding to the fragment sizes 222, 289, and 495 bp (*B. elkanii*) or 502 bp (*B. japonicum*) on PCR-RFLP could be clearly detected (Fig. 7d), whilst the rest of the bands over-ran during gel electrophoresis. Therefore, only the sequence of these fragments were used to confirm base changes detected by PCR-RFLP-SSCP (1006-1013 bp).

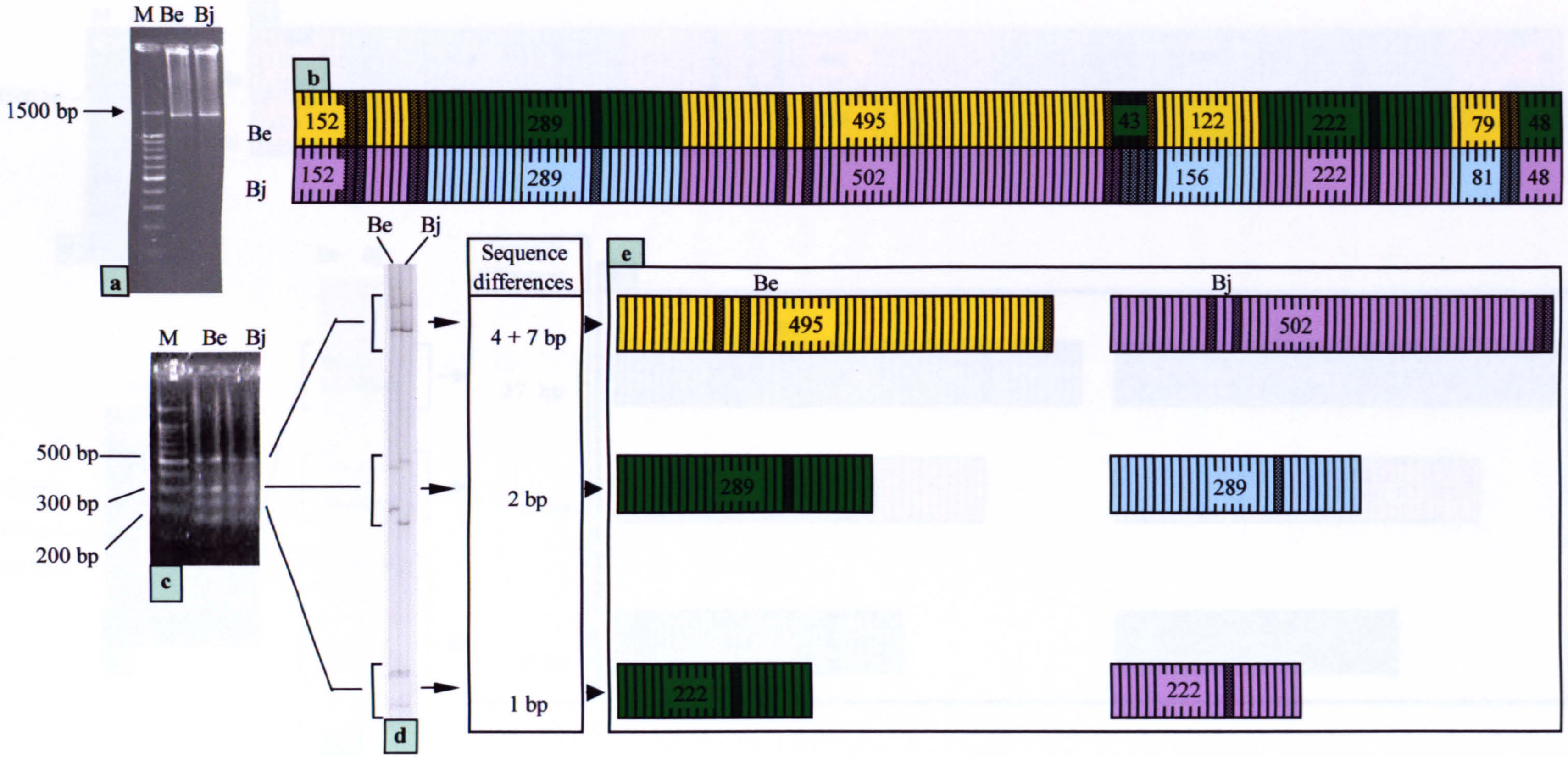


Figure 7. Comparison of the 16S rRNA gene of *B. elkanii* (Be) and *B. japonicum* (Bj). (a) PCR products of the amplified 16S rRNA gene electrophoresed on 2% (w/v) agarose gel, (b) a schematised 1450 bp 16S rRNA gene sequence of *B. elkanii* and *B. japonicum* representing the PCR product showing the position of *Msp*I-restriction fragments (different fragments in the same row coloured alternately) in the sequence, and approximate location of sequence differences between *B. elkanii* and *B. japonicum* (chequed). One block representing approximately 10 bases. Numbers inside fragments indicating the exact length of corresponding fragments. (c) the restriction products of the PCR products cut with a restriction enzyme *Msp*I run on 2% (w/v) agarose gel, (d) the restriction products as in (c), but run on an SSCP gel., and (e) schematised orders of the 3 uppermost restriction fragments in the agarose gel corresponding to the restriction product (c) and SSCP (d) extracted from the total sequence length (d). M= DNA marker.

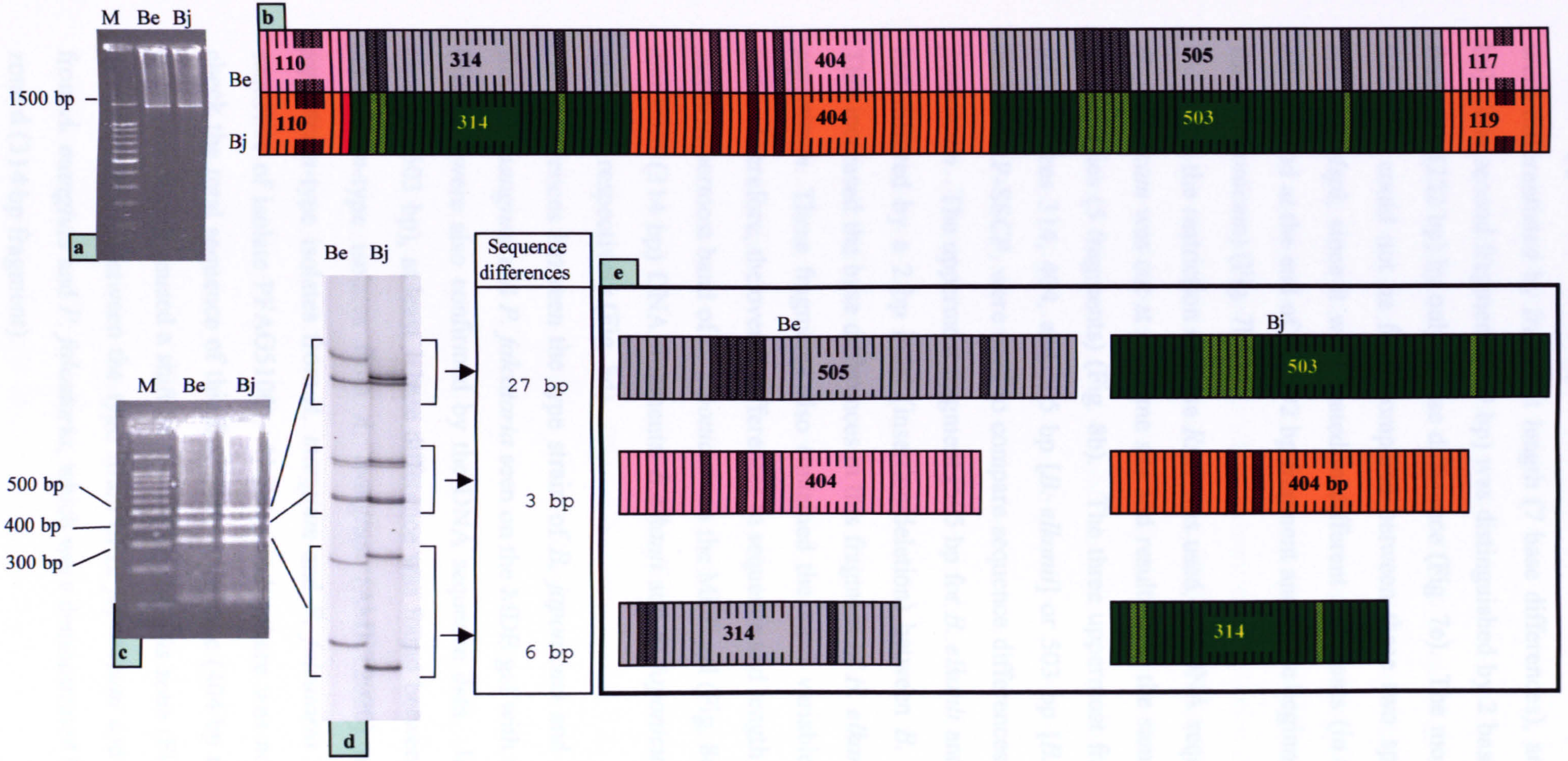


Figure 8. Comparison of the 16S rRNA gene of *B. elkanii* (Be) and *B. japonicum* (Bj). (a) PCR products of the amplified 16S rRNA gene electrophoresed on 2% (w/v) agarose gel, (b) a schematised 1450 bp 16S rRNA gene sequence of *B. elkanii* and *B. japonicum* representing the PCR product showing the position of *Rsa*I-restriction fragments (different fragments in the same row coloured alternately) in the sequence, and approximate location of sequence differences between *B. elkanii* and *B. japonicum* (chequed). One block representing approximately 10 bases sequence. Numbers inside fragments indicating the exact length (base) of corresponding fragments. (c) the restriction products of the PCR products cut with a restriction enzyme *Rsa*I run on 2% (w/v) agarose gel, (d) the restriction products as in (c), but run on an SSCP gel. (e) Schematised orders of the 3 uppermost restriction fragments on the agarose gel corresponding to the restriction product (b) and SSCP (c) extracted from the total sequence length (d). M= DNA ladder.

The uppermost DNA fragments from *B. elkanii* and *B. japonicum* on the MDE gel were differentiated by fragment length (7 base differences), and sequence differences (4 bp). The second fragment (289 bp) was distinguished by 2 base differences, and the third fragment (222 bp) by only 1 base difference (Fig. 7e). The most variable region (position 935-986) could not be fully compared between these two species using the restriction enzyme *Msp*I, since it was located in different fragments (in the 43 bp fragment for *B. elkanii*, and at the end of the 502 bp fragment and at the beginning of the 156 bp fragment for *B. japonicum*) (Fig. 7b).

When the restriction enzyme *Rsa*I was used, the DNA sequences from *B. elkanii* and *B. japonicum* was cut at the same site and resulting in the same number of fragments for both species (5 fragments) (Fig. 8b). The three uppermost fragments detected by PCR-RFLP (sizes 314, 404, and 505 bp [*B. elkanii*] or 503 bp [*B. japonicum*]), and also by PCR-RFLP-SSCP, were used to compare sequence differences between *B. elkanii* and *B. japonicum*. The uppermost fragment (505 bp for *B. elkanii* and 503 bp for *B. japonicum*) had differed by a 2 bp indel (insertion/deletion) between *B. elkanii* and *B. japonicum*. This increased the base differences in this fragment of *B. elkanii* to 27 bp from that of *B. japonicum*. These fragments also contained the most variable region of the 16S rRNA gene. Therefore, the overall differences in sequence and length caused the significant shift of the uppermost band of *B. japonicum* on the MDE gel (Fig. 8d). On the second (404 bp) and third (314 bp) DNA fragments, *B.elkanii* and *B. japonicum* were differentiated by 3 and 6 bp, respectively (Fig. 8d).

Differences between the type strain of *B. japonicum* and *B. japonicum*-type isolates from *A. mangium* and *P. falcataria* seen on the MDE gel with the restriction enzyme *Rsa*I (Fig. 5), were also confirmed by the DNA sequence data. In the uppermost fragments (zone b, 503 bp), at least 1 base difference was found between the DNA sequence of *B. japonicum*-type isolates from *A. mangium* (AMKT2020) and the most common *B. japonicum*-type isolates from *A. mangium* and *P. falcataria* (represented by the DNA sequence of isolate PFAG5100). Unfortunately, there was not enough sequence data to check the total sequence of this fragment. In zone c (404 bp fragment), there was 1 base difference which caused a shift on the top band at this zone (Fig. 5). Finally, there were 4 base differences between the type strain of *B. japonicum* and *B. japonicum*-type isolates from *A. mangium* and *P. falcataria*, which were demonstrated by a clear bands shifts of at zone d (314 bp fragment).

Discussion

Results of the diversity study on rhizobia isolated from *A. mangium* from different zones of Indonesia show that the isolates fell into at least 3 groups: one with similarity to *M. loti*, and the other two to either *B. elkanii* or *B. japonicum*. The occurrence of rhizobial isolates similar to *B. elkanii* and *B. japonicum* nodulating *A. mangium* has also been reported by Moreira *et al.* (1998) based on the partial sequence analysis of the 16S rRNA gene of two isolates from Brazil. The division of rhizobial isolates from *A. mangium* into three groups was also inferred in work by Frémont *et al.* (1999), based on the RFLP analysis of the rRNA operon, comprising the 16S and the 16S/23S intergenic spacer (IGS). The authors grouped 44 rhizobial isolates of *A. mangium* from Africa, Australia, Malaysia, and Singapore into two main groups of *Rhizobium* and *Bradyrhizobium*. The *Bradyrhizobium* group was further divided into two distinct clusters, which may reflect *B. elkanii*- and *B. japonicum*-type isolates observed in our work. Unfortunately, since no type strains or sequence data was included in that analysis, the affiliation of these isolates to the currently recognised species of rhizobia remains unknown. Nuswantara *et al.* (1997) studied the phylogeny of the rhizobial isolates from *A. mangium* isolated from 5 locations in West Java and 1 location in Sumatra, both in Indonesia, and concluded that these rhizobia were closely related to *B. elkanii*, but not to *B. japonicum*. This conclusion was drawn from the analysis of 10 isolates using REP-PCR, PCR-RFLP, and partial sequence analysis of the 16S rRNA gene. The main reason for the discrepancy between their result and that reported in our study was a much larger number of isolates was used from a wider geographical area. This gives a more comprehensive view of the diversity of rhizobia nodulating this tree species, showing an approximately equivalent split of 50:50 between *B. elkanii* and *B. japonicum*.

Results of the study suggested that *P. falcataria* is principally nodulated by rhizobia from the genus *Bradyrhizobium*. Similar results were obtained by other workers involving smaller numbers of isolates and based on phenotypic characterisation and total protein analyses (Turk and Keyser 1992; Moreira *et al.* 1993). Recently, Moreira *et al.* (1998) found that three rhizobial isolates from *P. falcataria* in Brazil, were closely related to *B. elkanii* based on a partial sequence of the 16S rRNA gene. Here, using a larger number of isolates from more diverse areas in Indonesia, *B. japonicum* isolates was also found in the root nodules of *P. falcataria* were found as well as *B. elkanii*-like isolates in the ratio 1:4 (*B. elkanii* : *B. japonicum*).

The grouping of the rhizobial isolates of *A. mangium* and *P. falcataria* isolated from Indonesia into three separate groups using PCR-RFLP-SSCP was supported by the sequence data for the 16S rRNA gene of the representative isolates of each group. In preliminary work, the potential use of PCR-RFLP-SSCP for discrimination of rhizobial isolates at species level, and even biovar level was shown by the observation that *Rhizobium leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii* could be clearly distinguished when their amplified 16S rRNA gene was digested with *Msp*I and run on an MDE gel (data not shown). This method provides a rapid, reliable and reproducible tool for identification of rhizobial isolates. The PCR products of the 16S rRNA gene of some isolates including the type strains have been run in the MDE gel at least three times on different occasions and under different conditions, and by different operators, yet the banding patterns obtained were always consistent for each restriction enzyme used. The only difference obtained between runs was the relative distance between bands, which was shorter or longer depending on time taken to run the gel. However, there was no problem if the type strains were included on each gel as standards. Compared with other PCR-based identification techniques for rhizobia, such as RFLP analyses of the 16S rRNA gene, REP-PCR, AP- or RAPD-PCR, PCR-RFLP-SSCP is simpler and yet is more reliable and reproducible. A single restriction enzyme was sufficient to identify the isolates to species level. In RFLP analysis several restriction enzymes were needed to obtain such results, and at least four restriction enzymes are needed to separate *B. elkanii* and *B. japonicum* (Nuswantara *et al.* 1997). The selection of restriction enzymes for RFLP analysis is also critical, since some of them have little power of discrimination (Nour *et al.* 1994). In contrast, the type of restriction enzyme being used to digest the 16S rRNA gene for SSCP analysis is not critical, because the aim of the DNA digestion was to reduce the size of the DNA fragment below 600 bp in order to increase the sensitivity of the SSCP to detect single base changes (Hayashi and Yandell 1993).

The work presented here has shown that minor base changes could be detected by PCR-RFLP-SSCP. Our data show that small base differences between *B. elkanii* and *B. japonicum*, as little as 1 base, could be detected by PCR-RFLP-SSCP analysis. This confirms the sensitivity of the SSCP to detect single base changes (Sheffield *et al.* 1993) compared with PCR-RFLP which could not detect differences between fragments even if there were 7 bp length or 27 base sequence differences. The sensitivity of PCR-RFLP-SSCP analysis is similar to that of sequence analysis of DNA (in this case the 16S rRNA gene sequence). Relatively small numbers of bands were used in this PCR-RFLP-SSCP

analysis, yet it did not reduce the sensitivity of the analysis to detect intraspecific differences. This contrasts with REP-, AP-, and RAPD-PCR, and PCR-RFLP which normally involve larger numbers of bands, and therefore need more complicated data analysis. Furthermore, Selenska-Pobell *et al.* (1995) pointed out that REP-, AP-, and RAPD-PCR were very sensitive to changes in primer, template DNA, nucleotide or *Taq* polymerase concentration. Therefore, the reproducibility of results between laboratories can be variable. It was noted during the initial screening that the selection of primers which could work for all isolates in RAPD-PCR was critical, and that this technique was sensitive to the type of thermocycler used (unpublished data). Furthermore, RAPD-PCR requires several single primers to resolve differences between isolates (Mathan *et al.* 1996; Young and Cheng 1998). In RAPD-PCR, Rep-PCR, and PCR-RFLP, the electrophoretic separation of the DNA fragments on the agarose gel is based on length and molecular weight, therefore fragments of DNA with similar length and weight, regardless of the DNA sequence, migrate to the same distance and thus give similar profiles.

Intraspecific diversity of rhizobia nodulating *A. mangium* and *P. falcataria* revealed by PCR-RFLP-SSCP was low, only 1 genotype was found within *B. elkanii* isolates, and 2 within *B. japonicum* isolates (AMKT2020 was the only one different from other *B. japonicum* isolates). This was expected since the 16S rRNA gene is a relatively conserved gene, so the number of informative sequence differences between closely related isolates is very small (Young 1996; Ludwig *et al.* 1998). If the objective is to reveal the interspecific diversity, a more variable region of DNA should be used, such as the IGS region (Gürtler and Stanisich 1996; Bennasar *et al.* 1998; Scheinert *et al.* 1996), or by combining other methods, such as AP-PCR with SSCP (McClelland *et al.* 1994).

Using PCR-RFLP-SSCP and the sequence data of the 16S rRNA gene, the rhizobia nodulating *A. mangium* were found to be more diverse than those of *P. falcataria*. Three species of rhizobia were able to nodulate *A. mangium* (*B. elkanii*, *B. japonicum*, and *M. loti*), and two species of rhizobia nodulate *P. falcataria* (*B. elkanii* and *B. japonicum*). The figure for *A. mangium* is also higher than that reported for *Acacia albida* from Senegal, which was found to be nodulated only by *B. elkanii* and *B. japonicum* (Dupuy *et al.* 1994), but lower than that of *Acacia saligna* from South-Western Australia, which was nodulated by 4 different species of rhizobia, *B. japonicum*, *B. lupini*, *R. leguminosarum*, and *R. tropici* (Marsudi *et al.* 1999).

In conclusion, the data show the potential of PCR-RFLP-SSCP analysis of the 16S rRNA gene to identify rhizobial isolates to the species level. The results obtained from

this analysis were in full agreement with the sequence data of the 16S rRNA gene of the bacteria. *Bradyrhizobium* spp. that nodulate *A. mangium* and *P. falcataria* are ubiquitous throughout the Indonesian archipelago. Since there was only one isolate obtained from Irian Jaya and from Lampung (both *B. elkanii*-type isolates), it is premature to suggest that *B. japonicum* isolates were absent from these areas. The fact that the rhizobial isolates of *A. mangium* and *P. falcataria* were ubiquitous, and more than one species could be found nodulating these tree species, indicates why this tree species could establish successfully outside its native range without the need for inoculation with indigenous rhizobia. Further investigation to examine the efficacy of these two species of *Bradyrhizobium* to promote plant growth and nitrogen fixation of *A. mangium* and *P. falcataria* is needed to obtained maximum benefit from this symbiosis.

References

Bennasar A, Guasp C, Tesar M, Lalucat J (1998) Genetic relationships among *Pseudomonas stutzeri* strains based on molecular typing methods. *Journal of Applied Microbiology*, **85**, 643-656.

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology*, **84**, 188-198.

Daffonchio D, De Biase A, Rizzi A, Sorlini C (1998) Interspecific, intraspecific and interoperonic variability in the 16S rRNA gene of methanogens revealed by length and single-strand conformation polymorphism analysis. *FEMS Microbiology Letters*, **164**, 403-410.

De Bruijn FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprinting the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology*, **58**, 2180-2187.

De Long EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs free-living marine bacterial assemblages. *Limnology and Oceanography*, **38**, 924-934.

De Oliveira VM, Coutinho HLC, Sobral BWS, Guimarães CT, VanElsas JD, Manfio GP (1999) Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE). *Letters in Applied Microbiology*, **28**, 137-141.

Dooley JJ, Harrison SP (1993) Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of random amplified polymorphic DNA profiles. *Canadian Journal of Microbiology*, **39**, 665-673.

Dupuy N, Willems A, Pot B, Dewettinck D, Vanderbruaene I, Maestrojuan G, Dreyfus B, Kersters K, Collin MD, Gillis M (1994) Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. *International Journal of Systematic Bacteriology*, **44**: 461-473.

Frémont M, Prin Y, Chauviere M, Diem HG, Pwee KH, Tan TK (1999) A comparison of *Bradyrhizobium* strains using molecular, cultural and field studies. *Plant Science*, **141**, 81-91.

Galiana A, Chaumont J, Diem HG, Dommergues YR (1990) Nitrogen-fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp. *Biology and Fertility of Soils*, **9**, 261-267.

Galiana A, Prin Y, Mallet B, Gnahoua G-M, Poitel M, Diem HG (1994) Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium* strains under field condition: long-term effect on plant growth and persistance of the introduced strains in soil. *Applied and Environmental Microbiology*, **60**, 3974-3980.

Gürtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology*, **142**, 3-16.

Hayashi K, Yandell DW (1993) Review Article: How sensitive is PCR-SSCP? *Human Mutation*, **2**, 338-346.

Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen.nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology*, **32**, 136-139.

Khbaya B, Neyra M, Normand P, Zerhari K, Filali-Maltouf A (1998) Genetic diversity and phylogeny of rhizobia that nodulate *Acacia* spp. in Morocco assessed by analysis of rRNA genes. *Applied and Environmental Microbiology*, **64**, 4912-4917.

Kishinevsky BD, Sen D, Yang G (1996) Diversity of rhizobia isolated from various Hedysarum species. *Plant and Soil*, **186**, 21-28.

Kumeda Y, Asao T. 1996. Single-strand conformation polymorphism analysis of PCR-amplified ribosomal DNA internal transcribed spacer to differentiate species of *Aspergillus* section *flavi*. *Applied and Environmental Microbiology*, **62**, 2947-2952.

Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp.nov. *Canadian Journal of Microbiology*, **38**, 501-505.

Lafay B, Burdon JJ (1998) Molecular diversity of rhizobia occuring on native shrubby legumes in Southeastern Australia. *Applied and Environmental Microbiology*, **64**, 3989-3997.

Laguerre G, Allard M-R, Revoy F, Amarger N (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Applied and Environmental Microbiology*, **60**, 56-63.

Laguerre G, Mavingui P, Allard M-R, Charnay M-P, Louvrier P, Mazurier S-I, Rigottier-Gois L, Amarger N (1996) Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology*, **62**, 2029-2036.

Laguerre G, VanBerkum P, Amarger N, Prévost D (1997) Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis*, and *Onobrychis*. *Applied and Environmental Microbiology*, **63**, 4748-4758.

Ludwig W, Amann R, Martinez-Romero E, Schönhuber W, Bauer S, Neef A, Schleifer K-H (1998) rRNA based identification and detection systems for rhizobia and other bacteria. *Plant and Soil*, **204**, 1-19.

Martawijaya A, Kartasujana I, Mandang YI, Prawira SA, Kadir K (1989) Atlas Kayu Indonesia, 2nd ed. Research and Development Agency, The Ministry of Forestry of Indonesia. Bogor, Indonesia.

Mathan N, Parani M, Parida A (1996) Random amplified polymorphic DNA analysis of root-nodulating bacterial strains from Arachis hypogaea with physiological characteristics of both fast and slow growers. *Letters in Applied Microbiology*, **23**, 89-92.

McClelland M, Arensdorf H, Cheng R, Welsh J (1994) Arbitrarily primed PCR fingerprints resolved on SSCP gels. *Nucleic Acids Research*, **22**, 1770-1771.

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA (1993) Characterisation of rhizobia isolated from different divergence groups of tropical leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology*, **16**, 135-146.

Moreira FMS, Haukka K, Young JPW (1998) Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. *Molecular Ecology*, **7**, 889-895.

Marsudi NDS, Glenn AR, Dilworth MJ (1999) Identification and characterisation of fast- and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. *Soil Biology Biochemistry*, **31**, 1229-1238.

Nielsen I, Guinet P, Baretta-Kuipers T (1983) Studies in the Malaysian, Australian and Pacific Ingeae (Leguminosae-Mimosoideae): the genera Archidendropsis, Wallaceo-dendron, Paraserianthes, Pararchidendron and Serianthes (part 2). *Bulletin du Museum National d'Histoire Naturelle, B Adansonia*, **5**, 335-360.

Nour SM, Cleyet-Marel J-C, Beck D, Effosse A, Fernandez MP (1994) Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.). *Canadian Journal of Microbiology*, **40**, 345-354.

Nour SM, Cleyet-Marel J-C, Normand P, Fernandez MP (1995) Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology*, **45**, 640-648.

Nuswantara S, Fujie M, Sukiman HI, Yamashita M, Yamada T (1997) Phylogeny of bacterial symbionts of the leguminous tree *Acacia mangium*. *Journal of Fermentation and Bioengineering*, **84**, 511-518.

Pinyopusarerk K, Liang SB, Gunn BV (1993) Taxonomy, distribution, biology, and use as an exotic. In: *Acacia mangium: growing and utilisation* (eds Awang K, Taylor D), pp. 1-19. Winrock International and the Food and Agriculture Organisastion of the United Nations, Bangkok, Thailand.

Scheinert P, Krausse R, Ullman U, Soller R, Krupp G (1996) Molecular differentiation of bacteria by PCR amplification of the 16S-23S rRNA spacer. *Journal of Microbiological Methods*, **26**, 103-117.

Schwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology*, **64**, 4870-4876.

Selenska-Pobell S, Gigova L, Petrova N (1995) Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. *Journal of Applied Bacteriology*, **79**, 425-431.

Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM (1993) The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics*, **16**, 325-332.

Souvannavong O, Galiana A. (1991). *Acacia mangium-Rhizobium* symbiosis: selection and propagation of the host plant and the microorganism. In: *Research on multipurpose tree species in Asia* (eds Taylor DA, MacDicken KG), pp. 216-222. Winrock International, Bangkok, Thailand.

Teaumroong N, Boonkerd N (1998) Detection of *Bradyrhizobium* spp. and *B. japonicum* in Thailand by primer-based technology and direct DNA extraction. *Plant and Soil*, **204**, 127-134.

Tokue Y, Sugano K, Noda T, saito D, Shimosato Y, Ohkura H, Kakizoe T, Sekiya T (1995) Identification of mycobacteria by non-radioisotopic single-strand conformation polymorphism analysis. *Diagnostic Microbiology and Infectious Disease*, **23**, 129-133.

Turk D, Keyser HH (1992) Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Canadian Journal of Microbiology*, **38**, 451-460.

Umali-Garcia M, Libuit JS, Baggayan RL (1988) Effects of rhizobium inoculation on growth and nodualtion of *Albizia falcataria* (L.) Fosh. and *Acacia mangium* Willd. In the nursery. *Plant and Soil*, **108**, 71-78.

Vinuesa P, Rademaker JLW, DeBruijn FJ, Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Applied and Environmental Microbiology*, **64**, 2096-2104.

Widjojoatmodjo MN, Fluit AC, Verhoef J (1994) Rapid identification of bacteria by PCR single-strand conformation polymorphism. *Journal of Clinical Microbiology*, **32**, 3002-3007.

Woese CR (1987) Bacterial evolution. *Microbiological Reviews*, **51**, 221-271.

Xu LM, Ge C, Cui Z, Li J, Fan H (1995) *Bradyrhizobium liaoningensis* sp.nov. isolated from the root nodules of soybean. *International Journal Systematic Bacteriology*, **45**, 706-711.

Yanagi M, Yamasato K (1993) Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiology Letters*, **107**, 115-120.

Yap EPH, McGee JO'D (1994) Non-isotopic single-strand conformation polymorphism (SSCP) analysis of PCR products. In: *PCR technology: current innovation* (ed Griffin HG), pp. 165-177. CRC Press Inc., Florida.

Young CC, Cheng KT (1998) Genetic diversity of fast- and slow-growing soybean rhizobia determined by random amplified polymorphic DNA analysis. *Biology and Fertility of Soils*, **26**, 254-256.

Young JPW (1996) Phylogeny and taxonomy of rhizobia. *Plant and Soil*, **186**, 45-52.

Young JPW, Haukka KE (1996) Diversity and phylogeny of rhizobia. *New Phytologist*, **133**, 87-94.

IV. Interactions between different species of arbuscular mycorrhizal fungi and rhizobia on the growth of *Acacia mangium* and *Paraserianthes falcataria*

Introduction

Acacia mangium and *Paraserianthes falcataria* are important leguminous tree species which have been planted extensively in Indonesia and other countries for many different purposes. The main use of these tree species in Indonesia is for industrial plantation, afforestation programmes and reclamation of mine sites (Setiadi, 1995; Otsamo *et al.*, 1997). Besides their multipurpose nature, these species, like other leguminous plants, also play important ecological roles in nitrogen (N) input to the soil and also in N-cycling (Umali-Garcia *et al.*, 1988). These trees form symbiotic relationships with N-fixing root-nodule bacteria, rhizobia, which give the tree access to ammonium substrates released by the bacteria. Because of this, the N-rich litters of leguminous trees will eventually improve the N status of the soil under the tree (Tomlinson *et al.*, 1995; Teklehaimanot & Animkwapong, 1996). There have been a few studies conducted on the N-fixation potential of *A. mangium* and *P. falcataria* (Umali-Garcia *et al.*, 1988; Galiana *et al.*, 1990, 1994; Binkley, 1997). These few studies showed that inoculation of *A. mangium* and *P. falcataria* with rhizobia could improve the seedling growth and N status of the trees. Galiana *et al.* (1998) reported that rhizobium inoculation had a positive effect on tree growth up to 39 months after tree planting. However, the capacity to fix N from atmosphere may vary depending on the isolate of rhizobia used (Umali-Garcia *et al.*, 1988; Souvannavong & Galiana, 1991; Frémont *et al.*, 1999).

The amount of nitrogen fixed by leguminous trees has been regarded as being relatively low (Yoneyama *et al.*, 1993), possibly relating to limitation of nutrients other than N (Sprent, 1995). Dommergues (1995) suggested three main approaches to increase N-fixation: clonal selection of trees combined with vegetative propagation; inoculation with effective rhizobium strains; and proper fertilisation, especially phosphorus (P). Giller & Cadisch (1995) noted that co-inoculation of rhizobium with AMF to improve N-fixation needed further study. This is important since AMF play a vital role in enhancing P uptake by plants, especially in tropical acid soils which have a high P-fixing capacity (Dodd *et al.*, 1990; Nurlaeny *et al.*, 1996). They also improve uptake of other nutrients (Vaast &

Zasoski, 1992), increasing tolerance to drought (Goicoechea *et al.*, 1995), and increasing resistance to root pathogens (Liu, 1995; Sharma *et al.*, 1995; Singh & Singh, 1995; Dar *et al.*, 1997) of the host plants. It has also been reported that mycorrhizal plants are more tolerant to salinity (Azcon & El Atrash, 1997) and heavy metal toxicity (Kaldorf *et al.*, 1999) than the non-mycorrhizal counterparts.

The availability of N and P within the plant has consequences on the development of both the mycorrhizal symbiosis and nodulation. For example, nitrogen fixation requires relatively large amounts of ATP. This is essential for breaking the triple bond of N_2 in the reduction reaction to produce NH_3 in N-fixation by rhizobia (Giller & Wilson, 1993). Thus the presence of AMF increases the P concentration in the plant, and thus facilitates an increase in N-fixing capacity. Kawai & Yamamoto (1986) reported that P content of nodules of mycorrhizal plant was generally greater than that of non-mycorrhizal plants. Similarly, the nitrogen demands of the fungus must be satisfied from the soil or from the host plant, either directly or indirectly. There is relatively little information regarding the role of N availability in regulating the development of the AM symbiosis. The tripartite symbiosis would increase N availability in the plant, thus enabling greater development of the fungal mycelium via increased carbon allocation from the plant photosynthate. Nodulation and mycorrhiza development can also be inhibited if N or P is in excess (Dommergues, 1995). However, tree legumes are usually planted in nutrient-poor soils in the tropics.

The tripartite symbioses between AMF, rhizobia and leguminous plants give a unique mechanism for the host plants to obtain adequate nutrition. As tree litters are mineralised, the amount of available N in the soil increases with time, this process leads to the cessation of N fixation (Dommergues, 1995). At this point, the mycorrhizal-leguminous trees will harvest the mineralised-N via the mycorrhiza. The main effect of co-inoculating AMF and rhizobium is to enhance N-fixation of the legumes by improved plant-P status (Dodd *et al.*, 1990; Azcon & Barea, 1992). The effect of inoculation on tropical forage legumes with AMF was more pronounced when the soil-P levels were low (Arias *et al.*, 1991). The ability of AMF to transport mineral N from soil to the host plant is also important as demonstrated by Johansen *et al.* (1993) using ^{15}N isotope-labelled ammonium and nitrate. Azcon-Aguilar *et al.* (1993) also found that mycorrhizal plants could use soil N sources, which were less available to non-mycorrhizal plants. The hyphal networks developed by AMF have also been reported to facilitate the transfer of symbiotically fixed-N from N-fixing to non-N-fixing plants (Hamel *et al.*, 1991; Frey &

Schuepp, 1992; Martensson *et al.*, 1998). These studies mainly focused on agriculturally-important food and forage crops, such as *Medicago* spp. (Azcon & Barea, 1992), *Stylosanthes capitata* and *Pueraria phaseoloides* (Arias *et al.*, 1991), and *Arachis hypogaea* (Khan *et al.*, 1995). In contrast, few studies have been conducted to explore the potential of the symbioses on leguminous tree species.

Results of studies on interactions between tree legumes and inoculation with their symbiotic partners have been variable to date. Stamford *et al.* (1997) studied *Mimosa caesalpiniaefolia* and found that co-inoculation of *Bradyrhizobium* sp. with AMF did not alter plant growth and N-fixation compared with inoculation with *Bradyrhizobium* sp. alone. This might be due to the incompatibility of the microsymbionts. Dela Cruz *et al.* (1988) found variation in the degree of compatibility between the two microsymbionts.

Studies of the tripartite symbiosis have usually only involved a limited number of AMF species and rhizobial isolates, e.g. one or two isolates of AMF and rhizobium (Ruiz-Lozano & Azcon, 1993; Khan *et al.*, 1995) or a single rhizobial isolate combined with several AMF species (Dela Cruz *et al.*, 1988; Ianson & Linderman, 1993). Azcon *et al.* (1991) used a combination of isolates of three species of AMF (*Glomus mosseae*, *Glomus fasciculatum* and *Glomus caledonium*) and six strains of *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) and demonstrated selective and specific compatibility between species and strains of the two types of microsymbiont, and also between the microsymbionts and the common host plant *Medicago sativa*. In the latter study, all of the AMF studied were *Glomus* species. However, in tropical soils AMF from all genera are found but there has been no attempt to investigate these fungi in previous studies.

The objectives of this study were: to investigate the interaction between three different AMF species (from unique genera) with different rhizobial isolates from Indonesia isolated from the tropical leguminous trees *A. mangium* and *P. falcataria*; to examine the effects of the interaction on plant growth, plant nutrient status (N and P), and the N-fixation by these two tree seedlings; and finally, to select the best combination of AMF and rhizobial isolates for the growth of seedlings of *A. mangium* and *P. falcataria* in Indonesia. In this study, unlike other previous studies, AMF spores were pre-germinated prior to inoculation to give all AMF an equal opportunity to colonise roots of the tree seedlings. This is important since delays in colonisation by AMF are often the result of dormancy of spores in the genera *Acaulospora* and *Scutellospora* (Boddington & Dodd, 1999).

Materials and Methods

Mycorrhizal species and rhizobial isolates

The AMF and rhizobial isolates used in this study are listed in Table 1. The same AMF species were used in the experiment with *A. mangium* and *P. falcataria*, but different rhizobial isolates were used for these plant species depending on the origin of the host of the isolates. All of the rhizobial isolates were isolated from Indonesia and were previously tested for their ability to nodulate their respective host (see Chapter 2). These isolates were identified, using PCR/RFLP/SSCP of their 16S rRNA gene (see Chapter 3), as either *Bradyrhizobium elkanii* or *Bradyrhizobium japonicum* (Table 1).

Media preparation

Terragreen, an inert attapulgite clay (Agsorb 8/16, Oil Dri, Wisbech, Cambs, UK) was washed with tap water then soaked 14 h in 5 mM MES (2-[N-Morpholino] ethanol-sulfonic acid) buffer solution (Vilariño *et al.*, 1997). The Terragreen was autoclaved at 121°C, for 20 min and added to 7.6 cm width x 6.8 cm height pots (120 g Terragreen pot^{-1}). Rock phosphate from Indonesia (13.9% P) was sterilised by autoclaving and added to each pot at the rate of 20 mg P kg^{-1} media, and mixed in thoroughly.

Seed surface sterilisation and germination

Seeds of *A. mangium* and *P. falcataria* were surface-sterilised by immersing the seeds in 95% (v/v) ethanol for 10 sec and then soaked in 3% (v/v) H_2O_2 for 5 min . Finally, the seeds were rinsed three times with sterile distilled water (dH_2O). To break dormancy, the seeds were soaked in sterile boiling water for 5 min and then in sterile cold dH_2O overnight. The seeds were germinated on fine Terragreen (1-2 mm diam. particles) in 1000-ml glass beakers covered with the lid of 18 cm diam. Petri dishes so that the germinating seeds could obtain sufficient light. The beakers were then kept in a Fitotron (Gallenkamp, UK) growth chamber, simulating tropical conditions (30°C, at 300 µmol m^{-2} sec^{-2} and 12/12 hr light/dark, RH 60%). Seven days after sowing the seedlings were ready for transplantation.

Table 1. Isolates of AMF and rhizobia used in this study.

Microsymbionts	**Host plant**	***B. elkanii/ B. japonicum***
Isolates of AMF		
Gigaspora rosea BEG111		
Glomus manihotis BEG112		
Scutellospora heterogama BEG40		
Rhizobial isolates		
PFAG5030	*P. falcataria*	*B. japonicum*
PFAG5040	*P. falcataria*	*B. japonicum*
PFAG5070	*P. falcataria*	*B. japonicum*
PFAG6030	*P. falcataria*	*B. japonicum*
PFAG6040	*P. falcataria*	*B. japonicum*
PFIR3040	*P. falcataria*	*B. elkanii*
PFJB1040	*P. falcataria*	*B. elkanii*
AMAG3010	*A. mangium*	*B. japonicum*
AMBG2030	*A. mangium*	*B. japonicum*
AMJB1010	*A. mangium*	*B. elkanii*
AMJB1020	*A. mangium*	*B. elkanii*
AMJB3010	*A. mangium*	*B. japonicum*

Spore extraction and surface sterilisation

Spores of *G. manihotis* (BEG112), *Gi. rosea* (BEG111), and *Sc. heterogama* (BEG40) were extracted from pot-cultures by sieving using a 500 µm mesh sieve placed on top of a 63 µm mesh sieve. The contents of the 63 µm sieve were subjected to sucrose gradient centrifugation (http://wwwbio.ukc.ac.uk/beg), washed and placed in 1.5 ml sterile microcentrifuge tubes. They were centrifuged for 2 min at 13 000 g using a microcentrifuge (Model 1-15, Sigma, Germany) to pellet the spores and remove excess water. One ml of 5% (v/v) commercial chloros (Hays Chemical Distribution Ltd. containing >5% and <16% available chlorine) was pipetted into the tubes and left for 2 min . The tubes were then centrifuged to pellet the spores and the sterilant was removed. Spores were washed five times with sterile dH_2O, then placed on sterile nitrocellulose papers, pore size 0.20 µm, 1 cm diam. (Whatman International Ltd., Maidstone, England). Spores were germinated on nitrocellulose papers [Brundrett & Juniper (1995) with some modification] in 18 cm diam. Petri dishes half-full with moist sterile fine terragreen (1-2 mm diam. particles), 5 spores per nitrocellulose paper and 150 nitrocellulose papers for each AMF species. The spores were then incubated at 30°C in the dark for 15-20 days. Only nitrocellulose papers containing 3-5 germinated spores with no apparent bacterial or/and non-AMF fungal contamination were used as inoculum.

Rhizobial inoculum

All rhizobial isolates were grown separately in 100 ml flasks containing 30 ml yeast extract mannitol broth (YMB) (Vincent 1970). The cultures were incubated shaken at 30°C for 7 days, and used directly as inoculum. One flask of uninoculated YMB was included and used to inoculate the control treatment.

Plant inoculation

The tap root of the seedling was put on the nitrocellulose paper as close as possible to the hyphal tip of the germinating spores. The seedling and nitrocellulose paper was then planted in the prepared pot (Fig. 1). At the same time, 1 ml of rhizobial inoculum containing 10^9 cells ml^{-1} was placed at the base of the seedlings and allowed to soak into the substrate. The pots were kept in the greenhouse simulating tropical conditions (min.

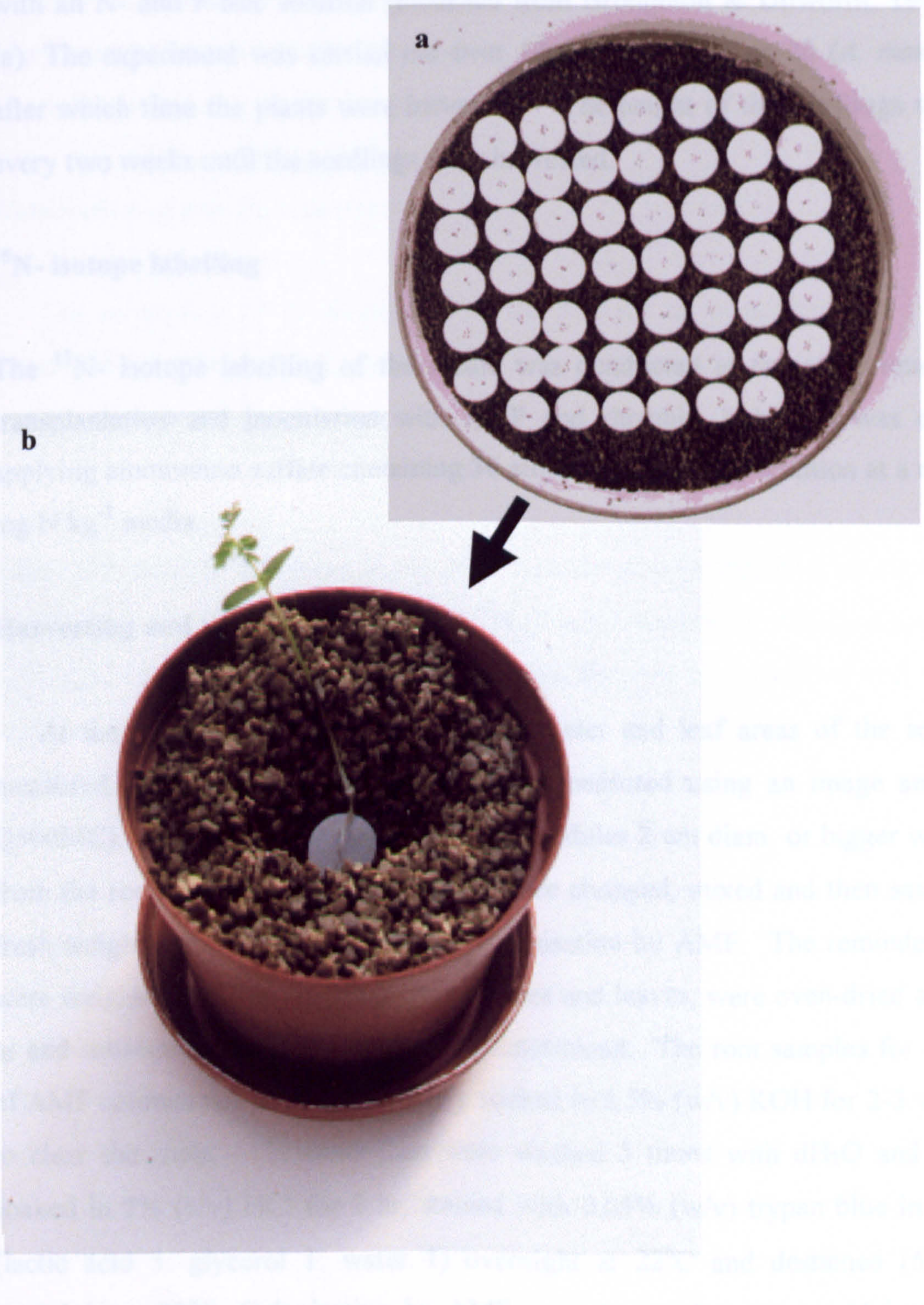


Figure 1. Inoculation of pre-germinated spores (*Sc. heterogama* BEG40) on a nitrocellulose paper (a) onto a *P. falcataria* seedling (b). Five spores were germinated on each nitrocellulose paper. The tip of the root was placed as close as possible to the germinated spores to facilitate contact between the fungus and the root to promote colonisation.

15°C/max 40°C, relative humidity 60-80%, light intensity 400-600 µmol $m^{-2}s^{-1}$). The seedlings were watered as needed using sterile deionised water (dH_2O) and fed fortnightly with an N- and P-free solution (modified from Broughton & Dilworth, 1971; Appendix Ia). The experiment was carried out over 17 (*P. falcataria*) or 20 (*A. mangium*) weeks after which time the plants were harvested. The height of the seedlings was measured every two weeks until the seedlings were harvested.

^{15}N- isotope labelling

The ^{15}N- isotope labelling of the media was conducted at the same time as seedling transplantation and inoculation with AMF and rhizobia. Labelling was conducted by applying ammonium sulfate containing 30 atom%^{15}N excess in solution at a rate equal to 2 mg N kg^{-1} media.

Harvesting and sample analyses

At the time of harvesting, the stem diameter and leaf areas of the seedlings were measured. Leaf areas of the seedlings were measured using an image analyser (Leica Q500MC). Root nodules were counted and nodules 2 cm diam. or bigger were separated from the roots. For each seedling, roots were chopped, mixed and then sampled (0.15 g fresh weight) to measure the extent of colonisation by AMF. The reminder of the roots were weighed and then, together with nodules and leaves, were oven-dried at 60°C for 48 hr and subsequently the dry weight was determined. The root samples for determination of AMF colonisation were immediately soaked in 2.5% (w/v) KOH for 2-3 weeks at 22°C to clear the roots. The roots then were washed 5 times with dH_2O and subsequently soaked in 2% (v/v) HCl for 2 hr, stained with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid 5: glycerol 1: water 1) overnight at 22°C and destained (50% glycerol) overnight at 22°C. Colonisation by AMF was measured using a grid intersect method (Giovannetti & Mosse, 1978). The grid intersect method was also used to estimate the total root lengths.

To determine the total N-concentration and N-fixation, the oven-dried shoot and root material were sampled proportionally, mixed and ground to pass through a 1 mm mesh sieve. The N content and ^{15}N enrichment of the material was determined using a CN analyser coupled to a mass-spectrometer (Cadisch *et al.* 1998). The same plant sample

were used to determine the P content of the plant tissue (Habte & Manjunath, 1991). Samples for P analyses were prepared by dry-ashing and acid digestion of the ground plant material. The P content of the plant tissues was assessed using the molybdate method (Anderson & Ingram 1986) and measuring absorbance at 880 nm (UV/VIS Perkin-Elmer, Lambda 1, UK).

Calculation of nitrogen fixation

The proportion of N obtained from the fixation of atmospheric N (% Ndfa) was calculated according to Goh *et al.* (1996) using the formula:

$$\% \text{ Ndfa} = (1 - c/a) \times 100$$

where c is atom%^{15}N excess in the plant inoculated with rhizobial bacteria singly or in combination with AMF species (atom%^{15}N rhizobial legume - 0.3663), and a is atom%^{15}N excess in the plant uninoculated with rhizobial bacteria or inoculated with AMF species only (atom%^{15}N non-rhizobial legume - 0.3663).

Total amount of N derived from atmosphere (Ndfa, mg) = (% Ndfa/100) x plant total N.

Experimental design

The study was carried out using a factorial design in randomised blocks. Two separate experimental designs were set up for *A. mangium* and *P. falcataria*. The experiments comprised 2 main factors (AMF and rhizobial treatments) replicated in 4 blocks. The mycorrhizal treatments were the same for both *A. mangium* and *P. falacataria*: no-AMF, *G. manihotis* BEG112, *Gi. rosea* BEG111, and *Sc. heterogama* BEG40. Six rhizobial treatments were used for *A. mangium* (non-rhizobial, AMAG3010, AMJB1010, AMJB1020, AMJB3010, and AMBG2030), and eight rhizobial treatments for *P. falcataria* (non-rhizobial, PFAG5070, PFAG5040, PFAG6040, PFIR3040, PFAG6030, PFJB1040, and PFAG5030).

Table 2. List of parameters of *A. mangium* and *P. falcataria* included for the cluster analysis of the combination treatments. The same parameters were used for both tree species unless stated.

No.	Parameters
1.	Total biomass
2.	Shoot dry weight
3.	Root dry weight
4.	Root length
5.	Leaf area
6.	Leaflet number (for *P. falcataria* only)
7.	Shoot/root ratio
8.	Height at final harvest
9.	Stem diameter
10.	% total P
11.	Total P content
12.	Shoot P content
13.	Root P content
14.	% Total N
15.	Total N content
16.	Shoot N content
17.	Root N content
18.	% Ndfa
19.	Total amount of Ndfa
20.	Shoot Ndfa
21.	Root Ndfa
22.	Nodule number (diam.< 1 mm) (for *A. mangium* only)
23.	Nodule number (diam. 1-2 mm) (for *A. mangium* only)
24.	Nodule number (diam.> 2 mm)
25.	Total nodule number
26.	Nodule dry weight
27.	% root length colonisation by AMF
28.	Actual root length colonisation by AMF

Statistical analyses

Data were analysed using analysis of variance (ANOVA) on Minitab version 11 for windows. When the F-value of the ANOVA was significant ($p<0.05$), treatment means were separated using the Least Significant Difference (LSD, $p= 0.05$). Cluster analyses based on 27 (*A. mangium*) and 26 (*P. falcataria*) parameters (Table 2) for the combination treatments were also performed using the Ward linkage method in the same statistical package.

Results

Paraserianthes falcataria

Seedling height and stem diameter

Significant ($p<0.05$) interactions between AMF and rhizobial treatments on the height of *P. falcataria* seedlings were noted at the two and thirteen week samplings. The effects were not significant ($p=0.06$) at the final sampling (week seventeen). At all other samplings there were no significant interactions between AMF and rhizobia. Figure 2 shows the effects of the interaction treatments over seventeen weeks. The combination of *Sc. heterogama* BEG40+PFAG5070 produced significantly smaller plants (Fig. 2a) than the no-AMF+PFAG5070 pairing after two weeks, but these became significantly larger than the latter combination thirteen weeks after inoculation (Fig. 2b). The significant effect on height was maintained until seventeen weeks after inoculation (Fig. 2c).

When interactions between AMF and rhizobia were not significant, main effects of AMF or rhizobia were significant. Inoculation with *Sc. heterogama* BEG40 significantly increased the height of the seedlings compared with non-inoculated seedlings and the pronounced increase in growth was noted after seven weeks and continued through to final sampling (Fig. 3). There was no significant effect on the height of plants inoculated with *G. manihotis* BEG112 or *Gi. rosea* BEG111 compared with non-mycorrhizal plants at any sampling. Inoculation with *Sc. heterogama* BEG40 also significantly increased stem diameter of seedlings at final harvest (Table 3).

Rhizobial isolates generally increased the height of seedlings, except for PFIR3040 and PFAG5030, which resulted in plants which were not significantly different from the non-rhizobial controls (Fig. 4). Thirteen weeks after inoculation, seedlings inoculated with rhizobia tended to grow better than the uninoculated counterpart, and by seventeen weeks after inoculation only isolate PFIR3040 did not significantly increase plant height compared with the non-rhizobial controls. Seventeen weeks after inoculation isolate PFJB1040 produced the tallest plants (Fig. 4). All rhizobial isolates significantly increased stem diameter of seedlings compared with the non-rhizobial controls (Table 3).

Shoot and root biomass, leaf areas and root length

Dual inoculation with AMF and rhizobia significantly increased the shoot biomass, leaf areas, shoot N and total N contents, shoot Ndfa (N derived from atmosphere) and total Ndfa, and shoot P contents of *P. falcataria* seedlings seventeen weeks after inoculation. Rhizobial isolates PFAG5070, PFAG5040, and PFJB1040 increased shoot biomass and leaf areas of seedlings when co-inoculated with *Sc. heterogama* BEG40, *Gi. rosea* BEG111, and *G. manihotis* BEG112, respectively, compared with no-AMF+non-rhizobial, or the corresponding AMF isolate+non-rhizobial, and to a lesser extent with the corresponding rhizobium isolate+no-AMF (Fig. 5). Co-inoculation of isolate PFJB1040+*G. manihotis* BEG112 increased seedling shoot biomass by 402%, 617%, and 31% compared with inoculation with non-rhizobial+no-AMF, *G. manihotis* BEG112 alone, or PFJB1040 alone, respectively (Fig. 5a). This trend was also similar for leaf areas where the same treatment increased the leaf areas of the seedlings by 437%, 747%, and 47% compared with the latter three treatments, respectively (Fig. 5b). Co-inoculation with AMF did not significantly increase the shoot biomass and leaf areas of seedlings inoculated with rhizobial isolates PFIR3040, PFAG6030, or PFAG5030 at final harvest compared with those inoculated with the corresponding rhizobial isolate alone. Interestingly, all AMF isolates increased the shoot biomass and leaf areas of seedlings when co-inoculated with isolate PFAG6040. However, only co-inoculation with *G. manihotis* BEG112 or *Gi. rosea* BEG111 significantly increased the shoot biomass (Fig. 5a), and only *G. manihotis* BEG112 significantly increased leaf areas compared with inoculation with isolate PFAG6040 alone (Fig. 5b). Figure 6 shows a comparison of leaf area and colour of seedlings of *P. falcataria* inoculated with combination of *G. manihotis*

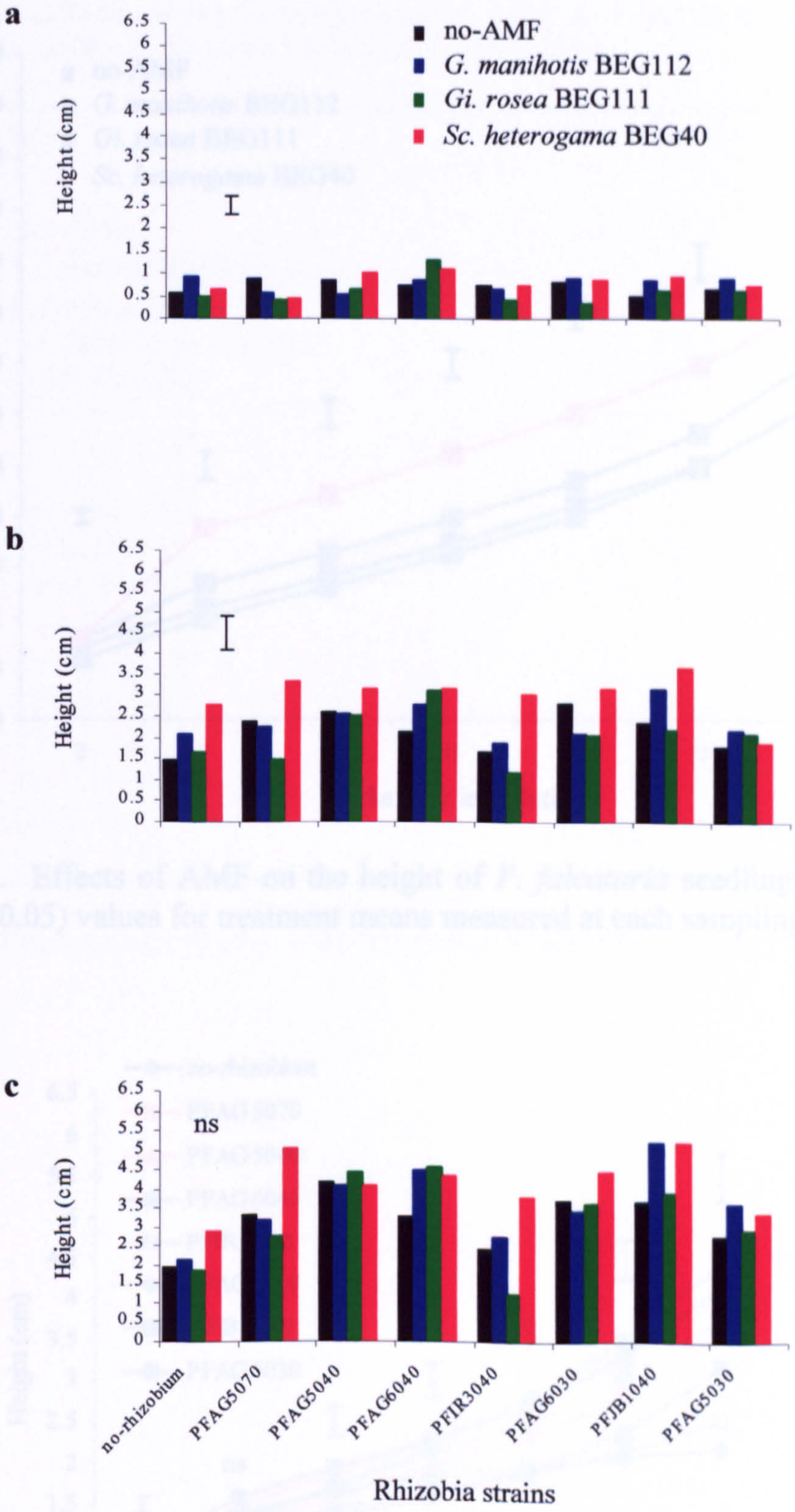


Figure 2. The effects of interaction of three AMF and rhizobia on the height of *P. falcataria*. The three graphs show the height at two weeks (a) thirteen weeks (b) and seventeen weeks (c) samplings. Bars are the LSD (p=0.05) values for the means of combination treatments. ns= the F-value was not significant ($p \geq 0.05$).

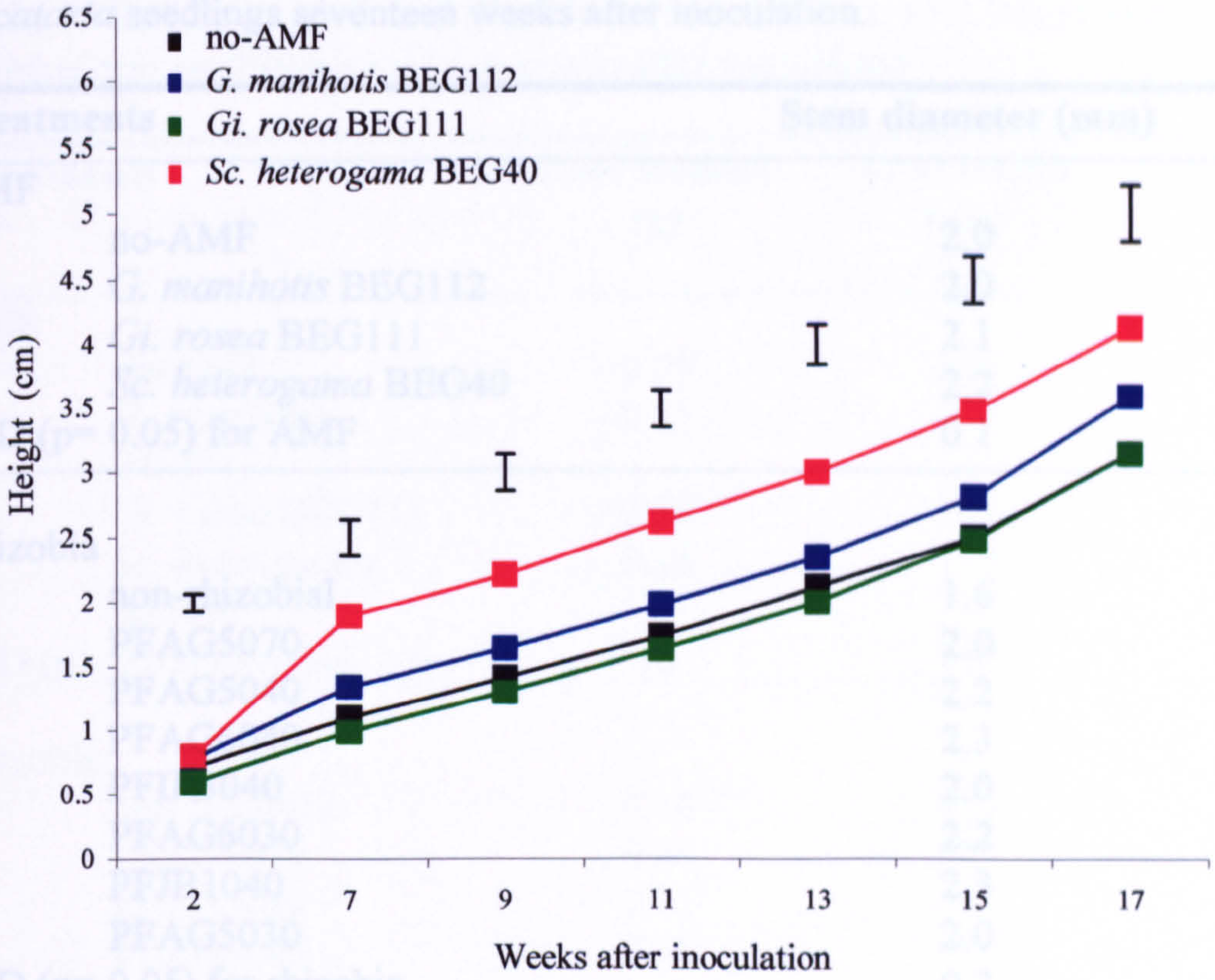


Figure 3. Effects of AMF on the height of *P. falcataria* seedlings over time. Bars are LSD (p=0.05) values for treatment means measured at each sampling.

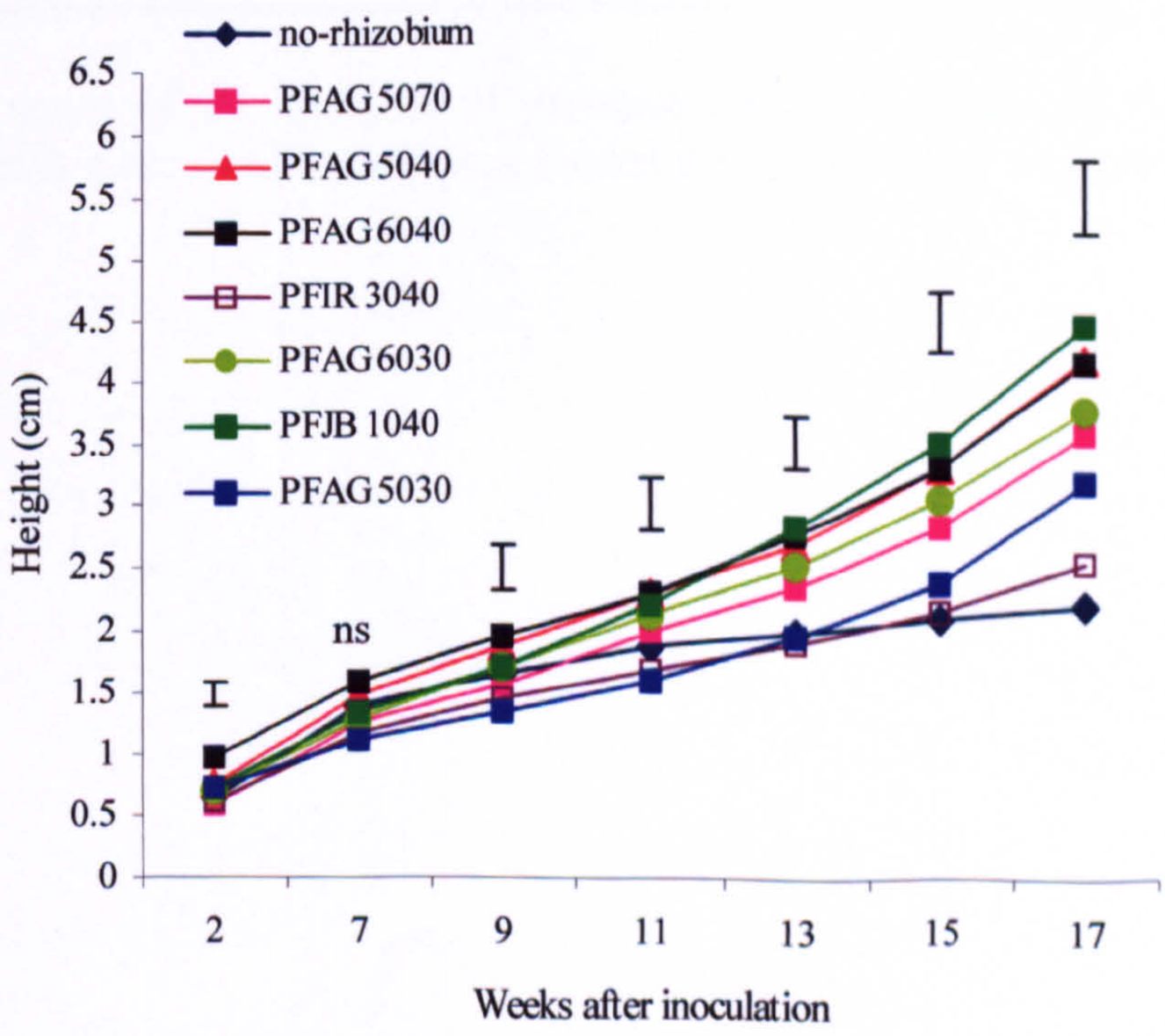


Figure 4. Effects of seven rhizobial isolates and an unioculated control on the height of *P. falcataria* seedlings over time. Bars are LSD (p= 0.05) values for treatment means measured at each sampling. ns= the F-value was not significant ($p \geq 0.05$).

Table 3. Effects of the main treatments, AMF and rhizobia, on stem diameter of *P. falcataria* seedlings seventeen weeks after inoculation.

Treatments	Stem diameter (mm)
AMF	
no-AMF	2.0
G. manihotis BEG112	2.0
Gi. rosea BEG111	2.1
Sc. heterogama BEG40	2.2
LSD (p= 0.05) for AMF	0.1
Rhizobia	
non-rhizobial	1.6
PFAG5070	2.0
PFAG5040	2.2
PFAG6040	2.3
PFIR3040	2.0
PFAG6030	2.2
PFJB1040	2.3
PFAG5030	2.0
LSD (p= 0.05) for rhizobia	0.2

The LSD values were used to compare means within the same treatment.

Table 4. Effects of AMF and rhizobia on root biomass, root length and shoot/root ratio of *P. falcataria* seedlings seventeen weeks after inoculation.

Treatments	Root biomass (g)	Root length (cm)	Shoot/root ratio
AMF			
no-AMF	0.25	664	2.7
G. manihotis BEG112	0.25	540	2.7
Gi. rosea BEG111	0.28	740	2.7
Sc. heterogama BEG40	0.28	716	2.7
LSD (p= 0.05) for AMF	ns	110	ns
Rhizobia			
non-rhizobial	0.18	450	1.4
PFAG5070	0.24	681	3.0
PFAG5040	0.31	663	2.9
PFAG6040	0.30	784	3.0
PFIR3040	0.25	636	2.1
PFAG6030	0.28	773	2.9
PFJB1040	0.32	728	3.5
PFAG5030	0.24	606	2.7
LSD (p= 0.05) for rhizobia	0.05	155	0.4

ns= the result of the analysis of variance (ANOVA) was not significant ($p \geq 0.05$). The LSD values were used to compare means within the same treatment.

BEG112+PFJB1040 with that receiving the no-AMF+non-rhizobial treatment. The leaves of the inoculated plants were much bigger and greener, whilst the controls were chlorotic. There was a significant effect of AMF on the root length of seedlings, but not root biomass and shoot/root ratio seventeen weeks after inoculation. *Glomus manihotis* BEG112 significantly reduced the root length of seedlings compared with the no-AMF controls, *Gi. rosea* BEG111, and *Sc. heterogama* BEG40 (Table 4). In contrast, rhizobial isolates significantly increased root biomass, root length, and the shoot/root ratio of *P. falcataria* seedlings seventeen weeks after inoculation (Table 4). Isolate PFJB1040 produced seedlings with a significantly higher shoot/root ratios compared with other rhizobial isolates. However, the greatest root length was obtained when seedlings were inoculated with isolate PFAG6040.

AMF colonisation and root nodule formation

Figure 7 shows the nodules formed by rhizobial isolates and root colonisation by AMF indicated by the presence of the extra-radical mycelium (ERM) and spores on the root of *P. falcataria* seedlings. Spores often found on the nodules. The root colonisation by AMF was confirmed by staining the roots with trypan blue, but no AMF colonisation was found in the nodules. The percentage of root length colonised by AMF and the actual length of colonised root were significantly affected by the AMF treatments alone. The % root length colonised by AMF and actual length of colonised root were greatest in the order *G. manihotis* BEG112>*Gi. rosea* BEG111>*Sc. heterogama* BEG40 (Fig. 8).

Generally, big nodules with diam. > 2 mm were formed by all isolates of rhizobia (Fig. 9a). These nodules were actively fixing N from atmosphere as indicated by the red colour of the leghaemoglobin when the nodules were cut open (Fig. 9a). Interestingly, nodules were also found in the roots of seedlings that were not inoculated with rhizobium, which might have been caused by contamination. However, these nodules were mostly small in diameter (< 2 mm) and inactive, not red inside (Fig. 9b). Nodule numbers and nodule dry weight were significantly ($p<0.05$) affected by rhizobial treatment only. Isolate PFJB1040 produced the heaviest nodules and nodule numbers with diam. bigger than 2 mm (Table 5). In contrast, isolate PFIR3040 produced nodules with the lowest dry weights and the smallest number of nodules with diam. bigger than 2 mm.

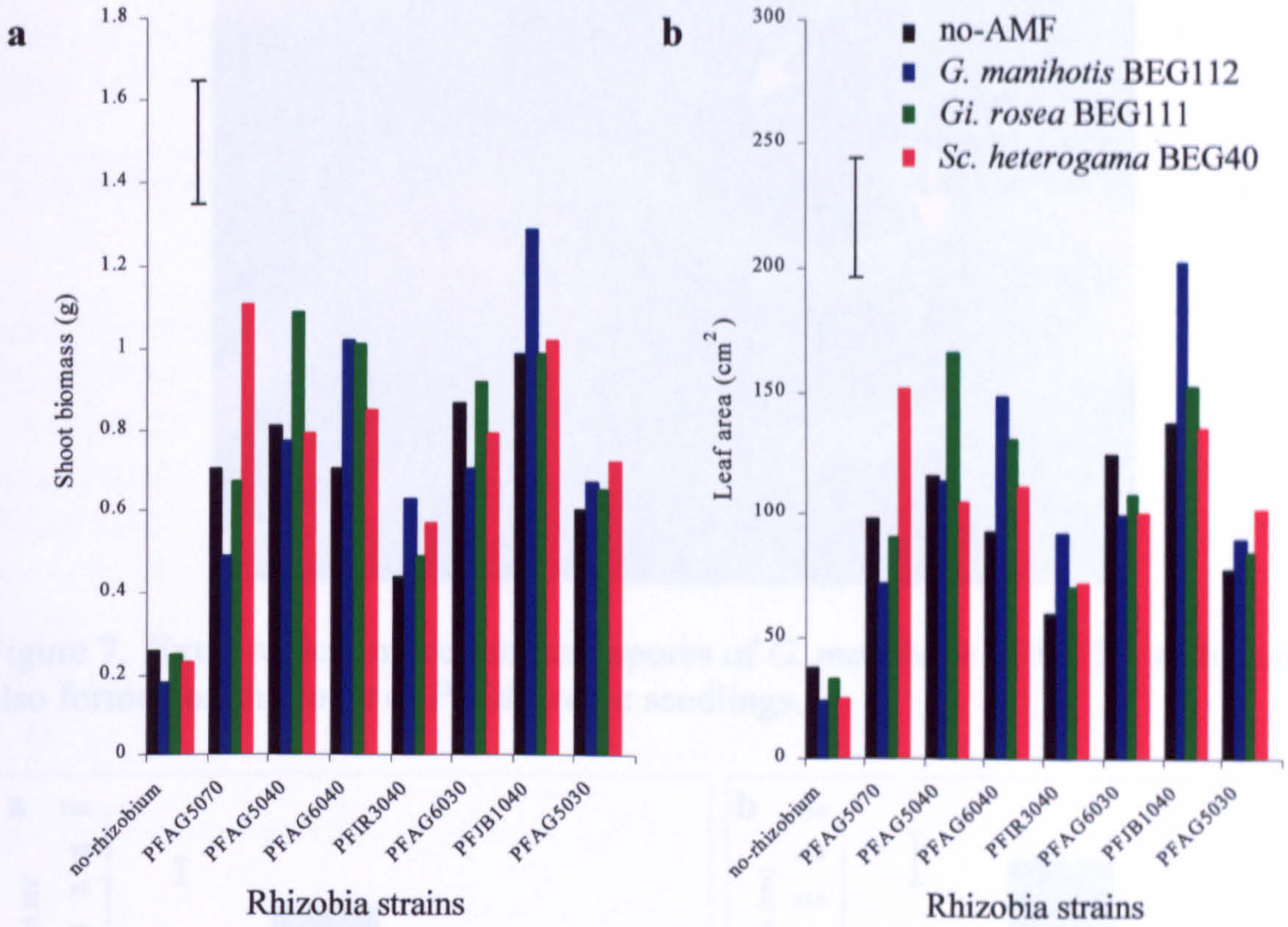


Figure 5. Effects of interaction between AMF and rhizobia on shoot biomass (a), and leaf areas (b) of *P. falcataria* at seventeen weeks sampling. Bars are the the LSD (p=0.05) values for the means of combination treatments.

Figure 6. The leaf area and colour of a *P. falcataria* seedling inoculated with the combination treatment of *G. manihotis* BEG112+PFJB1040 (top) compared with a control seedling uninoculated with either AMF or rhizobial isolate (bottom).

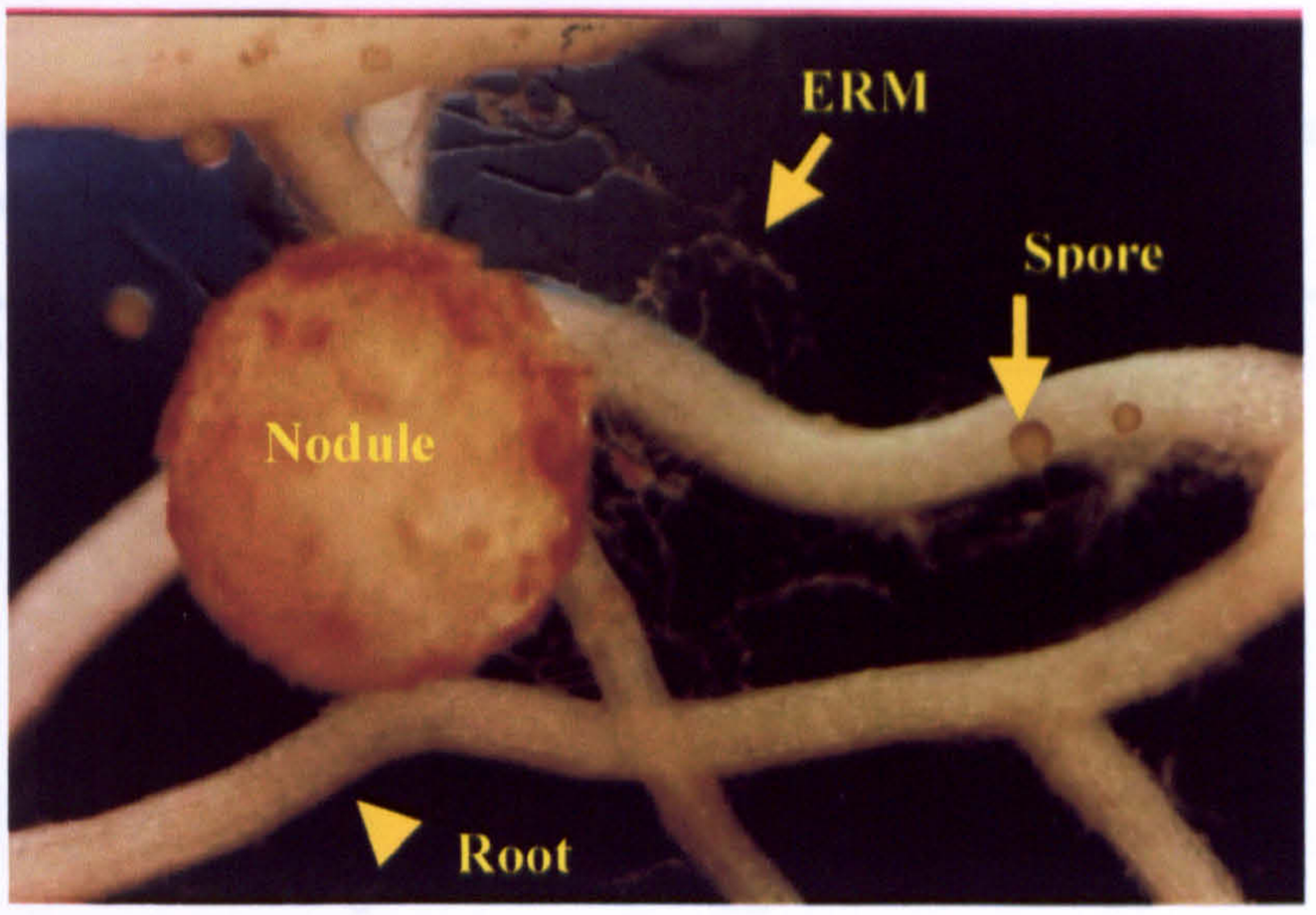


Figure 7. Extra-radical mycelium and spores of *G. manihotis* BEG112, with root nodules also formed on the roots of *P. falcataria* seedlings.

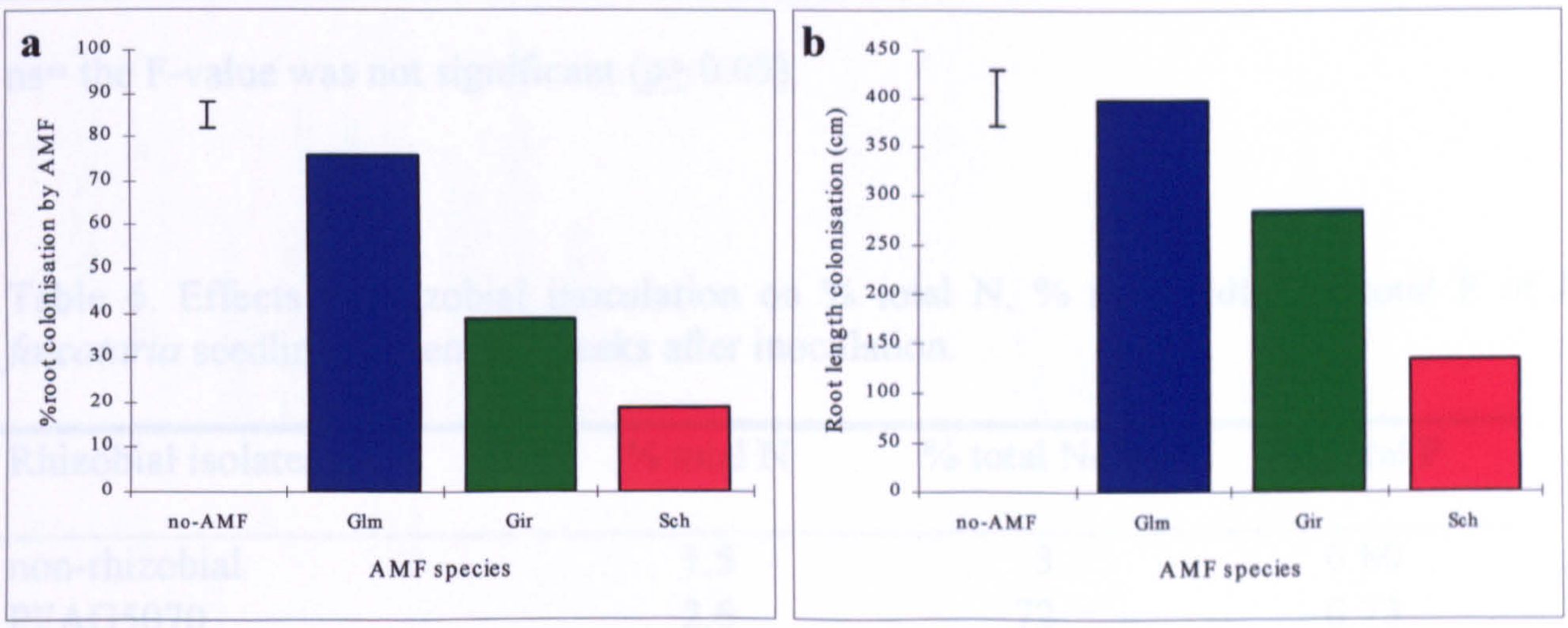


Figure 8. Effects of *G. manihotis* BEG112 (Glm), *Gi. rosea* BEG111 (Gir), *Sc. heterogama* BEG40 (Sch), and no-AMF control on (a) % root colonisation by AMF and (b) actual root length colonised by AMF of *P. falcataria* seedlings at seventeen weeks sampling. Bars are the LSD (p=0.05) values for treatment means.

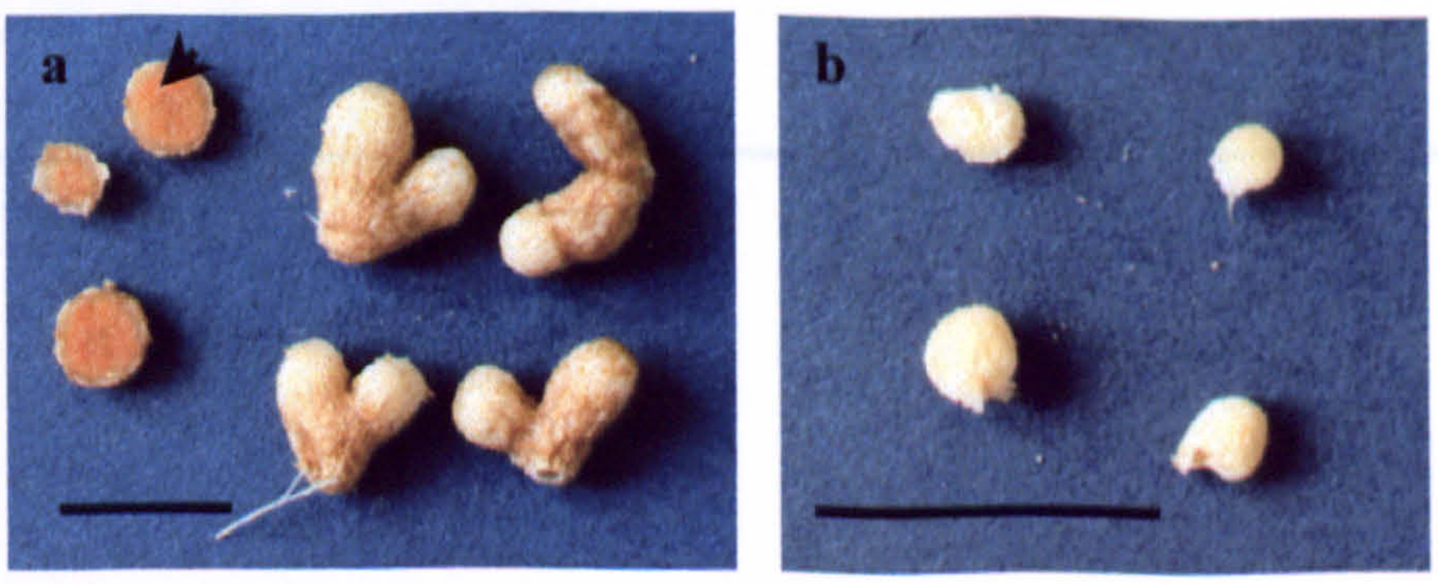


Figure 9. Nodules with indeterminate growth formed by rhizobial isolates inoculated on *P. falcataria* seedlings (a), compared with nodules formed on the uninoculated control seedlings (b). Active nodules were indicated by the red colour of the leghaemoglobin when the nodules were cut open (arrow). Bars= 1 cm.

Table 5. Effects of rhizobia on nodule numbers and nodule dry-weight from *P. falcataria* seedlings seventeen weeks after inoculation.

Rhizobial Isolate	Nodule numbers according to diameter			Nodule dry weight (mg)
	2 < mm	> 2 mm	Total	
non-rhizobial	13	2	15	10
PFAG5070	13	20	33	97
PFAG5040	10	23	33	104
PFAG6040	13	24	37	103
PFIR3040	6	11	17	89
PFAG6030	8	22	30	93
PFJB1040	9	27	36	120
PFAG5030	15	17	33	78
LSD (p=0.05)	ns	6	10	23

ns= the F-value was not significant ($p \geq 0.05$).

Table 6. Effects of rhizobial inoculation on % total N, % total Ndfa, % total P of *P. falcataria* seedlings seventeen weeks after inoculation.

Rhizobial isolates	% total N	% total Ndfa	% total P
non-rhizobial	1.5	3	0.80
PFAG5070	2.6	72	0.73
PFAG5040	2.6	81	0.71
PFAG6040	2.7	82	0.71
PFIR3040	2.2	62	0.72
PFAG6030	2.8	81	0.70
PFJB1040	2.6	87	0.70
PFAG5030	2.6	76	0.72
LSD (p=0.05)	0.3	5	0.06

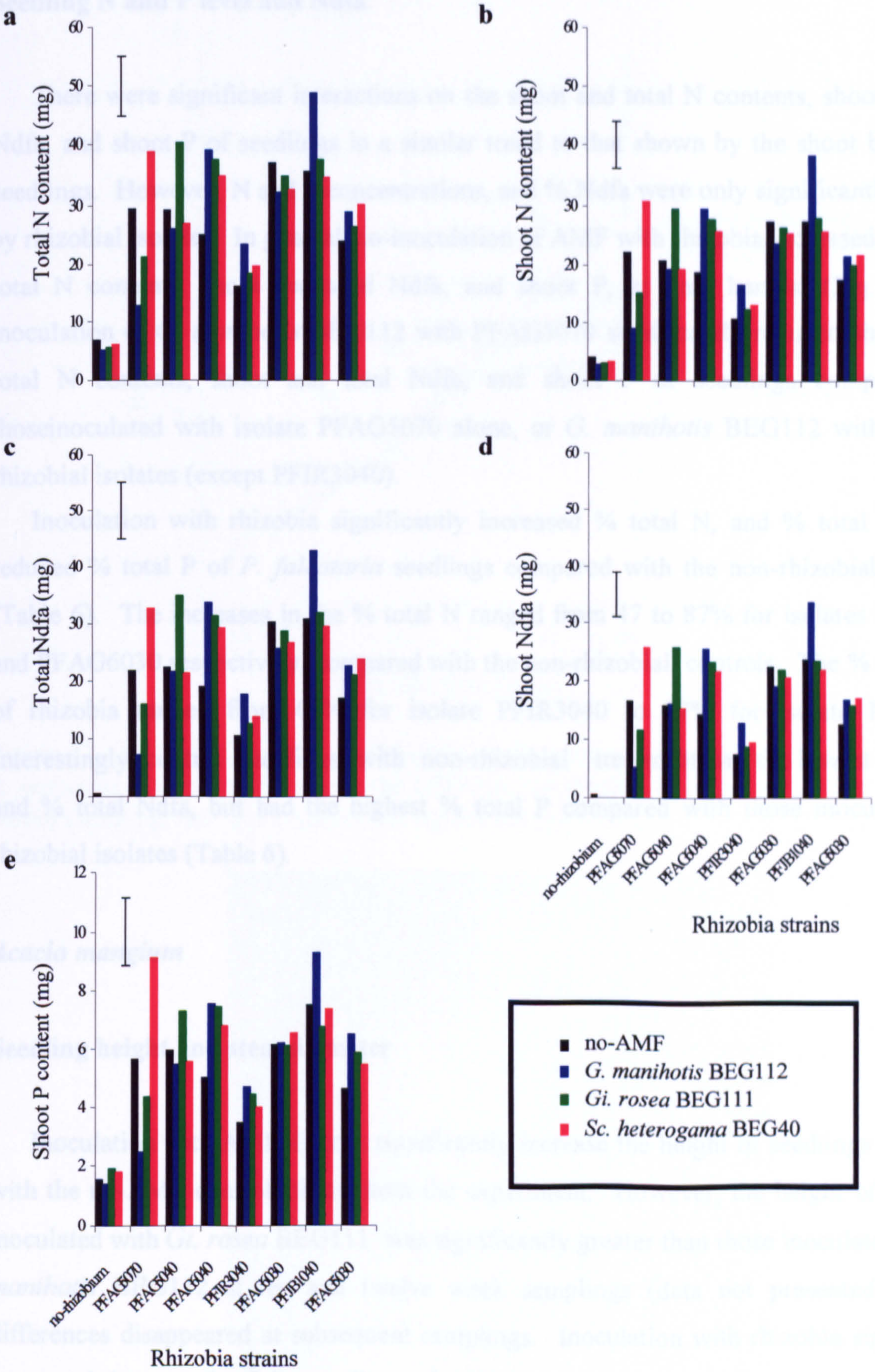


Figure 10. Effects of the interaction between AMF and rhizobia on total N content (a), shoot N content (b), total Ndfa (c), shoot Ndfa, and shoot P content (e) of *P. falcataria* seedlings seventeen weeks after inoculation. Bars are the LSD (p=0.05) values for the means of combination treatment.

Seedling N and P level and Ndfa

There were significant interactions on the shoot and total N contents, shoot and total Ndfa, and shoot P of seedlings in a similar trend to that shown by the shoot biomass of seedlings. However, N and P concentrations, and % Ndfa were only significantly affected by rhizobial isolates. In general, co-inoculation of AMF with rhizobia increased shoot and total N contents, shoot and total Ndfa, and shoot P, at final harvest (Fig. 10). Co-inoculation of *G. manihotis* BEG112 with PFAG5070 significantly reduced shoot N and total N contents, shoot and total Ndfa, and shoot P of seedlings compared with thoseinoculated with isolate PFAG5070 alone, or *G. manihotis* BEG112 with all other rhizobial isolates (except PFIR3040).

Inoculation with rhizobia significantly increased % total N, and % total Ndfa, but reduced % total P of *P. falcataria* seedlings compared with the non-rhizobial control (Table 6). The increases in the % total N ranged from 47 to 87% for isolates PFIR3040 and PFAG6030 respectively, compared with the non-rhizobial controls. The % total Ndfa of rhizobia ranged from 62% for isolate PFIR3040 to 87% for isolate PFJB1040. Interestingly, control seedlings with non-rhizobial treatment had the lowest % total N and % total Ndfa, but had the highest % total P compared with those inoculated with rhizobial isolates (Table 6).

Acacia mangium

Seedling height and stem diameter

Inoculation with AMF did not significantly increase the height of seedlings compared with the no-AMF controls throughout the experiment. However, the height of seedlings inoculated with *Gi. rosea* BEG111 was significantly greater than those inoculated with *G. manihotis* BEG112 at ten and twelve week samplings (data not presented). These differences disappeared at subsequent samplings. Inoculation with rhizobia significantly promoted the growth of the seedlings after the sixteen-week sampling, by which time the growth of seedlings without rhizobium had slowed (Fig. 11).

Inoculation with isolates AMJB1010 and AMJB1020 significantly increased the height of seedlings at the eighteen and twenty-week samplings compared with non-rhizobial controls (Fig. 12) and also with those inoculated with the other rhizobial isolates,

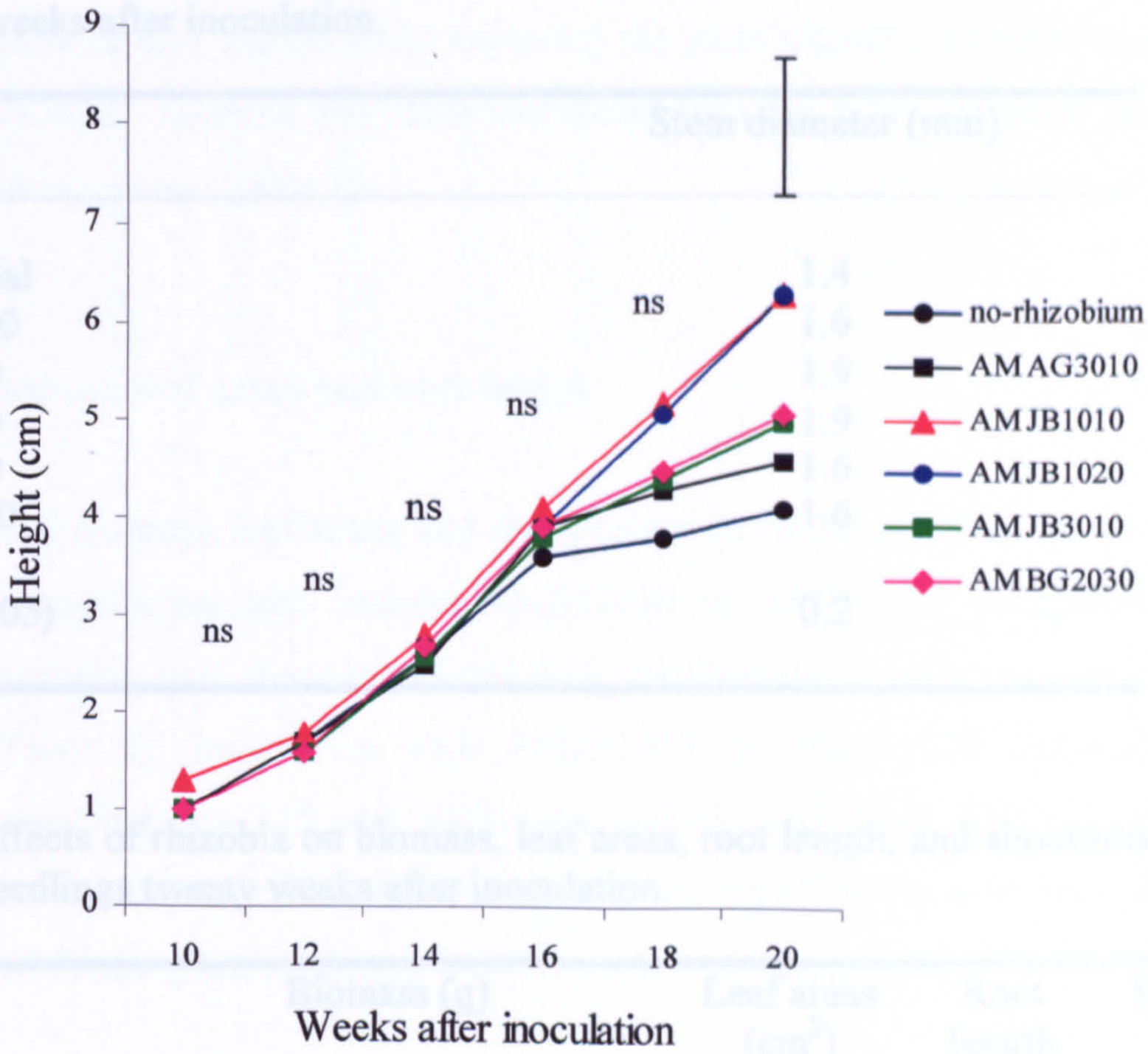


Figure 11. Effects of five rhizobial isolates and an uninoculated control on the height of *A. mangium* over time. The bar is the LSD (p= 0.05) value for treatment means at twenty weeks sampling. ns= the F-values not significant (p≥ 0.05).

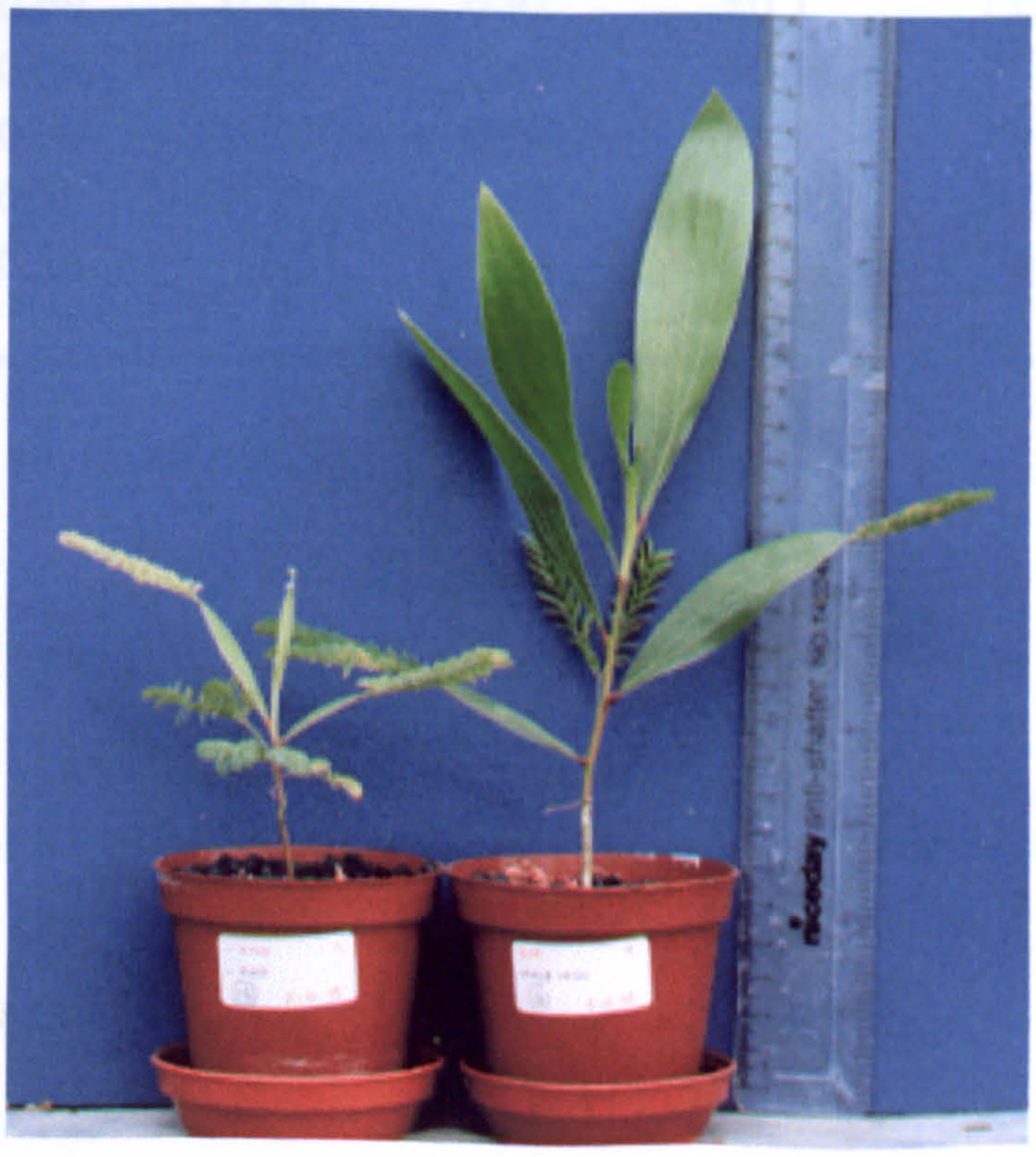


Figure 12. Comparison of the height of an *A. mangium* seedling inoculated with the combination treatment *Gi. rosea* BEG111+AMJB1020 (right) and a control seedling uninoculated with AMF and rhizobium (left) at the twenty-week sampling.

Table 7. Effects of AMF and rhizobia on stem diameter of *A. mangium* seedlings seventeen weeks after inoculation.

Treatments	Stem diameter (mm)
non-rhizobial	1.4
AMAG3010	1.6
AMJB1010	1.9
AMJB1020	1.9
AMJB3010	1.6
AMBG2030	1.6
LSD (p= 0.05)	0.2

Table 8. Effects of rhizobia on biomass, leaf areas, root length, and shoot/root ratio of *A. mangium* seedlings twenty weeks after inoculation.

Rhizobial isolate	Biomass (g)			Leaf areas (cm^2)	Root length (cm)	Shoot/root ratio
	Shoot	Root	Total			
non-rhizobial	0.25	0.18	0.43	32.2	1356	1.4
AMAG3010	0.33	0.18	0.51	39.1	1026	1.9
AMJB1010	0.69	0.23	0.92	80.1	1350	3.0
AMJB1020	0.68	0.22	0.89	80.9	1174	3.0
AMJB3010	0.39	0.20	0.59	49.1	1145	1.8
AMBG2030	0.32	0.17	0.50	37.8	1028	1.8
LSD (p=0.05)	0.16	ns	0.21	19.2	ns	0.4

ns= the F-value was not significant ($p \geq 0.05$).

AMAG3010, AMBG2030, and AMJB3010 twenty weeks after inoculation. Inoculation with these isolates also significantly increased the stem diameter of seedlings compared with non-rhizobial controls and those inoculated with the other rhizobial isolates at the twenty- week sampling (Table 7).

Seedling biomass, leaf areas and root length

Shoot and total biomass, leaf areas, and shoot/root ratio of the seedlings were significantly increased by inoculation with isolates AMJB1010 or AMJB1020 compared with non-rhizobial controls and those inoculated with the other rhizobial isolates at twenty-week sampling (Table 8). Inoculation with AMJB1010 or AMJB1020 increased the total biomass of seedlings by 113 and 106%, respectively compared with the non-rhizobial control. Inoculation with rhizobial isolates did not significantly alter root biomass and root length of *A. mangium* seedlings, twenty-week after inoculation.

AMF colonisation and root nodule formation

There were significant interactions between AMF and rhizobia on the % root length colonisation by AMF of *A. mangium* seedlings, but not the actual root length colonised by AMF. Co-inoculation of AMF with rhizobial isolates reduced the % root colonised by *G. manihotis* BEG112 and *Gi. rosea* BEG111 compared with inoculation with the AMF alone (Fig. 13). Isolates AMAG3010, AMJB1010 and AMJB1020 significantly reduced the % root length colonisation by these two AMF isolates (Fig. 13). The same rhizobial isolates tended to increase the % root colonised by *Sc. heterogama* BEG40 compared with inoculation with *Sc. heterogama* BEG40 alone. The main effect of AMF, but not rhizobia, significantly affected the actual root length colonised by AMF. The actual root length colonised by AMF was significantly higher when the seedlings of *A. mangium* were inoculated with *G. manihotis* BEG112 compared with those inoculated with *Gi. rosea* BEG111 or *Sc. heterogama* BEG40 (Fig. 14).

Inoculation of *A. mangium* seedlings with *G. manihotis* BEG112 or *Sc. heterogama* reduced the formation of big nodules (diam. > 2 mm) and nodule dry weight compared with seedlings uninoculated with AMF and seedlings inoculated with *Gi. rosea* BEG111. This reduction was statistically significant only if it was compared with the seedlings

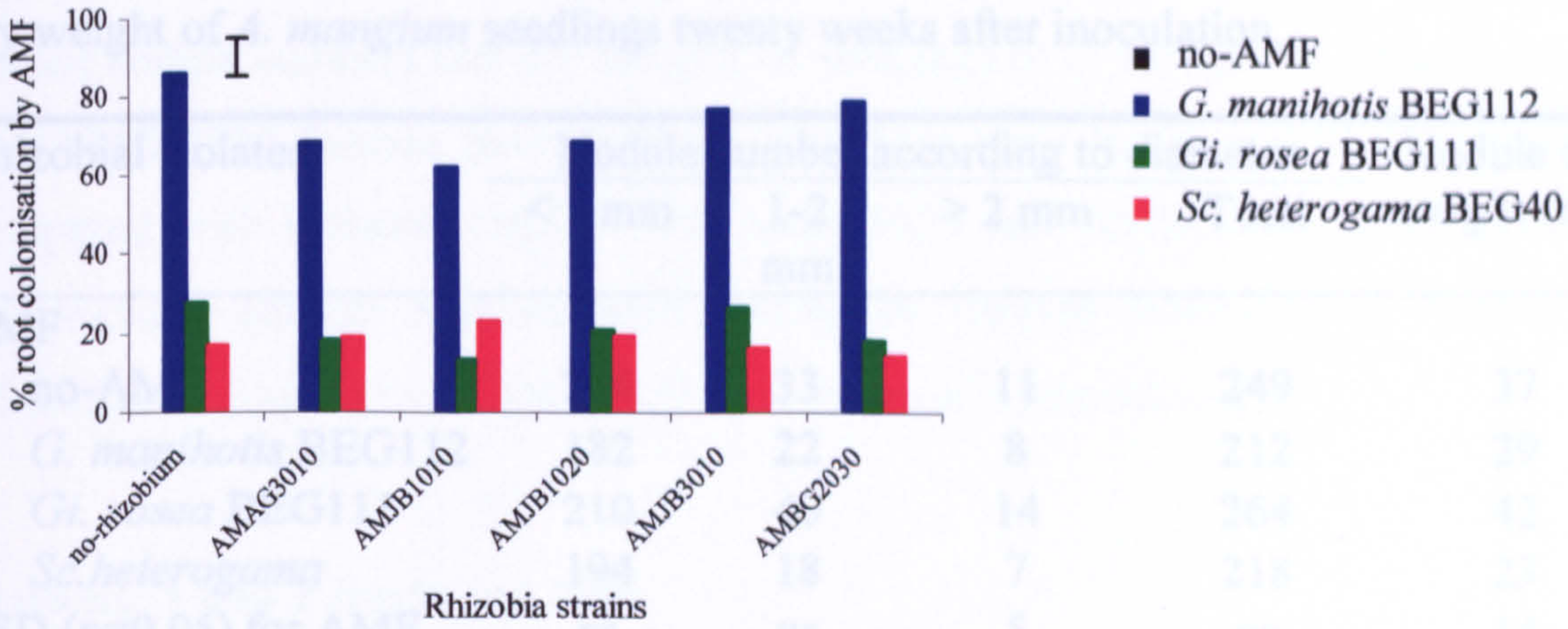


Figure 13. Interactions between AMF and rhizobia on % root colonisation by AMF of *A. mangium* seedlings at twenty weeks sampling. The bar is LSD (p=0.05) value for the means of combination treatments.

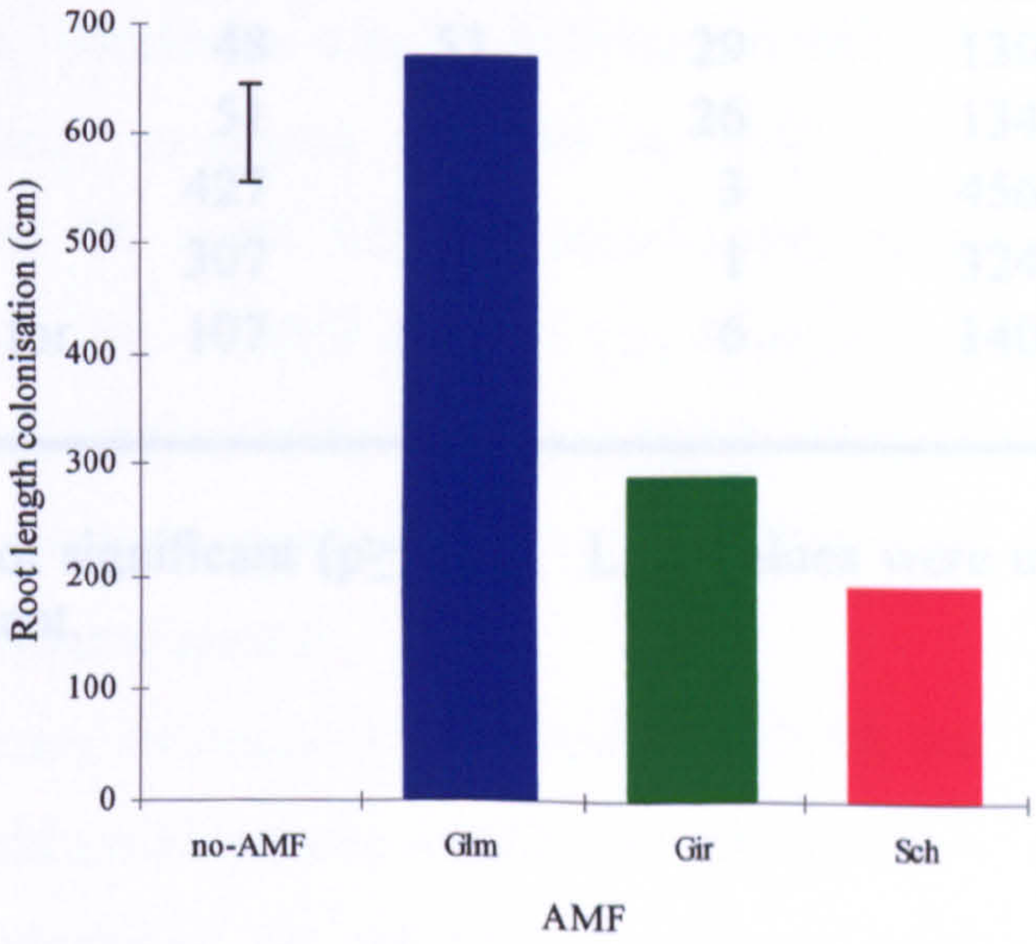


Figure 14. Actual root length colonisation of *A. mangium* seedlings by *G. manihotis* BEG112 (Glm), *Gi. rosea* BEG111 (Gir), and *Sc. heterogama* BEG40 (Sch) and an uninoculated control at twenty weeks sampling. The bar is LSD (p=0.05) value for treatment means.

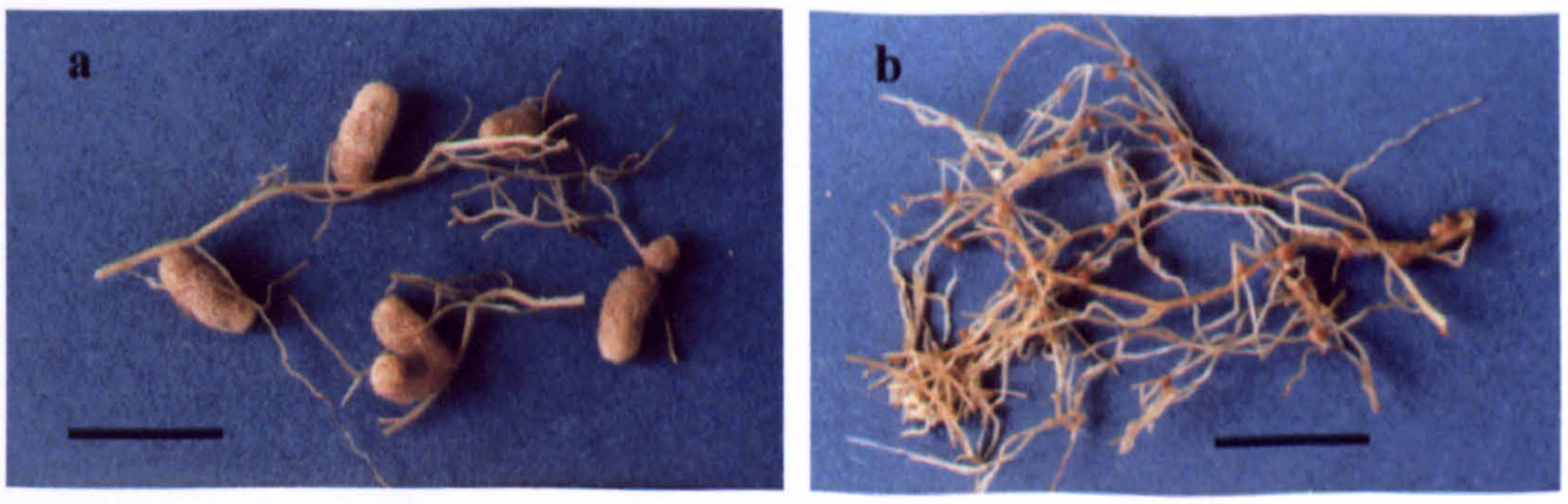


Figure 15. Size of nodules on the root of *A. mangium* seedlings formed by rhizobial isolates AMJB1010 and AMJB1020, which were mostly big with diam. > 2 mm (a), compared with those formed by isolates AMAG3010, AMBG2030 and AMJB3010 which were mostly small with diam. <2 mm (b). Bars= 1 cm.

Table 9. Effects of main treatments, AMF and rhizobia, on number of nodules and nodule dry weight of *A. mangium* seedlings twenty weeks after inoculation.

Rhizobial isolates	Nodule number according to diameter				Nodule dry weight (mg)
	< 1 mm	1-2 mm	> 2 mm	Total	
AMF					
no-AMF	204	33	11	249	37
G. manihotis BEG112	182	22	8	212	29
Gi. rosea BEG111	210	40	14	264	42
Sc.heterogama	194	18	7	218	23
LSD (p=0.05) for AMF	ns	ns	5	ns	14
Rhizobium					
non-rhizobial	0	0	0	0	0.0
AMAG3010	353	18	1	372	17.7
AMJB1010	48	53	29	130	31.4
AMJB1020	51	57	26	134	42.0
AMJB3010	427	26	3	456	23.5
AMBG2030	307	16	1	324	14.0
LSD (p=0.05) for	107	27	6	140	16.9

ns= the F-value was not significant ($p \geq 0.05$). LSD values were used to compare means within the same treatmant.

inoculated with *Gi. rosea* BEG111 (Table 9). Inoculation with rhizobia significantly altered nodule numbers and dry weights of seedlings of *A. mangium*. Isolates AMJB1010 and AMJB1020 formed fewer nodules, but these were larger and thus heavier nodules compared with isolates AMAG1010, AMJB3010 and AMBG2030 (Fig. 15; Table 9). Significantly smaller nodules were formed by isolates AMAG1010, AMJB3010 and AMBG2030 ranging from 304 to 427 nodules plant^{-1}, respectively.

Seedling N and P levels and Ndfa

At twenty-week sampling seedlings inoculated with *Gi. rosea* BEG111 tended to have higher N contents and Ndfa (shoot, root and total) compared with seedlings uninoculated with AMF. In contrast, inoculation with *Sc. heterogama* BEG40 had significantly lower N contents in the seedlings compared with the no-AMF controls (Table 10). *Glomus manihotis* BEG112 and *Gi. rosea* BEG111 significantly increased the % Ndfa of the seedlings compared with the no-AMF controls and those inoculated with *Sc. heterogama* BEG40 (Table 10). Inoculation with *Gi. rosea* BEG111 significantly improved total N contents and Ndfa of seedlings compared with inoculation with *Sc. heterogama* BEG40 but not with the uninoculated control.

There were significant increases in N contents and Ndfa when *A. mangium* seedlings were inoculated with rhizobial isolates AMJB1010 and AMJB1020 compared with those uninoculated with rhizobium or inoculated with rhizobial isolates AMAG3010, AMJB3010, and AMBG2030 twenty-week after inoculation (Table 11). Seedlings inoculated with AMJB1010 and AMJB1020 acquired most of their N through nitrogen fixation, which was indicated by relatively high % Ndfa values of 80 and 78%, respectively (Table 11).

Combinations of AMJB1010+no-AMF, AMJB1020+no-AMF, AMJB1010+*Gi. rosea* BEG111, AMJB1020+*Gi. rosea* BEG111 or AMJB3010+*Gi. rosea* BEG111 significantly increased P contents (shoot, root, and total) of seedlings of *A. mangium* compared with combinations of non-rhizobial, AMAG3010, or AMBG2030, with no-AMF or plus AMF twenty weeks after inoculation (Fig. 16a,b,c). Co-inoculation of *Sc. heterogama* BEG40 with AMJB1010 or AMJB1020 significantly reduced P contents of *A. mangium* seedlings compared with inoculation with the respective rhizobial isolates alone. In contrast, co-inoculation of *Gi. rosea* BEG111 with AMJB3010 significantly increased P contents of

Table 10. Effects of AMF on N contents and Ndfa of *A. mangium* seedlings twenty weeks after inoculation.

AMF species	N contents			Ndfa			
	Shoot (mg)	Root (mg)	Total (mg)	% Ndfa	Shoot (mg)	Root (mg)	Total (mg)
no-AMF	7.4	3.2	10.6	30	4.4	1.5	5.9
G.manihotis BEG112	6.5	2.8	9.3	46	4.4	1.6	6.0
Gi. rosea BEG111	9.0	3.5	12.5	45	6.2	2.2	8.4
Sc.heterogama BEG40	5.4	2.5	7.9	30	2.8	1.0	3.8
LSD (p=0.05)	2.2	0.6	2.8	11	2.1	0.7	2.8

Table 11. Effects of rhizobia on N contents and Ndfa of *A. mangium* seedlings twenty weeks after inoculation.

AMF species	N contents			Ndfa			
	Shoot (mg)	Root (mg)	Total (mg)	% Ndfa	Shoot (mg)	Root (mg)	Total (mg)
non-rhizobial	3.0	2.2	5.3	2	0.06	0.04	0.1
AMAG3010	3.0	1.9	4.9	11	0.4	0.2	0.6
AMJB1010	13.8	4.6	18.4	80	11.2	3.7	14.9
AMJB1020	13.2	4.2	17.5	78	10.7	3.4	14.1
AMJB3010	5.9	3.0	8.9	34	3.2	1.4	4.6
AMBG2030	3.6	2.2	5.6	22	1.1	0.6	1.7
LSD (p=0.05)	2.7	0.8	3.4	14	2.6	0.8	3.4

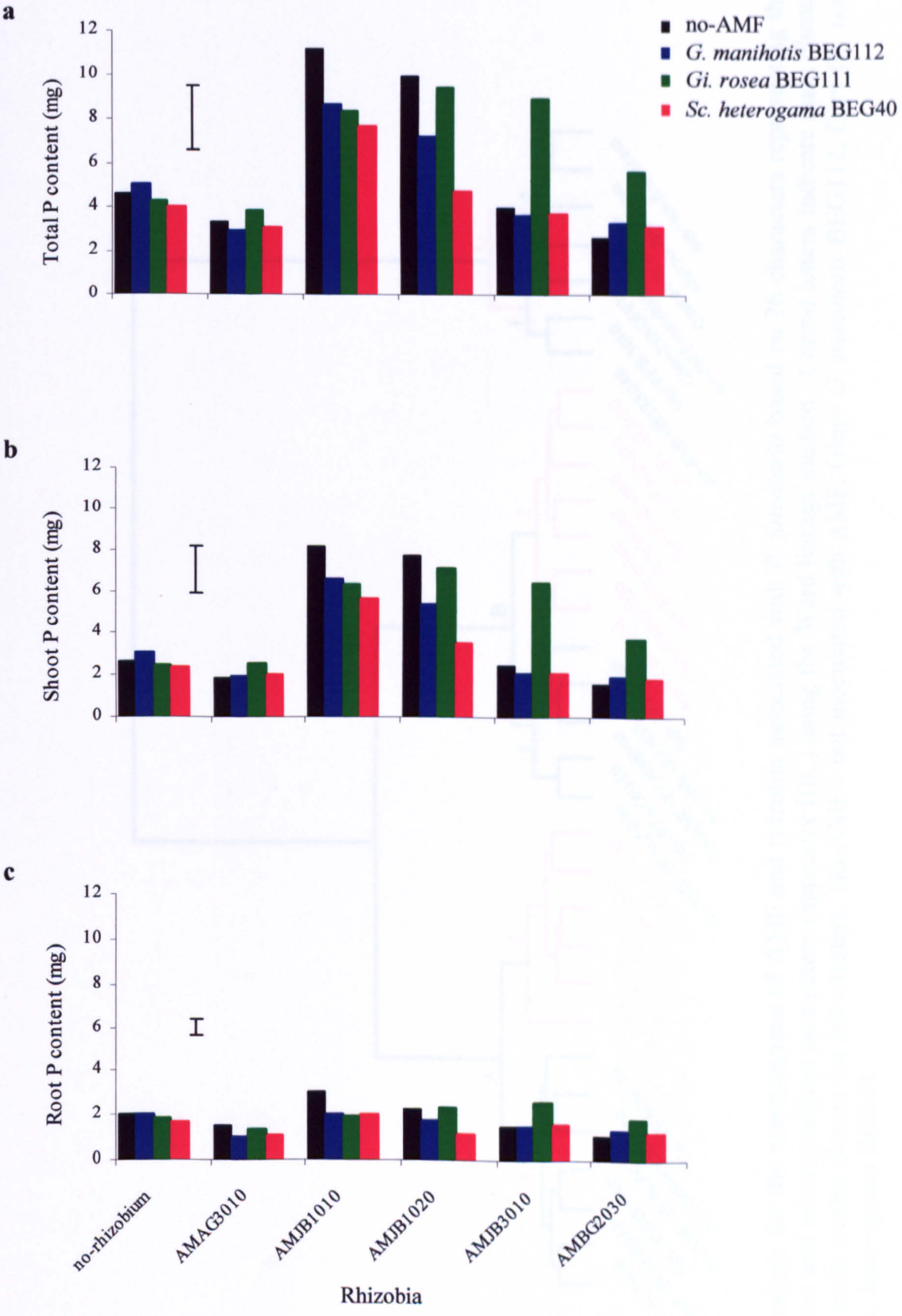


Figure 16. Effects of the interaction between AMF and rhizobia on total P content (a), shoot P content (b), and root P content (c) of *A. mangium* seedlings at twenty weeks sampling. Bars are the LSD (p= 0.05) values for means of combination treatments.

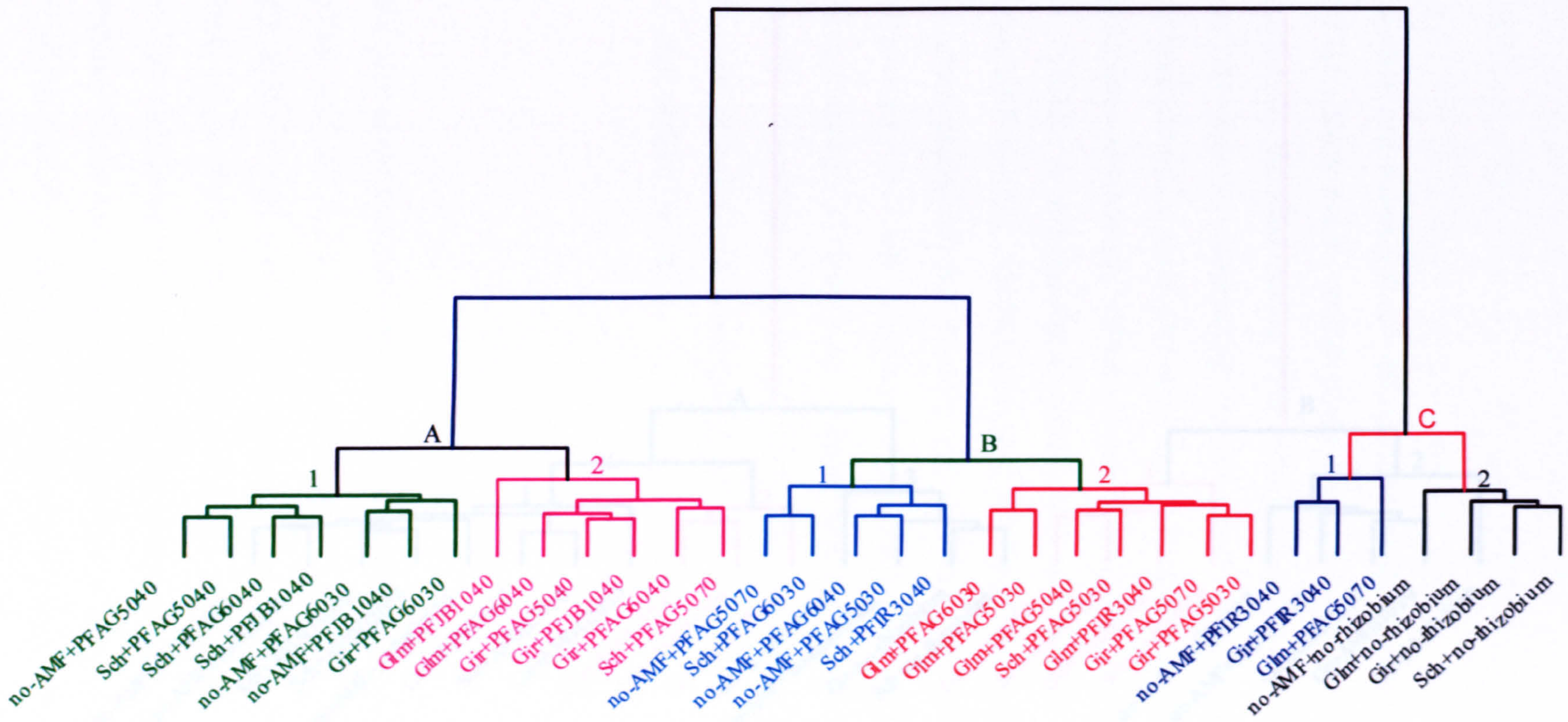


Figure 17. Cluster analysis for the combinations of AMF and rhizobia associated with *P. falcataria* based on 26 characters representing their effects on plant, nutrient and microsymbiont parameters (appendix IIIC) using the Ward linkage method. Capital letters indicate main clusters, and numbers in each main cluster denote the sub-clusters. No-AMF= not inoculated with AMF, Glm= *G. manihotis* BEG112, Gir= *Gi. rosea* BEG111, and Sch= *Sc. heterogama* BEG40.

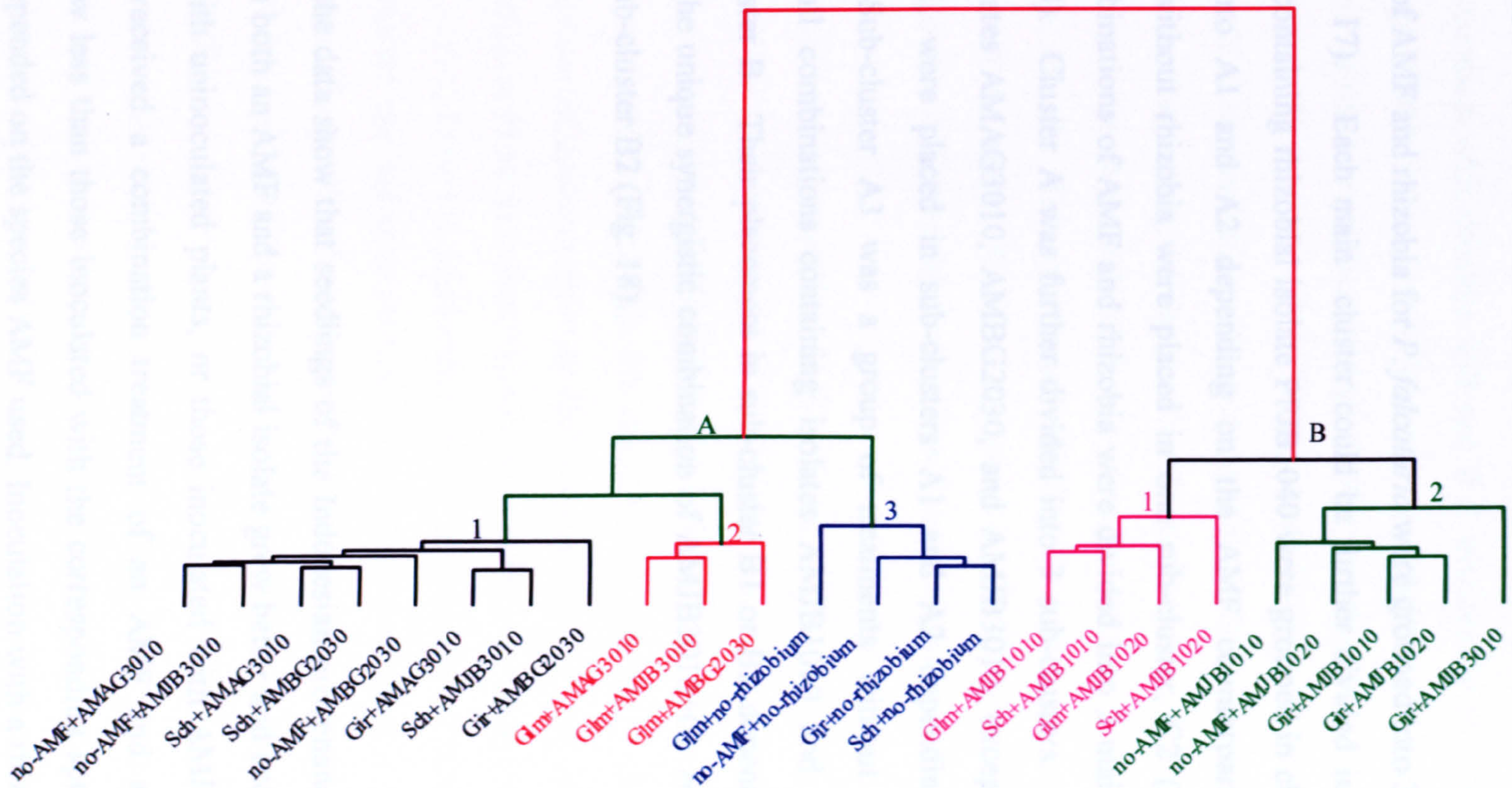


Figure 18. Cluster analysis for the combinations of AMF and rhizobia associated with *A. mangium* based on 27 characters representing their effects on plant, nutrient and microsymbiont parameters (appendix IIID) using the Ward linkage method. Capital letters indicate main clusters, and numbers in each main cluster denote the sub-clusters. No-AMF= not inoculated with AMF, Glm= *G. manihotis* BEG112, Gir= *Gi. rosea* BEG111, and Sch= *Sc. heterogama* BEG40.

the seedlings compared with co-inoculation of AMJB3010 with no-AMF, *G. manihotis* BEG112, or with *Sc. heterogama* BEG40.

Cluster analyses

Combinations of AMF and rhizobia for *P. falcataria* were grouped into 3 main clusters: A, B, and C (Fig. 17). Each main cluster could be further divided into 2 sub-clusters. Combinations containing rhizobial isolate PFJB1040 were grouped in cluster A, and were sub-grouped into A1 and A2 depending on the AMF counterpart. As expected, combinations without rhizobia were placed in one sub-cluster, C2 (Fig. 17). For *A. mangium*, combinations of AMF and rhizobia were divided into 2 main clusters only, A and B (Fig. 18). Cluster A was further divided into 3 sub-clusters. All combinations containing isolates AMAG3010, AMBG2030, and AMJB3010, except AMJB3010+*Gi. rosea* BEG111, were placed in sub-clusters A1 and A2 depending on their AMF counterpart. Sub-cluster A3 was a group of treatments without rhizobial isolate. Interestingly, all combinations containing isolates AMJB1010 and AMJB1020 were grouped in cluster B. Their placement in sub-cluster B1 or B2 depended on their AMF counterpart. The unique synergistic combination of AMJB3010+*Gi. rosea* BEG111 was placed in the sub-cluster B2 (Fig. 18).

Discussion

In general the data show that seedlings of the Indonesian provenance of *P. falcataria* inoculated with both an AMF and a rhizobial isolate grew better and acquired more N and P compared with uninoculated plants, or those inoculated with AMF alone. However, plants which received a combination treatment of an AMF and a rhizobial isolate sometimes grew less than those inoculated with the corresponding species of rhizobium alone. This depended on the species AMF used. Inoculation with a rhizobial isolate alone produced better growth to *P. falcataria* than inoculation with AMF alone under the prevailing environmental conditions. The improved growth of plants following inoculation with rhizobal isolates compared with inoculation with AMF agrees with the results of other studies (Soliman *et al.*, 1996; Rahman & Parsons, 1997). However, the results

contradict those of Colona *et al.* (1991) who found that inoculation with *G. mosseae* produced better growth and increased nodule dry weight in *Acacia senegal* seedlings compared with plants inoculated with *Rhizobium* only. These differences are explained by the fact that they used media with a low P availability, and thus the effect of inoculation with AMF may have produced a greater growth response in their experiment. It is well known that inoculation with rhizobia will fail if P availability in the media is limited (Morton & Yarger, 1990; Sprent, 1995; Vadez *et al.*, 1995). In the current study, P was applied as rock-P at the rate of 20-mg P kg^{-1} media, and this did not seem to limit plant growth or N fixation (Sun *et al.*, 1992; Vadez *et al.*, 1995), and all macro- and micro-nutrients, except N, were available in the nutrient solution (Broughton & Dilworth, 1971) supplied to the seedlings every 14 days. Therefore, the poor growth and low N and P content of seedlings inoculated with AMF, but not with rhizobia, may be due to a nutrient deficiency other than P. This was most probably N deficiency, as indicated by the yellowish colour of the leaves. This was supported by the fact that the % total N of the seedlings without rhizobial inoculation was significantly lower than those inoculated with rhizobial isolates.

Although it has been reported that AMF are able to transport N to the host (Vaast & Zasoski, 1992), in the current study, the N level in the medium was low and thus excess N for transport was not available. Unlike rhizobial isolates, AMF do not fix nutrients from the atmosphere and release them into the ecosystem. They do, however, increase the efficiency of the use of nutrients from available sources which may not be accessible to non-mycorrhizal plant roots. However, the results reported here, along with those of others (Colonna *et al.*, 1991; Ianson & Linderman, 1993), show that dual inoculation with AMF and rhizobial isolate can enhance plant growth and nutrient uptake even in N- and P-limited conditions. The results also emphasised the need for understanding the factors in the environment that limit the functioning of the plant-microbe symbioses as suggested by Colonna *et al.* (1991).

The effect of the two microsymbionts can differ in terms of plant growth stimulation, as shown by the sequential measurement of height data indicating that AMF could bring benefits earlier than the rhizobial isolate. However, when the nodules began to fix N from atmosphere (16 [*A. mangium*] or 13 [*P. falcataria*] weeks after inoculation) the growth of the plant increased markedly. An early stimulation of height of seedlings by AMF has also been reported by Rahman & Parsons (1997) in the symbiosis between an isolate of *Glomus mosseae*, *Azorhizobium caulinodans*, and a leguminous tree *Sesbania rostrata.*

This might be due to the early influence of the AMF as indicated by a detectable improvement in leaf P contents (Habte & Soedarjo, 1996) in *A. mangium* inoculated with an isolate of *Glomus aggregatum*.

Significant differences in plant growth, nodulation and N-fixation between isolates of *B. elkanii* and *B. japonicum* associated with the *P. falcataria* provenance indicate functional diversity of rhizobia with respect to this host plant. These data confirm the report of Turk & Keyser (1992) who stated that there was a high specificity of *P. falcataria* towards rhizobia, in terms of plant growth and N-fixation, and this tree species was nodulated effectively by *Bradyrhizobium* species. The functional diversity of the rhizobia was increased further when co-inoculated with different AMF. The study showed that unique combinations of AMF species with a particular rhizobial isolate could produce growth increases in *P. falcataria*. There was no single AMF species that uniquely gave maximum benefits for all rhizobial isolates tested or *vice versa*. For example, *Glomus manihotis* BEG112 was able to increase plant growth and nutrient acquisition when co-inoculated with rhizobial isolate PFJB1040 when compared with inoculation with isolate PFJB1040 alone, but decreased plant growth when co-inoculated with the rhizobial isolate PFAG5070 compared with inoculation with isolate PFJB1040 or PFAG5070 alone. Specific compatibility between AMF and rhizobia has also been shown earlier by Azcon *et al.* (1991) who inoculated *Medicago sativa* with three *Glomus* spp. and six isolates of *S. meliloti*. An isolate of *Glomus mosseae* significantly increased the shoot dry weight of the plant 70 days after inoculation, when co-inoculated with the rhizobial isolate Rm GR4 compared with an isolate of *G. caledonium* co-inoculated with the same rhizobial isolate. The outcome of the inoculation changed substantially when another rhizobial isolate (Rm104A14) was co-inoculated and *G. caledonium* was more effective in increasing shoot dry weight than *G. mosseae*.

The cluster analysis of growth parameters showed that 6 out of the 32 combinations of AMF and rhizobia tested were superior in promoting plant growth when all measured parameters (growth , nutrient contents, N-fixation, nodulation, colonisation by AMF) were considered. Within the 6 best combinations, *Sc. heterogama* BEG40 was compatible with the narrowest range of rhizobial isolates (only one compatible isolate, [PFAG5070]), whilst *Gi. rosea* BEG111 exhibited a wider range of compatible isolates (synergistic interaction with 3 rhizobial isolates [PFAG5040, PFAG6040 and PFJB1040]). *Glomus manihotis* BEG112 was intermediate in its range of compatible rhizobial partners (2 compatible rhizobial isolates [PFAG6040 and PFJB1040]). Two rhizobial isolates

(PFAG5040 and PFAG5070) showed a specific compatibility with only one AMF species (*Gi. rosea* BEG111 and *Sc. heterogama* BEG40 respectively) compared with the other two isolates (PFAG6040 and PFJB1040) which had synergistic partnerships with each of two species of AMF. Interestingly, the rhizobial isolates PFAG5040 and PFAG5070 were isolated from the same site in Kediri, East Java, yet their optimal activity occurred with different AMF, in our experiments. The result of the cluster analysis gives further evidence that relationships between AMF and rhizobial isolates for *P. falcataria* are specific and could not be predicted from the performance of the individual AMF and rhizobia inoculated separately. Further research should be focused on the effectiveness and the mechanisms of interactions of mixed combinations of AMF and rhizobia. Such multipartite interactions might produce either more stable and predictable symbiotic interactions or a greater range of variability. Both hypotheses need testing.

The data obtained using the *A. mangium* provenance from Indonesia, showed that the AMF and rhizobial isolates could promote plant growth, there were no significant statistical interactions between AMF and rhizobia. *Acacia mangium* shows a specific compatibility for its rhizobial symbionts, as indicated by its superior growth when inoculated with isolates of *B. elkanii* (AMJB1010 and AMJB1020), but not with the isolates of *B. japonicum* (AMAG3010, AMBG2030, and AMJB3010). Thus the rhizobial specificity for *A. mangium* is not only genus-specific as noted earlier by Turk & Keyser (1992), but species-specific. This opens the possibility that rhizobial isolates can be selected for *A. mangium* from those clustering with *B. elkanii* (done using PCR-RFLP-SSCP [see Chapter 3]). However, further tests involving more isolates from the two rhizobial types need to be done to verify this result. The fact that *Bradyrhizobium* spp. nodulating *A. mangium* comprised two distinct groups, with respect to their genetic characteristics (based on the RFLP analysis of the IGS regions) and effectiveness to promote plant growth, has been indicated by Frémont *et al.* (1999). However, in their work they did not characterise the isolates to species level and so comparisons cannot be made with the results obtained in this study.

The data summarised in the cluster analysis for the combination treatments for *A. mangium*, show that the performance of effective rhizobia (AMJB1010 or AMJB1020) could not be further improved by inoculation with AMF species. In contrast, there were examples where co-inoculation with AMF species reduced the effectiveness of the effective rhizobia, e.g. *Sc. heterogama* BEG40+AMJB1020. The lack of response of seedlings inoculated with effective rhizobia and co-inoculated with AMF showed the lack

of a synergistic compatibility between the rhizobial isolates and the AMF species tested. Another possibility is that *A. mangium* has a very low demand for P (3-6 mg P kg^{-1} media) for its growth and N-fixation as suggested by Vadez *et al.* (1995), thus the contribution of AMF to the growth of this provenance was not exhibited. This was also supported by data reported here, which clearly showed that *A. mangium* had similar root dry weights to *P. falcataria*, but had a significantly greater root length. Fitter *et al.* (1996) suggested that very well-branched systems of fine roots might not benefit as much from AMF through P uptake, but more through the improved protection against fungal pathogens in the root.

Gigaspora rosea BEG111 and *Sc. heterogama* BEG40 both belong to the same family in the Glomales the Gigasporaceae, but differ in their ability to stimulate plant growth and nutrient acquisition of *A. mangium*. *Gigaspora rosea* BEG111 was superior in this respect to *Sc. heterogama* BEG40. This confirms the results of Boddington & Dodd (1998) who compared *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 on a different forage legume *Desmodium ovalifolium*, and showed improved growth of plants was obtained by inoculation with *Gi. rosea* BEG111 compared with *Sc. heterogama* BEG40. However, when they were inoculated on *Pueraria phaseoloides*, *Sc. heterogama* BEG40 was superior to *Gi. rosea* BEG111 in promoting plant growth (Boddington & Dodd, 1998). In another study using an AMF isolate from the same genus (*Gigaspora margarita*) on ryegrass, Buwalda & Goh (1982) found that inoculation of *Gi. margarita* on ryegrass caused growth depressions. In their study they maintained the grass under low light intensity (one-third the intensity of full sunlight) for seven weeks, so the growth depressions could have been due to carbon drain by the fungus. This was supported by the fact that the % glucose and C/N ratio measured in the shoot of the plant were low compared with the uninoculated control (Buwalda & Goh, 1982). Dodd *et al.* (2000) suggested that the Gigasporaceae, which form much larger spores than those of *Glomus* species, may regulate P transfer to the plant to receive more carbon for spore formation over a longer period of time. This is supported by evidence that the ERM of *Gi. rosea* BEG111 accumulated polyphosphate, but none was observed in the ERM of *G. manihotis* BEG112 using 4',6-Diamidino-2-Phenylindole (DAPI) staining (Boddington & Dodd, 1999). This may indicate a storage of P within the ERM to restrict P transfer to the host, leading to a temporary depression of plant growth. Polyphosphate accumulation has also been observed in *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 (Vosatka & Dodd, unpublished data). Interestingly, our data show that *A. mangium* seedlings inoculated with *Sc. heterogama* BEG40 had lower P contents than those inoculated with *Gi. rosea*

BEG111. This reflects a different strategy of acquiring and using the resources between the species of AMF, even between those belonging to the same family.

Unique relationships between different AMF and rhizobial isolates for *A. mangium* and *P. falcataria* such as those described above, cannot be shown by studies employing a small number of AMF and rhizobial isolates. This approach has led to generalisations that all AMF and rhizobial interactions will be synergistic (Sieverding, 1991). The results reported here, allied to those obtained by Azcon *et al.* (1991), employing larger numbers of AMF species and rhizobial isolates, show that antagonistic, as well as synergistic relationships between AMF and rhizobial isolates can be demonstrated in the early stages of the development of tree seedlings.

The effectiveness of the two microbial symbionts on *A. mangium* seems to rely more on the rhizobial isolate used compared with *P. falcataria*, which was more responsive to both symbiont combinations, under the prevailing environmental conditions. The rhizobial isolates from *A. mangium* also show more specificity with the AMF symbiont (i.e. compatibility with *Gi. rosea* BEG111); therefore, screening for effective combinations of AMF and rhizobia might be easier than for *P. falcataria*. In contrast, the relationships between AMF and rhizobia for *P. falcataria* are specific, where different combinations of AMF and rhizobia varied in their ability to promote plant growth and nutrient acquisition, questions the reliability of screening for the effectiveness of each symbiont separately.

This is the first study on the tripartite symbiosis (AMF/rhizobia/tree legumes) which has involved a large number of microsymbionts (3 AMF species belonging to 3 unique genera, and 5 [*A. mangium* provenance] or 7 [*P. falcataria* provenance] isolates of rhizobia belonging to 2 species of *Bradyrhizobium*, on two different leguminous tree species). It is clear that different combinations of AMF and rhizobia within the tripartite symbiosis are unique for each tree species. This is important information to consider when attempting the management of the microsymbionts via inoculation of the different leguminous tree species. Studies on the symbioses between AMF, rhizobia, and *A. mangium* and *P. falcataria* have further emphasised the need for a greater understanding of the functional ecology of the microsymbionts to successfully apply these symbioses in reforestation and land rehabilitation programmes (Jeffries & Dodd, 1996).

References

Anderson JM, Ingram JSI. 1986. Tropical soil biology and fertility: A handbook of methods. CAB International, USA.

Arias I, Koomen I, Dodd JC, White RP, Hayman DS. 1991. Growth responses of mycorrhizal and nonmycorrhizal tropical forage species to different levels of soil phosphate. *Plant and Soil* **132**: 253-260.

Azcon R, El-Atrash F. 1997. Influence of arbuscular mycorrhizae and phosphorus fertilisation on growth, nodulation and N_2 fixation (N-15) in *Medicago sativa* at four salinity levels. *Biology and Fertility of Soils* **24**: 81-86.

Azcon R, Rubio R, Barea JM. 1991. Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2-fixation (N-15) and nutrition of *Medicago sativa* L. *New Phytologist* **117**: 399-404.

Azcon R, Rubio R, Barea JM. 1992. Nodulation, N_2 fixation (15N) and N nutrition relationships in mycorrhizal or phosphate-amended alfalfa plant. *Symbiosis* **12**: 33-41.

Azcon-Aguilar C, Alba C, Montilla M, Barea JM. 1993. Isotopic (N-15) evidence of the use of less available N-forms by VA mycorrhizas. *Symbiosis* **15**: 39-48.

Binkley D. 1997. Bioassays of the influence of *Eucalyptus saligna* and *Albizia falcataria* on soil nutrient supply and limitation. *Forest Ecology and Management* **91**: 229-234.

Boddington CL, Dodd JC. 1998. A comparison of the development and metabolic activity of mycorrhizas formed by arbuscular mycorrhizal fungi from different genera on two tropical forage legumes. *Mycorrhiza* **8**: 149-157.

Boddington CL, Dodd JC. 1999. Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life-cycle strategies. *New Phytologist* **142**: 531-538.

Broughton WJ, Dilworth MJ. 1971. Control of leghaemoglobin synthesis in snake beans. *Biochemistry Journal* **125**: 1075-1080.

Brundrett M, Juniper S. 1995. Non-destructive assessment of spore germination of VAM fungi and production of pot cultures from single spores. *Soil Biology & biochemistry* **27**: 85-91.

Buwalda JG, Goh KM. 1982. Host-fungus competition for carbon as a cause of growth depressions in vesicular-arbuscular mycorrhizal ryegrass. *Soil Biology & biochemistry* **14**: 103-106.

Cadisch G, Handayanto E, Malama C, Seyni F, Giller KE. 1998. N recovery from legume prunings and priming effects are governed by the residue quality. *Plant and Soil* **205**: 125-134.

Colonna JP, Thoen D, Ducousso M, Badji S. 1991. Comparative effects of *Glomus mosseae* and P fertiliser on foliar mineral composition of *Acacia senegal* seedlings inoculated with *Rhizobium*. *Mycorrhiza* **1**: 35-38.

Dar GH, Zargar MY, Beigh GM. 1997. Biocontrol of *Fusarium* root rot in the common bean (*Phaseolus vulgaris* L.) by using symbiotic *Glomus mosseae* and *Rhizobium leguminosarum*. *Microbial Ecology* **34**: 74-80.

DeLa Cruz RE, Manalo MQ, Aggangan NS, Tambalo JD. 1988. Growth of three legume trees inoculated with VA mycorrhizal fungi and *Rhizobium*. *Plant and Soil* **108**: 111-115.

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

Dommergues YR. 1995. Nitrogen fixation by trees in relation to soil-nitrogen economy. *Fertiliser Research* **42**: 215-230.

Fitter AH, Newsham KK, Merryweather JW. 1996. Diversity and function of arbuscular mycorrhizas in natural ecosystems. In Azcon-Aguilar C, Barea JM, eds. *Mycorrhizas in integrated systems from genes to plant development. Proceedings of the fourth European symposium on mycorrhizas, Granada 11-14 July 1994.* European Commission. Brussels, Luxemburg. 58-62.

Frémont M, Prin Y, Chauviere M, Diem HG, Pwee KH, Tan TK. 1999. A comparison of *Bradyrhizobium* strains using molecular, cultural and field studies. *Plant Science* **141**: 81-91.

Frey B, Schuepp H. 1992. Transfer of symbiotically fixed nitrogen from berseem (*Trifolium alexandrinum* L.) to maize via vesicular arbuscular mycorrhizal hyphae. *New Phytologist* **122**: 447-454.

Galiana A, Chaumont J, Diem HG, Dommergues YR. 1990. Nitrogen-fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp. *Biology and Fertility of Soils* **9**: 261-267.

Galiana A, Gnahoua GM, Chaumont J, Lesueur D, Prin Y, Mallet B. 1998. Improvement of nitrogen fixation in *Acacia mangium* through inoculation with rhizobium. *Agroforestry Systems* **40**: 297-307.

Galiana A, Prin Y, Mallet B, Gnahoua G-M, Poitel M, Diem HG. 1994. Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium* strains under field condition: long-term effect on plant growth and persistence of the introduced strains in soil. *Applied and Environmental Microbiology* **60**: 3974-3980.

Giller KE, Cadisch G. 1995. Future benefit from biological nitrogen fixation: An ecological approach to agriculture. *Plant and Soil* **174**: 255-277.

Giller KE, Wilson KJ. 1993. *Nitrogen fixation in tropical cropping systems.* C.A.B. International. Wallingford.

Giovannetti M, Mosse B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* **84**: 489-500.

Goh KM, Mansur I, Mead DJ, Sweet GB. 1996. Biological nitrogen fixing capacity and biomass production of different understorey pastures in a *Pinus radiata*-pasture agroforestry system in New Zealand. *Agroforestry Systems* **34**: 33-49.

Goicoechea N, Dolézal K, Antolín MC, Strnad M, Sánchez-Díaz M. 1995. Influence of mycorrhizae and *Rhizobium* on cytokinin content in drought-stressed alfalfa. *Journal of Experimental Botany* **46**: 1543-1549.

Habte M, Manjunath A. 1991. Categories of vesicular-arbuscular mycorrhizal dependency of host species. *Mycorrhiza* 1: 3-12.

Habte M, Soedarjo M. 1996. Response of *Acacia mangium* to vesicular-arbuscular mycorrhizal inoculation, soil pH, and soil P concentration in an oxisol. *Canadian Journal of Botany* **74**: 155-161.

Hamel C, Barrantes-Cartín U, Furlan V, Smith DL. 1991. Endomycorrhizal fungi in nitrogen transfer from soybean to maize. *Plant and Soil* **138**: 33-40.

Ianson DC, Linderman RG. 1993. Variation in the response of nodulating pigeonpea (*Cajanus cajan*) to different isolates of mycorrhizal fungi. *Symbiosis* **15**: 105-119.

Jaizme-Vega MC, Azcon R. 1995. Responses of some tropical and subtropical cultures to endomycorrhizal fungi. *Mycorrhiza* **5**: 213-217.

Jeffries P, Dodd JC. 1996. Functional ecology of mycorrhizal fungi in sustainable soil-plant systems. In Azcon-Aguilar C, Barea JM, eds. *Mycorrhizas in integrated systems from genes to plant development. Proceeding of the fourth European symposium on*

mycorrhizas, Granada 11-14 July 1994. European Commision. Brussels, Luxemburg. 497-501.

Johansen A, Jacobsen I, Jensen ES. 1993. Hyphal transport by a vesicular arbuscular mycorrhizal fungus of N applied to the soil as ammonium or nitrate. *Biology and Fertility of Soils* **16**: 66-70.

Kaldorf M, Kuhn AJ, Schroder WH, Hildebrandt U, Bothe H. 1999. Selective element deposits in maize colonised by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. *Journal of Plant Physiology* **154**: 718-728.

Kawai Y, Yamamoto Y. 1986. Increase in the formation and nitrogen fixation of soybean nodules by vesicular-arbuscular mycorrhiza. *Plant Cell Physiology* **27**: 399-405.

Khan MK, Sakamoto K, Yoshida T. 1995. Dual inoculation of peanut with *Glomus* sp. and *Bradyrhizobium* sp. enhanced the symbiotic nitrogen fixation as assessed by ^{15}N-technique. *Soil Science and Plant Nutrition* **41**: 769-779.

Li X-L, Marschner H, George E. 1991. Acquisition of phosphorus and copper by VA-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant and Soil* **136**: 49-57.

Liu R -J. 1995. Effect of vesicular-arbuscular mycorrhizal fungi on *Verticillium* wilt of cotton. *Mycorrhiza* **5**: 293-297.

Martensson AM, Rydberg I, Vestberg M. 1998. Potential to improve transfer of N in intercropped systems by optimising host-endophyte combinations. *Plant and Soil* **205**: 57-66.

Morton JB, Yarger JE. 1990. Soil solution P concentrations necessary for nodulation and nitrogen fixation in mycorrhizal and non-mycorrhizal red clover (*Trifolium pratense* L.). *Soil Biology & biochemistry* **22**: 127-128.

Nurlaeny N, Marschner H, George E. 1996. Effects of liming and mycorrhizal colonisation and soil phosphate depletion and phosphate uptake by maize (*Zea mays* L.) and soybean (*Glycine max* L.) grown in two tropical acid soils. *Plant and Soil* **181**: 275-285.

Otsamo A, Adjers G, Hadi TS, Kuusipalo J, Vuokko R. 1997. Evaluation of reforestation potential of 83 tree species planted on *Imperata cylindrica* dominated grassland - A case study from South Kalimantan, Indonesia. *New Forest* **14**: 127-143.

Rahman MK, Parsons JW. 1997. Effects of inoculation with *Glomus mosseae*, *Azorhizobium caulinodans* and rock phosphate on the growth of and nitrogen and phosphorus accumulation in *Sesbania rostrata*. *Biology and Fertility of Soils* **25**: 47-52.

Ruiz-Lozano JM, Azcon R. 1993. Specificity and functional compatibility of VA mycorrhizal endophytes in association with *Bradyrhizobium* strains in *Cicer arietinum*. *Symbiosis* **15**: 217-226.

Setiadi Y. 1995. The practical application of arbuscular mycorrhizal fungi for reforestation in Indonesia. PhD thesis. University of Kent at Canterbury. England.

Sharma MP, Bhargava S, Varma MK, Adholeya A. 1995. Effect of vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum* on survival and development of root-knot nematode *Meloidogyne incognita* in tomato. In Adholeya A, Singh S, eds. *Mycorrhizae: biofertilizer for the future, Proceedings of the third national conference on mycorrhiza.* Tata Energy Research Institute, New Delhi. 115-118.

Singh RS, Singh D. 1995. Effect of *Glomus aggregatum* inoculation and P-amendments on *Fusarium oxysporum* f.sp. *ciceri* causing chickpea wilt. In Adholeya A, Singh S, eds. *Mycorrhizae: biofertilizer for the future, Proceedings of the third national conference on mycorrhiza.* Tata Energy Research Institute, New Delhi. 119-123.

Sieverding E. 1991. Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Technical co-operation, Federal Republic of Germany. Eschborn.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis.* 2nd edition. London: Academic Press,

Soliman S, El-Ghandour IA, Khandra-Abbady A. 1996. Effects of nitrogen and phosphorus supply, and of *Rhizobium* and VAM fungus inoculation on dinitrogen fixation in soybean. *Folia Microbiologica* **41**: 197-200.

Souvannavong O, Galiana A. 1991. *Acacia mangium-Rhizobium* symbiosis: selection and propagation of the host plant and microorganism. In: Taylor DA, MacDicken KG, eds. *Research on multipurpose tree species in Asia.* Winrock International, Bangkok. Thailand, 216-222.

Sprent JL. 1995. Legume trees and shrubs in the tropics-N_2 fixation in perspective. *Soil Biology & biochemistry* **27**: 401-407.

Stamford NP, Ortega AD, Temprano F, Santos DR. 1997. Effects of phosphorus fertilisation and inoculation of *Bradyrhizobium* and mycorrhizal fungi on growth of *Mimosa caesalpiniaefolia* in acid soil. *Soil Biology & biochemistry* **29**: 959-964.

Sun JS, Simpson RJ, Sands R. 1992. Nitrogenase activity of two genotypes of *A. mangium* as affected by phosphorus nutrition. *Plant and Soil* **144**: 51-58.

Teklehaimanot Z, Animkwapong G. 1996. The potential of *Albizzia zygia* (DC) Macbride for soil amelioration. *Applied Soil Ecology* **3**: 59-68.

Tomlinson H, Teklehaimanot Z, Traore A, Olapade E. 1995. Soil amelioration and root symbioses of *Parkia biglobosa* (Jacq.) Benth in West Africa. *Agroforestry Systems* **30**: 145-159.

Turk D, Keyser HH. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Canadian Journal of Microbiology* **38**, 451-460.

Umali-Garcia M, Libuit JS, Baggayan RL. 1988. Effects of rhizobium inoculation on growth and nodulation of *Albizia falcataria* (L.) Forsh, and *Acacia mangium* Willd. in the nursery. *Plant and Soil* **108**: 71-78.

Vaast Ph, Zasoski RJ. 1992. Effects of VA-mycorrhizae and nitrogen sources on rhizosphere soil characteristics, growth and nutrient acquisition of coffee seedlings (*Coffea arabica* L.). *Plant and Soil* **147**: 31-39.

Vadez V, Lim G, Durand P, Diem HG. 1995. Comparative growth and symbiotic performance of four *Acacia mangium* provenances from Papua New Guinea in response to the supply of phosphorus at various concentration. *Biology and Fertility of Soils* **19**: 60-64.

Vincent JM. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publication, Oxford.

Yoneyama T, Muraoka T, Murakami T, Boonkerd N. 1993. Natural abundance of N-15 in tropical plants with emphasis on tree legumes. *Plant and Soil* **153**: 295-304.

V. Establishment of mycorrhizas of four different species of arbuscular mycorrhizal fungi observed using a Petri-dish observation chamber (PDOC)

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligately symbiotic fungi which colonise the roots of most terrestrial plant families. These fungi cannot be cultured without the presence of host plants (Smith & Read, 1997). This makes it difficult to follow the development of the fungi, the interaction with the plant, and the life-cycle. The fungi are generally propagated using open pot-cultures maintained in a growth chamber or greenhouse adjusted to conditions which favour the interaction between the fungus and the host plant (Menge, 1984). Using these methods, the development of extra-radical structures of the AMF can be observed from samples taken from the mycorrhizal system. Thus much of the data concerning AMF-plant interactions are based on single time point observations rather than continuous development (De Souza & Berbara, 1999). There is also a problem in that some structures are destroyed during sampling, especially the extra-radical mycelium (ERM).

An alternative technique has been the use of non-transformed or transformed root-organ cultures (Mosse & Hepper, 1975; Chabot *et al.*, 1992). This is the most advanced technique used to propagate AMF, and to observe the development of AMF without destructive sampling. Roots of a host plant, such as *Trifolium repens* (Mosse & Hepper, 1975; De Souza & Berbara, 1999), *Daucus carota*, *Lycopersicon esculentum* (Chabot *et al.*, 1992), or *Medicago sativa* (Douds Jr *et al.*, 1998), were grown in nutrient agar media in a Petri-dish then inoculated with surface-sterilised spores of AMF and incubated at 27 to 32°C (Chabot *et al.*, 1992; Jolicoeur *et al.*, 1998) depending on the AMF species used. Using this technique some isolates of species of AMF, such as *Glomus clarum* CNPAB 5 (De Souza & Berbara, 1999), *Glomus intraradices* DAOM 197198 (Chabot *et al.* 1992), could complete their life-cycle in the system. Spore germination and the subsequent development could be monitored easily and regularly without destroying any fungal structures. Bago *et al.* (1998a) used this technique to study the development of the ERM of *Glomus intraradices* DAOM 197198 and the pH changes in the medium caused by the

mycelium. The fungus formed branched absorbing structures (ABS) at the end of hyphal tips, structures which had not been noticed in destructive sampling of cultures.

However, root-organ culture is highly artificial and may not represent the true events seen in soil (Hepper & Mosse, 1975). When comparing the structures of AMF developed in root-organ culture and buried slides, Hepper and Mosse found that the "rhizoid-like" structures formed by the AMF in root-organ cultures were not found using a buried slide technique. However, it is not known which represents the soil equivalent as in both techniques the AMF was grown on agar. Pawlowska *et al.* (1999) reported that an isolate of *Glomus etunicatum* propagated using a transformed root-organ culture produced spores with a significantly smaller diameter and thicker spore walls compared with those of spores extracted from pot-cultures. Few species of AMF have so far been successfully propagated using root-organ culture and these are mainly isolates of *Glomus*, such as *G. clarum* CNPAB 5 (De Souza & Barbera, 1999), *G. etunicatum* (Pawlowska *et al.*, 1999), *G. intraradices* DAOM 197198 (Chabot et al., 1992; Bago *et al.*, 1998a), *G. mosseae* (Mosse & Hepper, 1975), and *G. versiforme* (Declerck *et al.*, 1996). In additional, *Gigaspora gigantea* (Gemma & Koske, 1988) and *Gigaspora margarita* DAOM194757 (Becard & Fortin, 1993) have also been grown in root-organ culture. The technique is expensive, and the facilities needed may not be available in many laboratories.

Several simpler methods had been used to study the development of AMF and the interaction with the plant (Hepper, 1984). For example, test-tube culture (Mosse, 1962; Mosse & Phillips, 1971) using various artificial supports for plant roots, such as filter papers and water agar. Other techniques involved the use of deep dishes (Allen *et al.*, 1979). The latter technique was used to study the growth of *Glomus fasciculatum*, including the growth of the intra- and extra-radical mycelia (ERM). The development of ERM along the root, and the internal hyphal and arbuscular development behind root tips, and at the base of root branches, could be followed. Hepper & Mosse (1975) used a "buried slide" technique to investigate germ-tube development and colonisation of roots by AMF. They found that germination occurred by means of long relatively unbranched hypha, however, the hypha frequently crossed roots without initiating colonisation. Setiadi (1995) used a test-tube culture with terragreen as the supporting medium for roots to produce pure cultures of AMF from single spores. If the plants, once inoculated with spores of AMF, were inserted next to the wall of the test tube, the germination of the spore and the subsequent fungal development could be observed (Mansur, unpublished). However, the test tube culture could not support the growth of the plant for long.

Therefore, the aim of this study was to test several observation chamber designs to study AMF-plant interactions. The requirements: 1) were suitable for most AMF species, 2) were easy to prepare, maintain, and check regularly with little disturbance to fungal structures , 3) were reproducible and 4) required simple facilities which can be adopted by most laboratories. Four species of AMF belonging to four different genera were tested.

Materials and Methods

Several techniques were tested including test-tubes and Petri-dish observation chambers (PDOC); however, only the PDOC proved promising to study the development of AMF and only those data were reported here.

Media preparation

Terragreen, an inert attapulgite clay (Agsorb 8/16, Oil Dri, Wisbech, Cambs, UK) was washed with tap water then soaked 14 h in 5 mM MES (2-[N-Morpholino] ethanol-sulfonic acid) buffer solution (Vilariño *et al.*, 1997). The Terragreen was autoclaved at 121°C for 20 min then was distributed into 9 and 18 cm diameter Petri-dishes. A small hole (0.5 x 0.5 cm) was made at one side of each Petri-dish to insert the seedling (Fig. 1).

Seed surface-sterilisation and germination

Seeds of *Desmodium ovalifolium* and *Paraserianthes falcataria* were surface-sterilised by immersing the seeds in 95% (v/v) ethanol for 10 sec and then soaking them in 3% (v/v) H_2O_2 for 5 min. Finally, the seeds were rinsed three times with sterile distilled water (dH_2O). To break dormancy, the seeds were soaked in sterile boiling water for 5 min and then in sterile cold dH_2O 14 h. The seeds were germinated on fine terragreen (1-2 mm diameter particles) in a 250 ml glass beaker and kept in a Fitotron growth chamber (Gallenkamp, UK) simulating tropical conditions (30°C, 12 hr light at 300 µmol m^{-2} sec^{-1} and 12 hr dark, RH 60%).

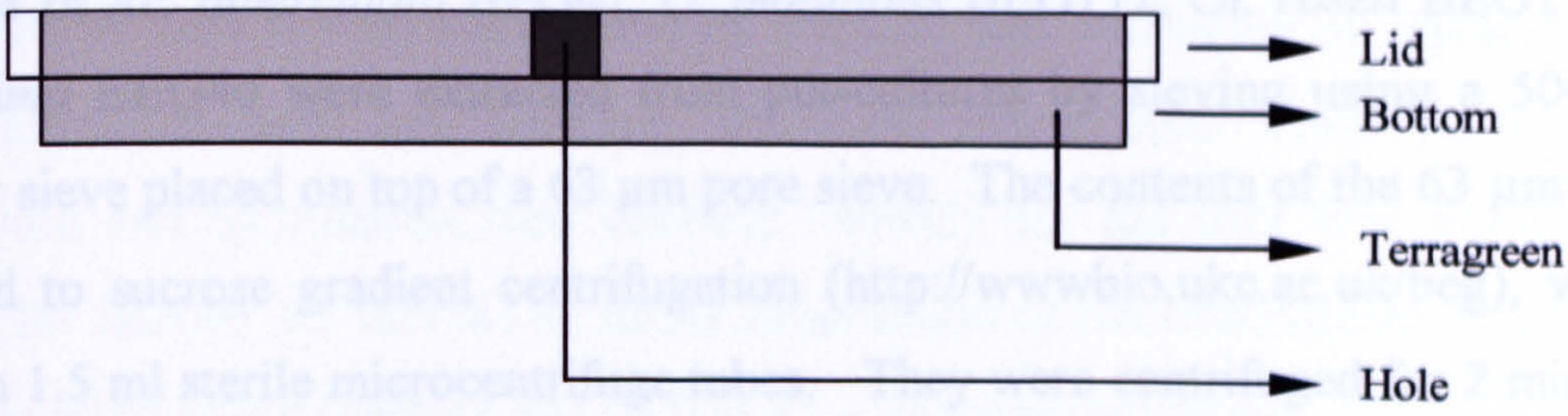


Figure 1. A 9 or 18 cm diameter Petri-dish microcosm. A 0.5 x 0.5 cm hole was made to facilitate the growth of the shoot. Sterile Terragreen was used as a medium to support the plant root and the fungi.

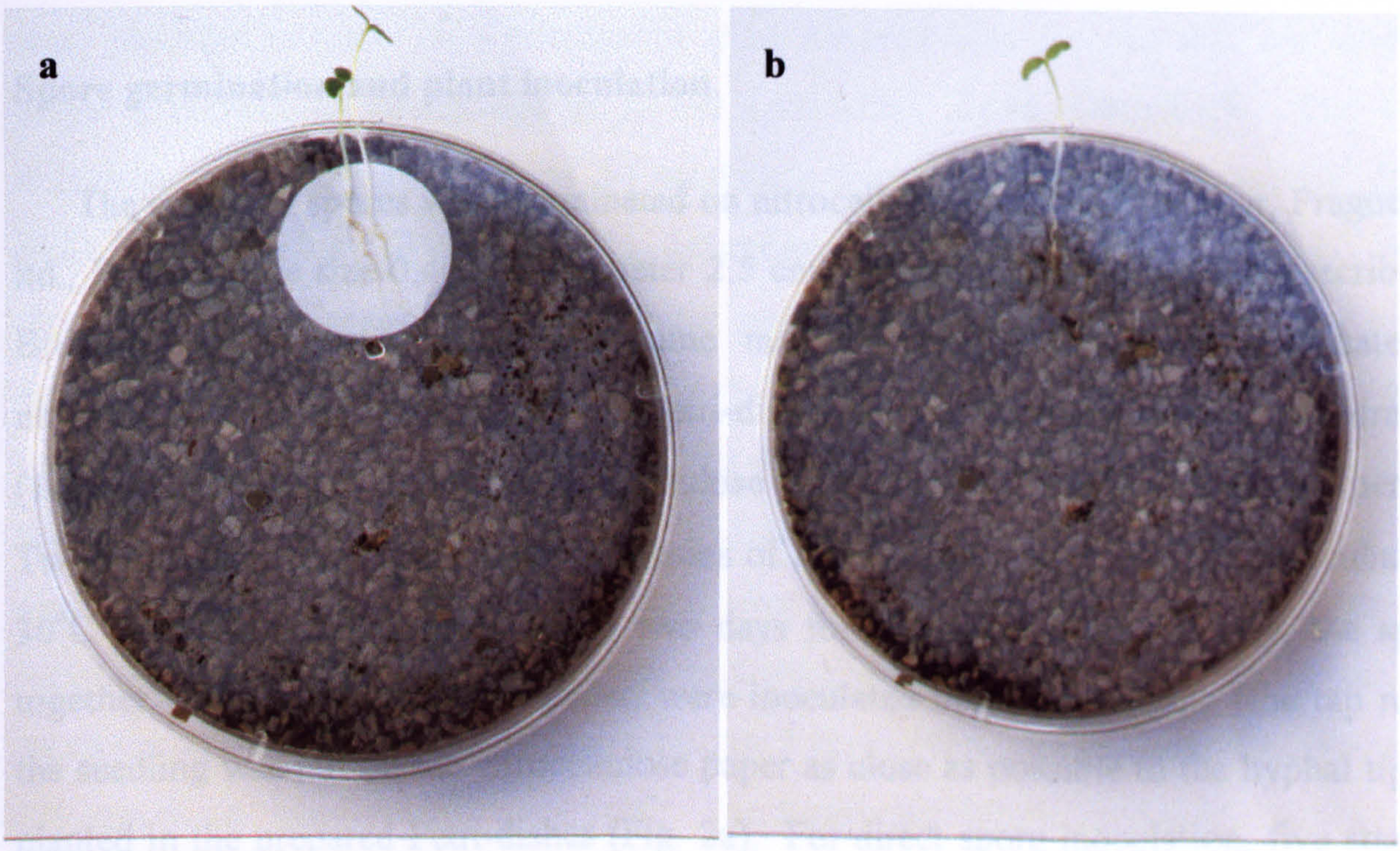


Figure 2. Inoculation and planting of seedlings of *Desmodium ovalifolium* in the Petri-dish microcosm (PDM); a) inoculation with pre-germinated spores on nitrocellulose paper, b) spores direct inoculation onto the seedling root.

Spore extraction and surface-sterilisation

Spores of *Ac. tuberculata* BEG41, *G. manihotis* BEG112, *Gl. rosea* BEG111, and *Sc. heterogama* BEG40 were extracted from pot-cultures by sieving using a 500 µm pore diameter sieve placed on top of a 63 µm pore sieve. The contents of the 63 µm sieve were subjected to sucrose gradient centrifugation (http://wwwbio.ukc.ac.uk/beg), washed and placed in 1.5 ml sterile microcentrifuge tubes. They were centrifuged for 2 min at 13 000 g using a microcentrifuge model 1-15 (Sigma, Germany) to pellet the spores and remove excess water. One ml of 5% (v/v) commercial Chloros (Hays Chemical Distribution Ltd., UK) containing >5% and <16% chlorine was pipetted into the tube and left for 2 min. The tube was then centrifuged to pellet the spores and the sterilant removed. Finally, spores were washed five times with sterile dH_2O.

Spore germination and plant inoculation

The sterilised spores were germinated on nitrocellulose papers (Pragopor, Pragochema ltd., Prague; pore size 0.40 µm, diameter 2.5 cm), according to the method described by Brundrett & Juniper (1995) with some modification. Spores were germinated on nitrocellulose papers in 9 cm diameter Petri-dishes half full of moist sterile fine-terragreen (1-2 mm diameter), 5 spores per nitrocellulose paper and 10 nitrocellulose papers per dish. Two replicates were set-up for each species of AMF. The spores were then incubated at 30°C in the dark and checked every two days for 20 days. Subsequently, the spores, together with the nitrocellulose paper, were inoculated onto the seedling (the tap root of the seedling was put on the nitrocellulose paper as close as possible to the hyphal tip) and planted in the prepared Petri-dishes (Fig. 2a). For direct spore inoculation, five sterilised spores of the AMF species were arranged on the tap root of the seedling close to the tip, and the seedlings were then planted in the Petri-dish (Fig. 2b). The Petri-dish was sealed with PVC tape leaving the side of the dish opposite to the hole for the plant untaped. The Petri-dishes were then arranged vertically in square plastic containers containing water, such that the water would be absorbed by the medium to keep it moist but not waterlogged. Cultures containing different species of AMF were kept in separate boxes to avoid cross-contamination. The systems were placed in the tropical section of a greenhouse (min. 15°C/max 40°C, relative humidity 60-80%, light intensity 400-600 µmol

m^{-2} sec^{-1}). Water was added to the plastic container as needed and the plants were fed fortnightly with 1.4 g l^{-1} Vitafeed 102 fertiliser (Vitax Ltd., Leicester, UK) containing 18% N, 0% P, 36% K with trace elements. Feeding was conducted by replacing the water in the container with the fertiliser solution. Mycorrhizal development, including colonisation and sporulation, was checked weekly for the first four months and then monthly for another six months by direct observation of the observation chamber under a dissecting microscope (Nikkon SMZ-U) at 15 - 100x magnification.

Measurement of growth parameters of AMF

Ten months after inoculation, sampling was conducted to measure spore diameters, hyphal diameters and length. Twenty five spores per AMF species from 5 Petri-dishes were mounted in water on a microscope slide, covered with a cover slip, and measured under a compound microscope at x 100 (Axioskop, Zeiss, Germany). The hyphal diameters were determined from 50 randomly selected fragments of hyphae directly picked up from the PDOC under a dissecting microscope (Nikkon SMZ-U) at 20x magnification and mounted in water as for spores. Measurement was conducted under a compound microscope at x 1000 (Axioskop, Zeiss, Germany). To measure total hyphal lengths, 4 cores (1 cm diam.) were taken to the bottom of the dish from 4 random positions in each Petri-dish, mixed, and used as a composite sample. Five replicates were used for each AMF species. The samples were agitated in 100 ml of deionised water (dH_2O) by stirring for 1 min at 200 rpm and were then allowed to settle for 10 sec. Five ml of the solution containing hyphae was then filtered onto nitrocellulose membrane filter (25 mm diam. and 0.45 µm pore size). The membrane filters, containing hyphae, were transferred to microscope slides, and subsequently two drops of 0.05% (w/v) trypan blue in lacto-glycerol (lactic acid: glycerol: dH_2O; 5:1:1) were added to the filter. The entire membrane was scanned under a compound microscope (Axioskop, Zeiss, Germany) for mycelium and estimations of mycelial length were made using an eyepiece graticule at a magnification of x 100. The length of mycelium was expressed in metres of total hyphae in 1 gram of dry substrate (oven-dried for 3 h at 105°C).

Results

This system was effective for *G. manihotis* BEG112, *Gi. rosea* BEG111 and *Sc. heterogama* BEG40. However, due to the low germination percentage and consequent problems of contamination, pre-germinated spores were not used for *Ac. tuberculata* BEG41. Instead fresh crude inoculum, containing spores, mycelia and root fragments, from six-month-old pot-cultures of the fungus was used to fill the 1/3 volume of the 18 cm diameter Petri-dish around the root of the plant. Another alternative tried was to transplant *D. ovalifolium* (6-month-old) colonised by the fungus into 18 cm diameter Petri-dish containing the medium. Using this modification *Ac. tuberculata* BEG41 could be established in the PDOC.

The ease of use and simplicity of the PDOC allowed the early stages of development of the AMF symbiosis to be studied. In all four AMF, spore germination, root colonisation and development of the ERM, and spore ontogeny could be observed.

Spore germination

Spores of the different species of AMF from the same age of pot-cultures germinated at different rates, with spores of *G. manihotis* BEG112 being the fastest, producing germ-tubes within 4 ± 1 days after inoculation onto the nitrocellulose paper (Table 1). Generally, spores inoculated on the nitrocellulose paper started to germinate earlier than those inoculated directly onto the root. *Acaulospora tuberculata* BEG41 and *Sc. heterogama* BEG40 germinated through their germination shield (Fig. 3a,e). A single germ-tube was formed by spores of *Ac. tuberculata* BEG41 and *G. manihotis* BEG112 in contrast to *Gi. rosea* BEG111 and *Sc. heterogama* BEG40, which produced multiple germ-tubes. In all cases, the germ-tube produced secondary branches after germination. When germinated on the nitrocellulose paper without the presence of the host plant *Ac. tuberculata* BEG41 and *G. manihotis* BEG112 produced immature sporer or "jouvenile spores" (terminology *sensu* De Souza & Berbara, 1999) on the secondary branches (Fig. 3b,c), whilst *Gi.rosea* BEG111 and *Sc. heterogama* BEG40 produced auxiliary cells (Fig. 3d,f). The immature spores were not found when the spores were inoculated directly onto roots. In contrast, auxiliary cells were also formed when spores were inoculated directly onto roots.

Root colonisation

The germination hyphae in the directly-inoculated treatments were not tropic toward the root but grew in random directions, often away from the roots. The entry points formed by *Sc. heterogama* BEG40 were the easiest to observe due to their colour (brown pigment) and the fact that they formed a distinct intensive proliferation of hyphae on the surface of roots (Fig. 4). Colonisation of the roots by pre-germinated spores was not detected until 2-3 weeks for *G. manihotis* BEG112 and *Gi. rosea* BEG111, 3-4 weeks for *Ac. tuberculata* BEG41 (inoculated with a crude inoculum), and 6-7 weeks for *Sc. heterogama* BEG40. Successful colonisation resulted in the growth of ERM. Where nitrocellulose inoculum was used, this was particularly evident as the extension of mycelium beyond the nitrocellulose paper. If colonisation was not successful, there were no signs of mycelium growing beyond the nitrocellulose paper.

The architecture of the extra-radical mycelia (ERM)

In all cases, the ERM comprised germ-tubes, infection networks, runner hyphae, hyphal networks and hyphal bridges (terminology *sensu* Friese & Allen, 1991) in the observation chamber. Runner hyphae either grew away from the root, or grew alongside the colonised root. The former type of runner hypha of *Ac. tuberculata* BEG41 and *G. manihotis* BEG112 formed a hyphal network in the substrate and subsequently produced dichotomous branching of infection networks when approaching another root (Fig. 5a). Many branches of the runner hyphae passed over the root and did not attempt to penetrate. The runner hyphae that grew alongside the colonised root formed entry points on the same root and also produced branches that formed a hyphal bridge that connected adjacent roots (Fig. 5b) and colonised them.

The diameter and length of the hyphae formed by AMF varied among species (Table 2). *Glomus manihotis* BEG112 formed significantly thicker and longer hyphae compared with *Ac. tuberculata* BEG41, *G. rosea* BEG111, and *Sc. heterogama* BEG40. The colour of the hyphae was generally hyaline, except for *Sc. heterogama* BEG40 which had dark red-brown hyphae main channel hyphae and hyaline ephemeral hyphae.

Table 1. Time (days after inoculation) for spores of different AMF species to germinate on either nitrocellulose paper or on roots.

AMF species	**On nitrocellulose paper (dai)**	**On root (dai)**
Ac. tuberculata BEG41	12±5*	nd
Gi. rosea BEG111	6±2	17±5
G. manihotis BEG112	4±1	18±5
Sc. heterogama BEG40	11±4	>144

dai= day after inoculation. *Most of these germinated spores were contaminated by either bacteria or other fungi and were not used for further experimentation. nd= no data, *Ac. tuberculata* BEG41 was inoculated using crude inoculum or a transplanted seedling colonised by this fungus.

Table 2. The diameter and length of hyphae formed by four species of AMF representing four different genera.

AMF species	**Diameter (µm)**			**Length (cm g^{-1} substrate)**
	minimum	**mean**	**maximum**	
Ac. tuberculata	1.5	4.5[c]	11.0	7.1[c]
G. manihotis BEG112	2.0	9.5[a]	25.0	48.7[a]
Gi. rosea BEG111	2.0	6.8[b]	11.0	21.1[b]
Sc. heterogama	1.0	4.8[c]	12.0	4.3[c]

Values in the same column followed by the same letter were not significantly different (LSD test [$p \geq 0.05$]).

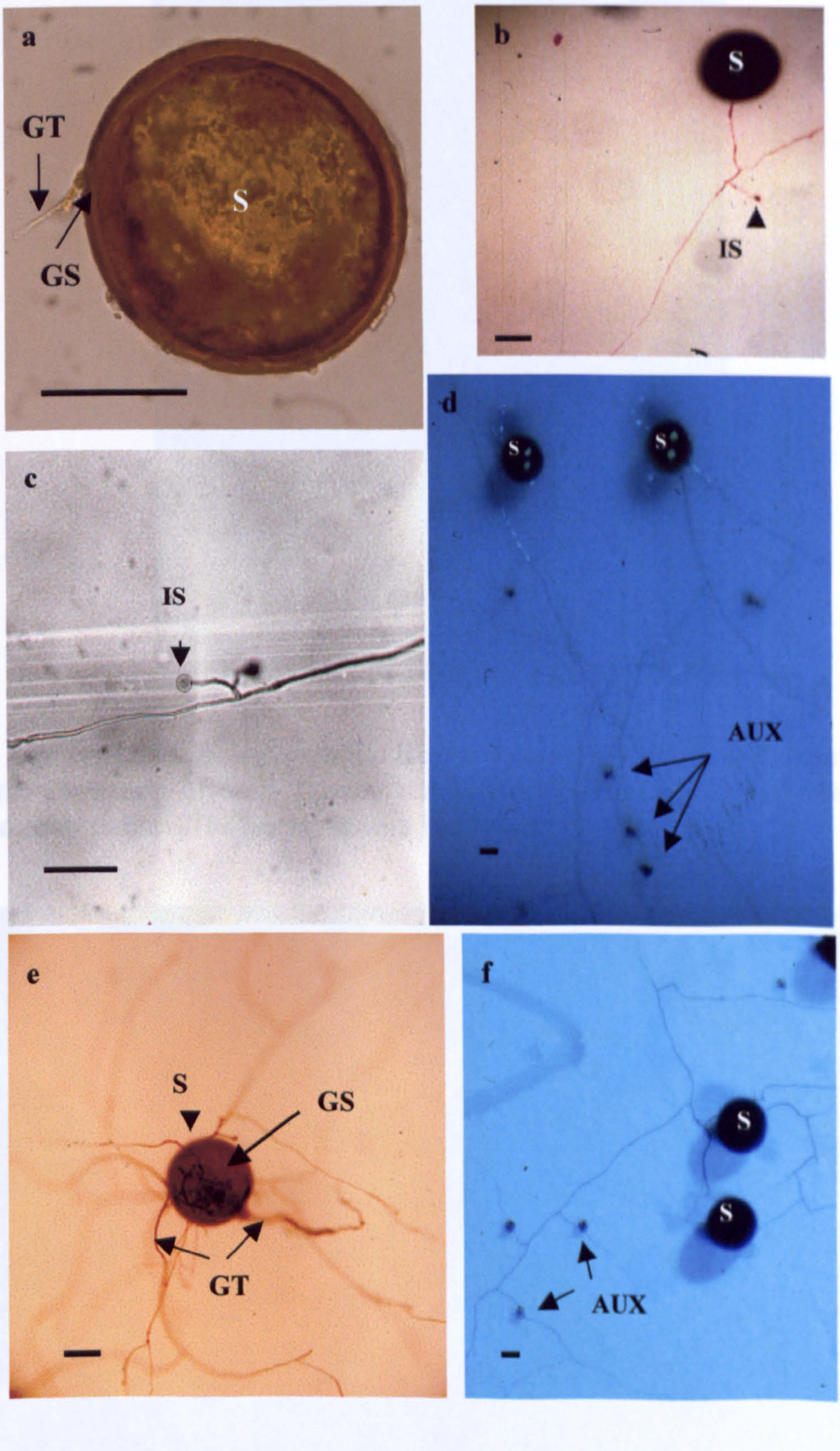


Figure 3. Spore germination of *Ac. tuberculata* BEG41 (a,b), *G. manihotis* BEG112 (c), *Gi. rosea* BEG111 (d), and *Sc. heterogama* BEG40 (e,f). *Acaulospora tuberculata* BEG41 germinated through a germination shield (GS) (a), and subsequently produced secondary branches and "immature spores" (IS) (b). Immature spores were produced by *G. manihotis* BEG112 (c). *Gigaspora rosea* BEG111 (d) and *Sc. heterogama* BEG40 (f) produced multiple germ tubes (GT), and subsequently secondary branches and auxiliary cells (AUX) were formed. *Scutellospora heterogama* BEG40 germinated through a germination shield (e). Bars represent 100 µm.

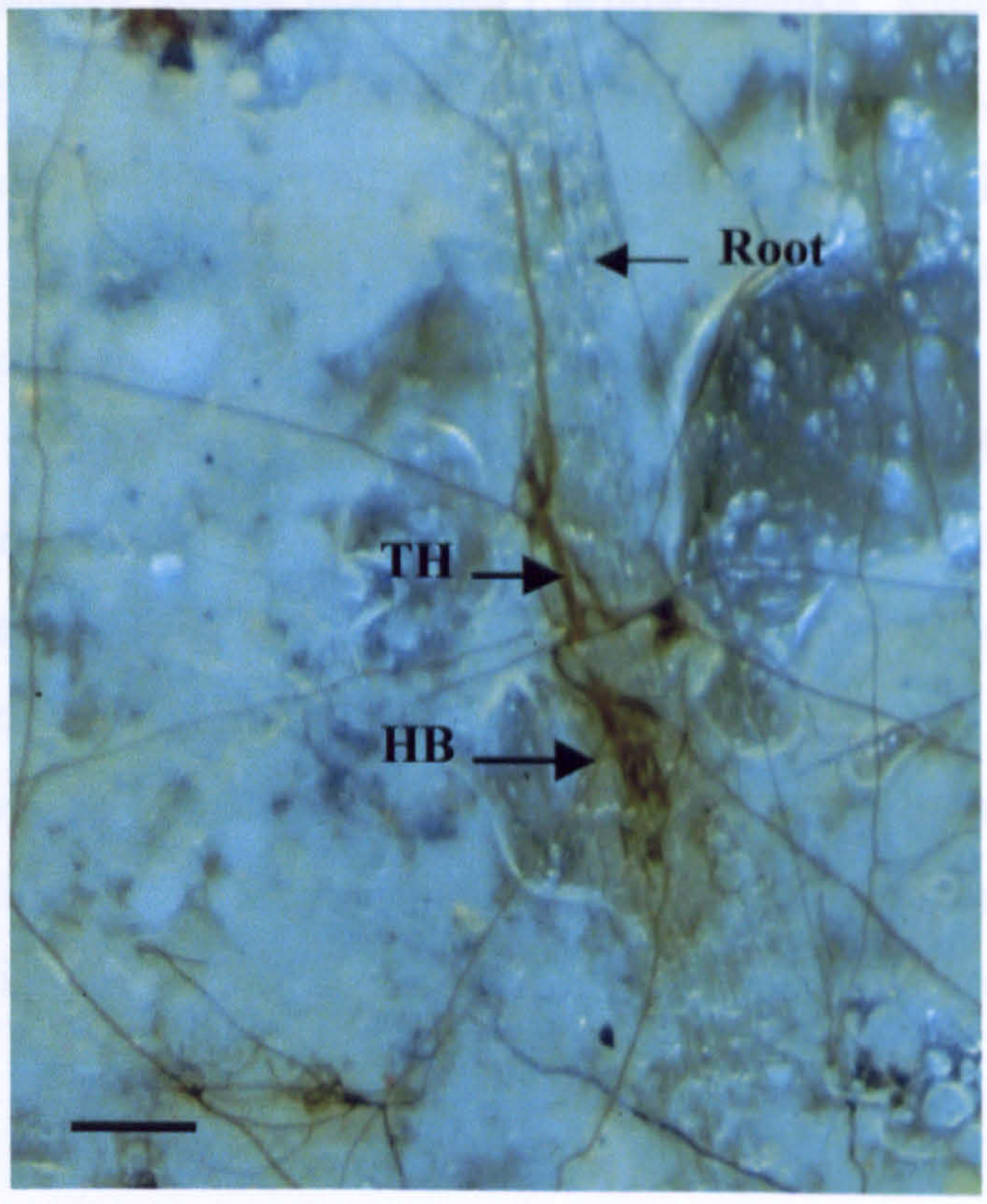


Figure 4. The hyphae of *Sc. heterogama* BEG40 became thickened and pignmented (TH) prior to the formation of entry points, and produced intensive hyphal branches (HB) around the entry points. The bar represents 100 µm.

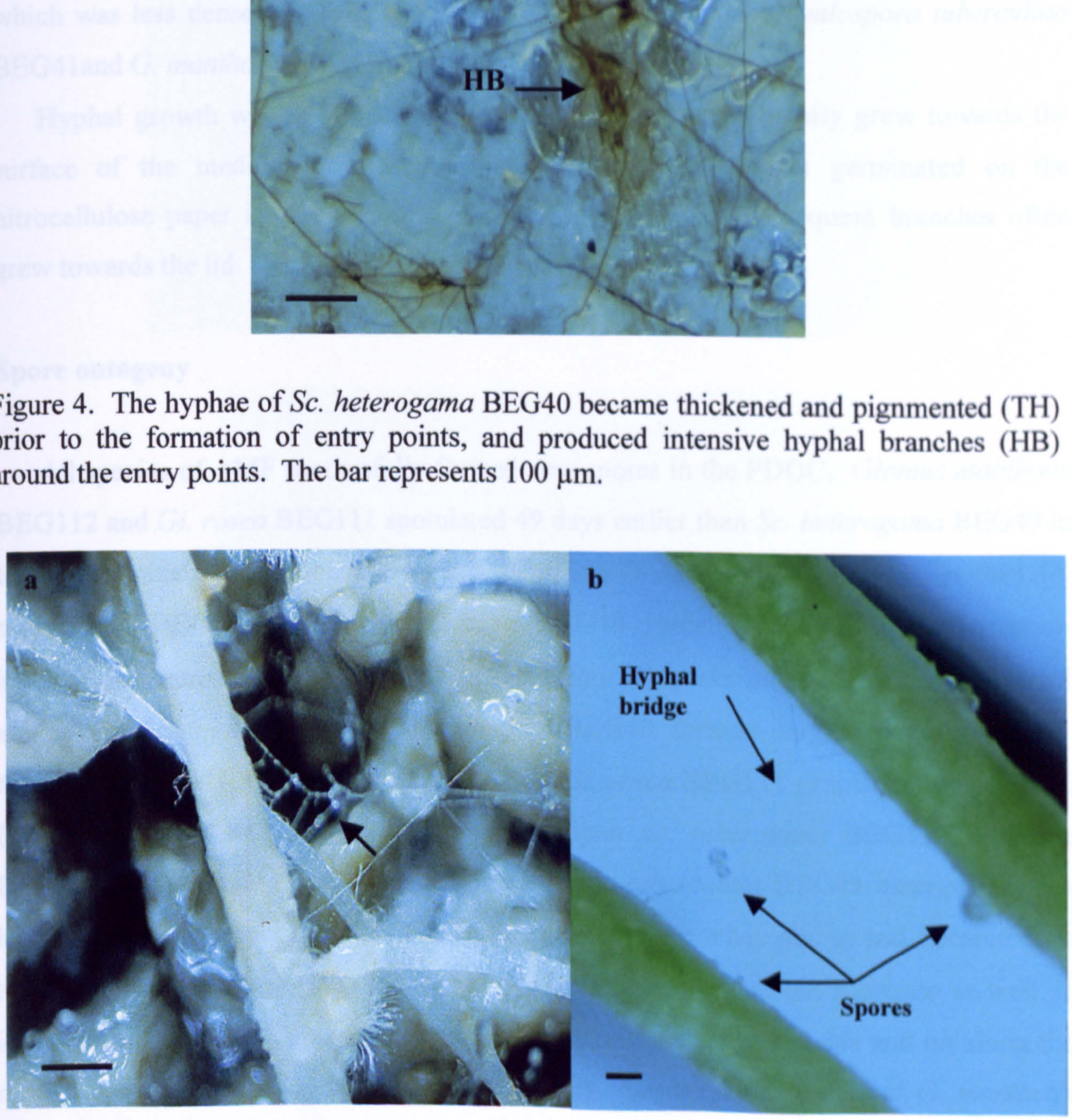


Figure 5. The ERM of of *G. manihotis* BEG112 formed dichotomous branches (arrow) when approaching a root (a), and also formed a hyphal bridge, which connected two adjacent mature, suberised roots of *P. falcataria* (4-month-old culture) (b). Spores were formed at the hyphal bridge as well as on the root around the bridge indicating that the hyphal bridge was formed at the earlier during colonisation, and the structure was maintained even when the root formed secondary growth. Bars represent 100 µm.

Short and fine intensive hyphal branching ("arbuscule-like" structures [ALS] or latter called branched absorbing structure [BAS] (*sensu* Bago *et al.* 1998a,b) was formed at the tips of hyphal branches in all AMF, but the shape and size varied (Fig. 6). *Acaulospora tuberculata* BEG41 formed a more intensive and shorter branching type (Fig. 6a) compared with *G. manihotis* BEG112, *Gi. rosea* BEG111, and *Sc. heterogama* BEG40. The BASs were also found around newly-formed spores of *G. manihotis* BEG112 within the substrate (6b). *Gigaspora rosea* BEG111 and *Sc. heterogama* BEG40 formed BASs which was less dense and had longer branches compared with *Acaulospora tuberculata* BEG41and *G. manihotis* BEG112 (Fig. 6b).

Hyphal growth was not positively geotropic, as hyphae frequently grew towards the surface of the medium, towards the root collar. When spores germinated on the nitrocellulose paper in the Petri-dish, the germ-tubes or the subsequent branches often grew towards the lid.

Spore ontogeny

All species of AMF successfully formed new spores in the PDOC. *Glomus manihotis* BEG112 and *Gi. rosea* BEG111 sporulated 49 days earlier than *Sc. heterogama* BEG40 in the pre-germinated-spore-inoculation treatment, and 78 days and 211 days earlier than *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 respectively, in the direct-spore-inoculation treatment (Table 3). The size and colour of spores formed by the AMF varied among species. *Scutellospora heterogama* BEG40 formed larger spores than *Ac. tuberculata* BEG41, *G. manihotis* BEG112, or *Gi. rosea* BEG111 (Table 4). In contrast, *G. manihotis* BEG112 formed smaller spores than *Ac. tuberculata* BEG41, *Gi. rosea* BEG111 or *Sc. heterogama* BEG40. Spores of *Ac. tuberculata* BEG41 emerged from a hyaline sporiferous saccule (7a). The spores were hyaline when young and became dark red-brown when mature (Fig. 7a). The spores were formed on the substrate as well as inside roots (Fig. 7b). *Glomus manihotis* BEG112 formed spores inside and on along the root (7c), and later also in the substrate (Fig. 6c). Some mature spores of *G. manihotis* BEG112 caused a splitting of the older roots close to the root collar. Spores were hyaline when young, and became white to yellow-brown when mature. Spores of *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 were formed singly in the soil at the end of hyphae and were formed from bulbous suspensors (Fig. 7d). Some of the newly formed spores of *Gi. rosea* BEG111 were found to germinate after reaching maturity. These spores still

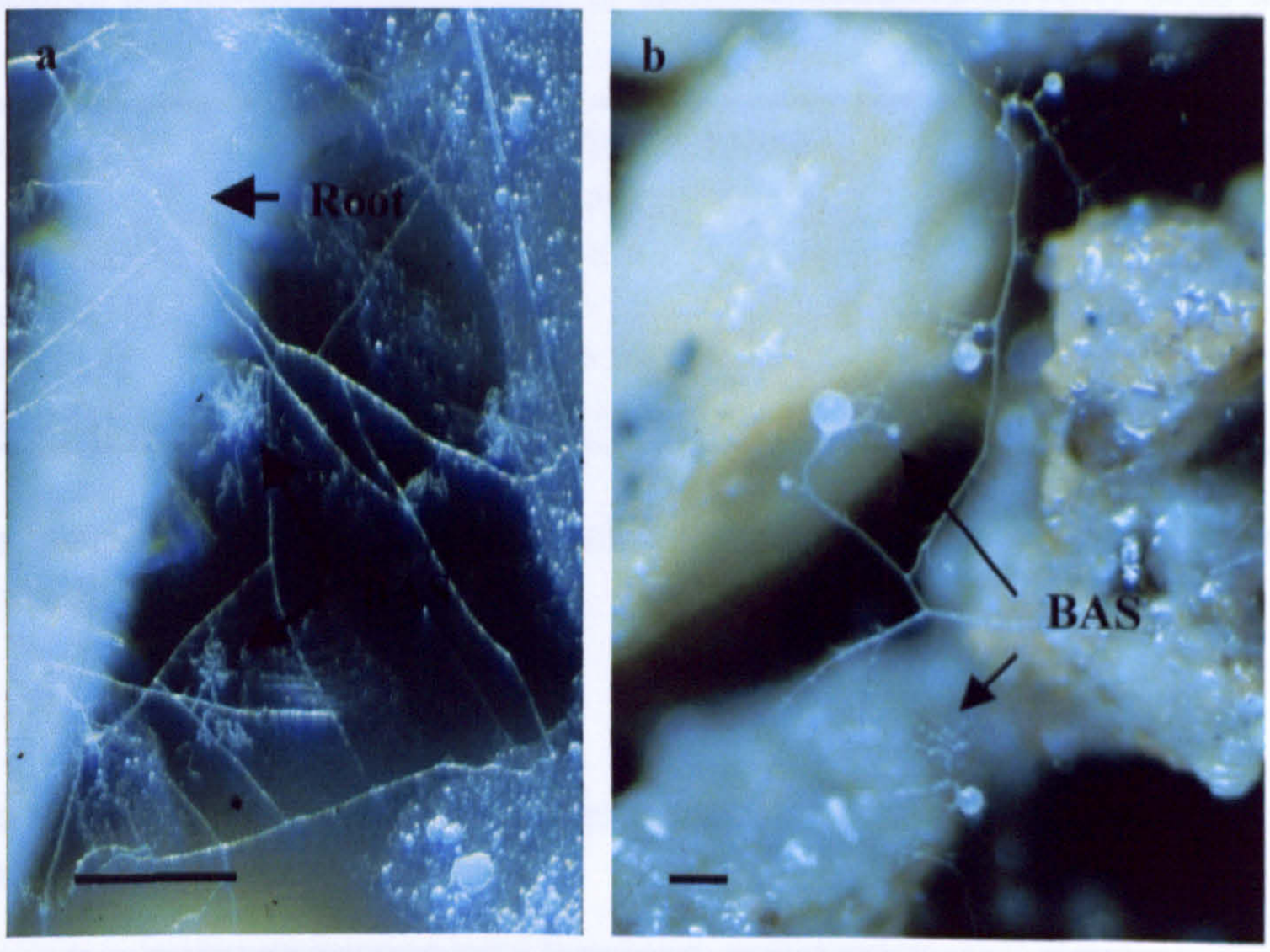


Figure 6. Branched absorbing structures (BAS) formed by different species of AMF. (a) *Ac. tuberculata* BEG41 formed intensive and short branching at hyphal tips. (b) Similar structures with less intensive branching were also produced around the newly formed spores of *G. manihotis* BEG112. (c) BAS of *Gi. rosea* BEG111 and *Sc. heterogama* BEG40; the box showing fine hyphal branching of BAS formed by *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 which was longer and less intensive compared with *Ac. tuberculata* BEG41. MH= main channel hyphae. Bars represent 100 μm.

Table 3. Time (days) from inoculation for the detection of mature new spores in the PDOC formed by four different species of AMF. Inoculation was conducted either with pre-germinated spores on nitrocellulose paper, or spores inoculated directly onto the root.

AMF species	Pre-germinated spore (dai)	Direct inoculation (dai)
Ac. tuberculata BEG41	nd	177+24*
G. manihotis BEG112	64+15	99+15
Gi. rosea BEG111	64+15	99+15
Sc. heterogama BEG40	113+15	310+24

dai=day after inoculation. *transplanted colonised-plant or inoculated with crude inoculum. nd=do data.

Table 4. The size of spores formed by different species of AMF belonging to four different genera in the PDOC.

AMF species	Spore size (μm)		
	minimum	mean	maximum
Ac. tuberculata BEG41	170	210c	260
G. manihotis BEG112	100	170d	240
Gi. rosea BEG111	160	230b	300
Sc. heterogama BEG40	210	280a	330

The values of the mean followed by the same letter were not significantly different (LSD test [$p \geq 0.05$]).

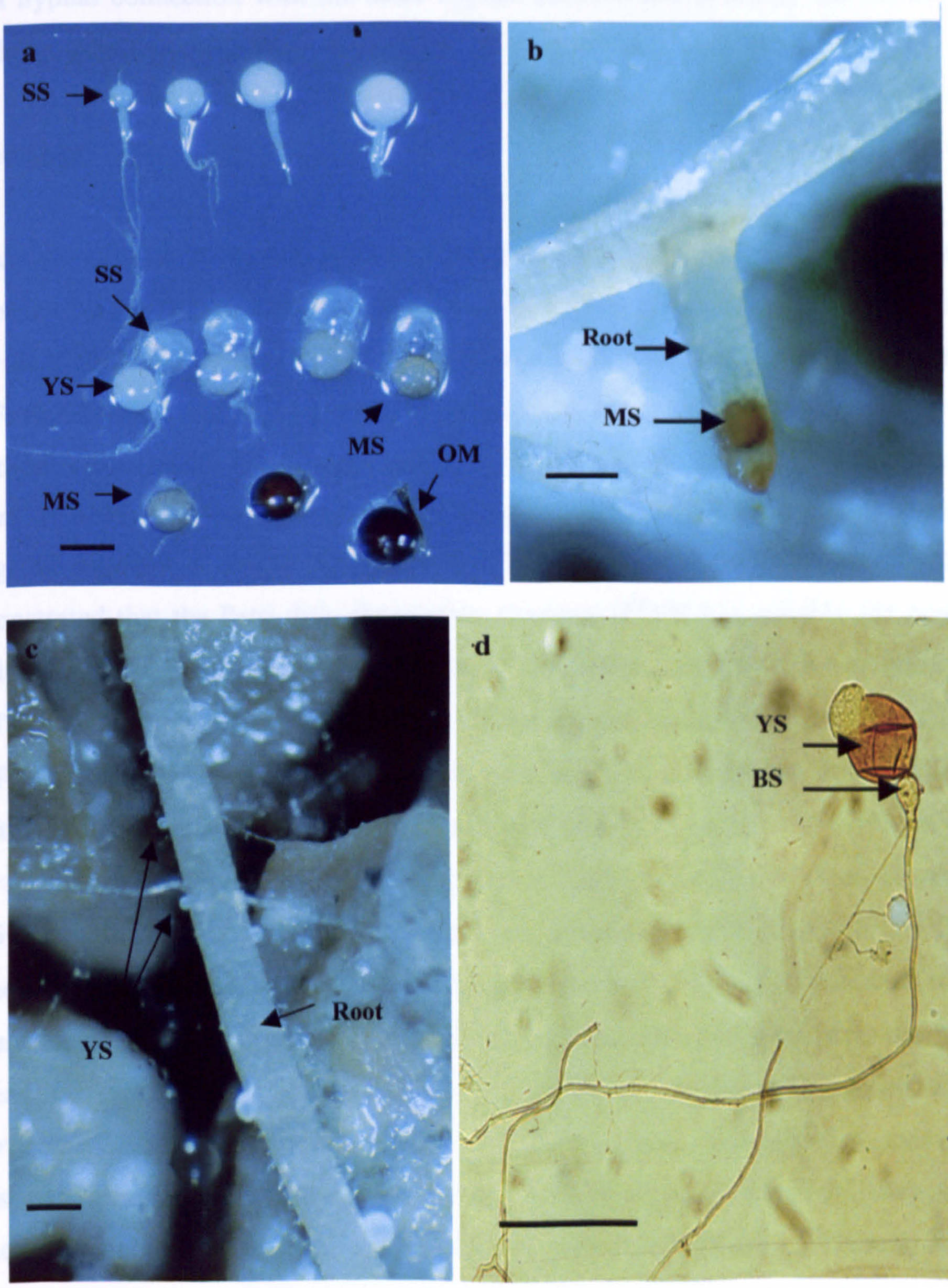


Figure 7. Spore production by different species of AMF. (a) Spores maturation of *Ac. tuberculata* BEG41 formed on the media initiated with sporiferous saccule (SS) (top row) then followed by the formation of the young spore (middle row) and finally the saccule collapse when the spore became mature and over-matured (OM) (bottom row); from left to right showing a stage of maturation. (b) Spores of *Ac. tuberculata* BEG41 were also formed inside roots. (c) *Glomus manihotis* BEG112 produced spores on the media and on along the roots. (d) *Gigaspora rosea* BEG111 and *Sc. heterogama* BEG40 formed spores singly on the substrate on bulbous suspensors. YS= young spore, MS= mature spore, and BB= bulbous suspensor. Bars represent 200 μm.

had a hyphal connection with the older hyphal network and probably the mother spore, which had already collapsed.

Discussion

A comparison of the life-cycles of AMF species from different genera has been hampered by the fact that these fungi cannot be grown axenically in the absence of the host plant (Siqueira *et al.*, 1985). Even for the most advanced technique for culturing AMF, transformed root organ culture, few species of AMF have been successfully grown, and these were mainly *Glomus* spp. along with two *Gigaspora* spp. Species from other genera of AMF, i.e. *Acaulospora* and *Scutellospora*, have not been reported as culturable using this method (S. Declerck, pers. comm.). However, the results reported here have demonstrated that the Petri-dish observation chamber (PDOC) is suitable for continuous monitoring of the establishment of 4 species of AMF belonging to 4 different genera.

The PDOC facilitated the observation of the growth and architecture of the ERM of AMF in an attapulgite clay. The data show that the ERM architecture that was described by Friese & Allen (1991), using a mixed inoculum of AMF, was predominantly of *Glomus* spp. (Dodd *et al.*, 2000) based on comparisons made with the ERM of *G. manihotis* BEG112 in this investigation. Both *Ac.tuberculata* BEG41 and *G. manihotis* BEG112 formed dichotomous hyphal branches when approaching the root ("infection network" *sensu* Friese & Allen, 1991). The other two species did not show this morphology. Instead, *Gi. rosea* BEG111 formed simple nondichotomising hyphal branches, while *Sc. heterogama* BEG40 had profuse randomly branching hyphae. The spatial growth of germ-tubes and subsequent branching observed in the PDS revealed that there was no tropic attraction shown towards roots, confirming the previous findings of Hepper & Mosse (1975) for an isolate of *G. mosseae* in root-organ culture and buried slide methods. Germ-tubes grew in all directions and often were negatively geotropic as observed previously by Watrud *et al.* (1978) for an isolate of *Gi. margarita.*

The PDOC allowed, for the first time, observation of the formation of "branched absorbing" structures (BAS, *sensu* Bago *et al.*, 1998b) by species of AMF belonging to different genera and maintained in non-agar media. Previous descriptions of the delicate hyphal structures called “rhizoid-like” structures (Hepper & Mosse 1975), or “arbuscule-like” structures (Declerck *et al.*, 1996; Bago *et al.*, 1998a); later called “branched-

absorbing" structures by Bago *et al.* (1998b) were based on observations made in root-organ cultures using *Glomus* species (*G. intraradices* DAOM 197198 and isolates of *G. mosseae* and *G. versiforme*). In previous studies, these structures might have been destroyed during sampling in conventional soil-based culture, or ignored or regarded as contaminants (Dodd, 1994). The formation of BAS in all species of AMF across the genera tested could be considered as distinctive morphological markers of successful AMF establishment, and they might play an important role in localised nutrient acquisition as suggested by Bago *et al.* (1998a).

Intensive hyphal exploration of the growth medium has been regarded as an important measure of successful colonisation (Smith & Read, 1997) and reflects a functioning mycorrhizal network in soils (Degens *et al.* 1996). However, if different ERM architectures are produced by AMF from unique genera the hyphal length measurements, often used to reflect effective mycorrhizas (Jacobsen *et al.*, 1992; Green *et al.*, 1994), may be misleading. Substantial differences in hyphal distribution in the PDOCs were observed in these studies (Dodd *et al.*, 2000) and unique patterns of spreading of ERM of *Ac. tuberculata* BEG41, *G. manihotis* BEG112, *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 in Terragreen have been observed. The ERM of the first two isolates tended to proliferate in the substrate around the roots, whilst the ERM of the last two isolates could spread rapidly to areas unoccupied by roots.

The spread of the ERM of AMF in the soil has been studied extensively by Jakobsen *et al.* (1992) who used two-compartment microcosms (root and hyphal [root-free] compartments) to study the spread of the ERM of *Acaulospora laevis* WUM 11(4), *Glomus* sp. WUM 10(1) and *Scutellospora calospora* WUM 12(2). They found that each of these AMF had different patterns for their hyphal spread in the soil. Using non-destructive sampling, our observations using different species of *Acaulospora*, *Glomus*, and *Scutellospora* in the PDOC at the early stage of AMF establishment, contradicted the findings of Jakobsen *et al.* (1992). Jakobsen *et al.* (1992) noted that the spread of the hyphae of *Scutellospora* declined more rapidly in the substrate away from roots compared with species of *Acaulospora*, which tended to be more consistent across the hyphal chamber. The cause for the differences between their results and the current study was in the type of observation chamber used and the nature of observation. In their system, the hyphal spread was determined by sampling the media at different positions in the hyphal compartment relative to the root compartment. In our observation chamber, the spread of the hyphae was determined by direct observation of the areas, which were not occupied by

roots in the observation chamber. It was clear that in these areas *Ac. tuberculata* BEG41 formed a very small volume of hyphal network. In contrast, a more intensive hyphal network was found for *Sc. heterogama* BEG40. Since the size of the Petri-dish was 9 or 18 cm diam., the average radial distance hypha could grow from colonised roots was 4.5 and 9 cm, respectively. In the study of Jakobsen *et al.* (1992), interestingly, the length density of the hyphae of *Sc. calospora* was much higher than that of *Ac. laevis* within 4.5 cm from the root compartment. Considering that the cultures of *Sc. calospora* and *Ac. laevis* planted in the root compartment were of the same age, the data indicate that the hyphae of *Sc. calospora* may spread quicker than *Ac. laevis*. In our study, the observation chamber allowed observation of the ERM spread and interaction with roots. Therefore, not only hyphal length, as in Jakobsen *et al.* (1992), but also the ERM architecture was different between species of AMF. Interestingly, the results of our observations on *G. manihotis* BEG112 confirmed the results of Jakobsen *et al.* (1992), where the spread of hyphae declined sharply away from roots. The spread of the ERM of *G. manihotis* BEG112 may have been facilitated by the presence of roots. This could be clearly observed in the PDOC where hyphal bridges connected roots to form new colonisation. Jakobsen *et al.* (1992) also found plants inoculated with *Ac. laevis* WUM 11(4) accumulated significantly higher P than those inoculated with *Glomus* sp. WUM 10(1) and *Sc. calospora* WUM 12(2), and this coincided with the fact that *Ac. laevis* WUM 11(4) had a higher hyphal length density in the hyphal compartment than the other two AMF isolates. In our studies, *Ac. tuberculata* BEG41 formed BASs with very intensive fine hyphal branching filling the gaps which were not explored by the main channel hyphae. This kind of BASs structure might have increased the efficiency of *Acaulospora* in absorbing nutrients in the substrate close to the roots of the host.

Incubation of spores of AMF on nitrocellulose membranes in Petri-dishes at 30°C facilitated the germination of all species tested, despite the subsequent contamination of *Ac. tuberculata* BEG41 by other fungi and bacteria. Temperature and moisture are important environmental factors for spore germination as suggested by other workers (Daniels & Trappe, 1980; Haugen & Smith, 1992; see also Smith & Read, 1997), especially for isolates of tropical species of AMF, e.g. *G. clarum* (Louis & Lim, 1988) and *G. intraradices* (Haugen & Smith, 1992), which germinated quicker and at higher percentages when incubated at 30°C. *Glomus manihotis* BEG112 and *Gi. rosea* BEG111 generally germinated quicker than *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40. The reason for the slow germination of *Ac. tuberculata* BEG41 and *Sc. heterogama*

BEG40, could have been spore dormancy (Tommerup, 1983; Gazey *et al.*, 1993; Boddington & Dodd, 1999). Tommerup (1983) found that spores of isolates of AMF *Acaulospora laevis*, *Scutellospora calospora*, *Glomus caledonium* and *Glomus monosporum* were innately dormant when first formed. However, the length of the period of dormancy varied among the AMF isolates, with the two *Glomus* spp. being the shortest (6 weeks), *Sc. calospora* (12 weeks), and the longest dormancy was experienced by the *Ac. laevis* isolate (6 months). The observations of strong dormancy factors in *Acaulospora* and *Scutellospora* support our data (Tommerup, 1983).

The data in our studies show that spores inoculated directly onto the plant roots in the PDOC were slower to germinate than those pre-germinated on nitrocellulose membranes in the absence of the plant and subsequently inoculated onto root in the PDOC. The differences in the time taken for spores to germinate between the genera of AMF, thus inoculation with pre-germinated spores is recommended for experimental purposes. Inoculation with pre-germinated spores can also increase the rate of colonisation of seedlings (Tommerup, 1984). The effects of the plant on spore germination of AMF reported so far have been equivocal. The enhancement of spore germination when host plant exudates were accessible have been reported (El-Atrach *et al.*, 1989; Gianinazzi-Pearson *et al.* 1989). Plant exudates have been shown to affect hyphal growth rather than spore germination (Carr *et al.*, 1985; Paula & Siqueira, 1990; Nair *et al.*, 1991). However, the effect of flavonoids in the exudates on hyphal growth of AMF seems to be specific. For example, Biochanin A which had a stimulatory effect on hyphal growth of *Glomus* sp. (Nair *et al.*, 1991) had inhibitory effects on spore germination and subsequent hyphal growth of *Gi. margarita* DAOM 194757 (Chabot *et al.*, 1992). Vosatka & Dodd (1998) also noted that the percentage of germination of *Ac. tuberculata* BEG41 was significantly reduced by the presence of the host plant. This was probably due to inhibitory compounds released by the host plant (Vierheilig & Ocampo, 1990). This variation in response of spores from AMF in different genera to plant presence may reflect their differing ecological strategies.

Acaulospora tuberculata BEG41 and *G. manihotis* BEG112 produced immature spores after germination on nitrocellulose membranes. The production of these structures in other species of *Glomus* has been reported, e.g. *G. clarum* CNPAB 5 (De Souza & Berbara, 1999), and *G. intraradices* (Haugen & Smith, 1992). De Souza & Berbara (1999) noted that the juvenile spores would develop into mature spores if colonisation was

successful. However, these structures have not previously been reported for *Acaulospora*, probably due to difficulties in germinating these spores.

Descriptions of spore ontogeny of AMF to date have been based on single time-point observations by sampling of pot-cultures or continuous observation using root-organ cultures. Using the latter technique, single isolates of AMF species were investigated, and comparisons between species of AMF across different genera were never made. Using the PDOC, the spore ontogeny of species of AMF from four different genera could be followed. As expected, *Ac. tuberculata* BEG41, *G. manihotis* BEG112, *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 differed in their spore ontogeny. The earlier sporulation of *G. manihotis* BEG112 and *Gi. rosea* BEG111 compared with *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 was probably caused by the greater delay in spore germination of the latter species of AMF and hence root colonisation. The result of delayed colonisation would be a lack of carbon for both ERM development and the production of spores. The large size of the spores of *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 would have added to the carbon requirement from the host plant. Dodd *et al.* (2000) suggested that the Gigasporaceae regulate phosphorus transfer to the plant in order to receive greater amounts of carbon for spore formation over a longer period of time. This is supported by evidence that the ERM of *Gi. rosea* BEG111 accumulated polyphosphate, but none was observed in the ERM of *G. manihotis* BEG112 using 4',6-diaminido-2-phenylindole (DAPI) staining technique (Boddington & Dodd, 1999). Similar observations of polyphosphate accumulation was found in *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 (Vosatka & Dodd, unpublished data). This reflects a different strategy of acquiring and using the resources between species of AMF.

The importance of understanding the development, structure, functioning and activities of the ERM of individual species of AMF has been highlighted (Dodd, 1994; Smith & Read, 1997; Dodd *et al.*, 2000). The PDOC developed in this study could provide a useful means to study the dynamics of ERM development and architecture over time. It is also clearly shown that AMF from different genera not only differ in their spore morphology, but also differ in their requirements for spore germination, strategies to colonise roots, in the spread of their ERM in the substrate, ERM architecture, and spore production, which might suggest clear differences in their ecological strategies and their function in the ecosystem. It is often assumed that all species of AMF have a similar function because of the ubiquity of the arbuscular mycorrhizal symbiosis and the fact that all AMF occupy the same plant/soil niche (Dodd *et al.*, 2000). The PDOC also has potential for studying the

interaction between AMF, rhizobia and leguminous plants. The pattern of AMF establishment as well as nodule formation will be easily observed using this technique. In addition, the PDOC can be used for culture production and maintenance. It is relatively easy to keep free of contamination and insects. However, if the technique is going to be used for spore production, optimisation of the system, such as choice of media, host, nutrients and other environmental conditions, especially light intensity, may be needed for each AMF species under the prevailing production conditions.

References

Allen MF, Moore Jr TS, Christensen M, Stanton N. 1979. Growth of vesicular-arbuscular mycorrhizal and non-mycorrhizal *Bouteloua gracilis* in a defined medium. *Mycologia* **71**: 666-669.

Bago B, Azcón-Aguilar C, Piché Y. 1998a. Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. *Mycologia* **90**: 52-62.

Bago B, Azcón-Aguilar C, Goulet A, Piché Y. 1998b. Branched absorbing structures (BAS): a feature of extra-matrical mycelium of symbiotic arbuscular mycorrhizal fungi. *New Phytologist* **139**: 375-388.

Bartolome-Esteban H, Schenck NC. 1994. Spore germination and hyphal growth of arbuscular mycorrhizal fungi in relation to soil aluminum saturation. *Mycologia* **86**: 217-226.

Boddington CL, Dodd JC. 1998. A comparison of the development and metabolic activity of mycorrhizas formed by arbuscular mycorrhizal fungi from different genera on two tropical forage legumes. *Mycorrhiza* **8**: 149-157.

Boddington CL, Dodd JC. 1999. Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life-cycle strategies. *New Phytologist* **142**: 531-538.

Brundrett M, Juniper S. 1995. Non-destructive assessment of spore germination of VAM fungi and production of pot cultures from single spores. *Soil Biology & Biochemistry* **27**: 85-91.

Carr GR, Hinkley MA, Le Tacon F, Hepper CM, Jones MGK, Thomas E. 1985. Improved hyphal growth of two species of vesicular-arbuscular mycorrhizal fungi in the presence of suspension-cultured plant cells. *New Phytologist* **101**: 417-426.

Chabot S, Bécard G, Piché Y. 1992. Life-cycle of *Glomus intraradix* in root organ culture. *Mycologia* **84**: 315-321.

Daniels BA, Trappe JM. 1980. Factors affecting spore germination of the vesicular-arbuscular mycorrhizal fungus, *Glomus epigaeus*. *Mycologia* **72**: 457-471.

De Souza FA, Berbara RLL. 1999. Ontogeny of *Glomus clarum* in Ri T-DNA transformed roots. *Mycologia*, **91**: 343-350.

Declerck S, Strullu DG, Plenchette C. 1996. In vitro mass production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. *Mycological Research* **100**: 1237-1242.

Degens BP, Sparling GP, Abott LK. 1996. Increasing the length of hyphae in a sandy soil increases the amount of water-stable aggregates. *Applied Soil Ecology* **3**: 145-159.

Dodd JC. 1994. Approaches to the study of the extra-radical mycelium of arbuscular mycorrhizal fungi. In: Gianinazzi S, Schüepp H, eds. *Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems*. Basel, Switzerland: Birkhäuser, 147-166.

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I. 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant and Soil.* (in press)

Douds Jr DD, Galvez L, Bécard G, Kalpunik Y. 1998. Regulation of arbuscular mycorrhizal development by plant host and fungus species in alfalfa. *New Phytologist* **138**: 27-35.

El-Atrach F, Vierheilig H, Ocampo JA. 1989. Influence of non-host plants on vesicular-arbuscular mycorrhizal infection of host plants and on spore germination. *Soil Biology & Biochemistry* **21**: 161-163.

Friese CH, Allen MF. 1991. The spread of VA mycorrhizal fungal hyphae in the soil: inoculum type and external hyphal architecture. *Mycologia* **83**: 409-418.

Gazey C, Abbott LK, Robson AD. 1993. VA mycorrhizal spores from three species of Acaulospora: germination, longevity and hyphal growth. *Mycological Research* **97**: 785-790.

Gemma JN, Koske RE. 1988. Pre-infection interactions between roots and the mycorrhizal fungus *Gigaspora gigantea*: chemotropism of germ tubes and root growth response. *Transaction of the British Mycological Society* **91**: 123-132.

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S. 1989. In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. *Symbiosis* **7**: 243-255.

Giovannetti M, Sbrana C, Logi C. 1994. Early processes involved in host recognition by arbuscular mycorrhizal fungi. *New Phytologist* **127**: 703-709.

Giovannetti M, Sbrana C, Citernesi AS, Avio L. 1996. Analysis of factors involved in fungal recognition responses to host-derived signals by arbuscular mycorrhizal fungi. *New Phytologist* **133**: 65-71.

Green DC, Vilariño A, Newsam R, Jeffries P, Dodd JC. 1994. Quantrification of mycelial development of arbuscular mycorrhizal fungi using image analysis. *Mycorrhiza* **5**: 105-113.

Grey WE. 1991. Influence of temperature on colonisation of spring barleys by vesicular-arbuscular mycorrhizal fungi. *Plant and Soil* **137**: 181-190.

Haugen LM, Smith SE. 1992. The effect of high temperature and fallow period on infection on mung bean and cashew roots by the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. *Plant and Soil* **145**: 71-80.

Hepper C.M. 1984. Isolation and culture of VA mycorrhizal (VAM) fungi. In: Powell CL, Bagyaraj DJ, eds. *VA mycorrhiza*. Florida: CRC Press, 95 – 112.

Hepper CM, Mosse B. 1975. Techniques used to study the interaction between *Endogone* and plant roots. In: Sanders FE, Mosse B, Tinker PB, eds. *Endomycorrhizas: Proceedings of a symposium held at the university of Leeds*, July 22-25, 1974. London, Academic Press: 65-75.

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

Jarstfer AG, Farmer-Koppenol P, Sylvia DM. 1998. Tissue magnesium and calcium affect arbuscular mycorrhiza development and fungal reproduction. *Mycorrhiza* **7**: 237-242.

Jolicoeur M, Germette S, Gaudette M, Perrier M, Bécard G. 1998. Intracellular pH in arbuscular-mycorrhizal fungi: a symbiotic physiological marker. *Plant Physiology* **116**: 1279-1288.

Louis I, Lim G. 1988. Effect of storage of inoculum on spore germination of a tropical isolate of *Glomus clarum*. *Mycologia* **80**: 157-161.

Maschke M, Berta G, Gianinazzi S, Moncousin C. 1996. Effects of endomycorrhizal infection on root system development in the apple rootstock (*Malus domestica* Borkh) M26. In: Azcón-Aguilar C, Barea JM, eds. *Mycorrhizas in integrated systems from genes to plant development. Proceeding of the fourth European symposium on mycorrhizas.* Brussels: European Commission, 349-352.

Menge JA. 1984. Inoculum production. In: Powell CL, Bagyaraj DJ, eds. *VA Mycorrhiza*. Florida: CRC Press, 187-203.

Morton JB. 1990. Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia* **82**: 192-207.

Mosse B. 1959. Observations on the extra-matrical mycelium of a vesicular-arbuscular endophyte. *Transaction of the British Mycological Society* **42**: 439-448.

Mosse B. 1962. The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. *Journal of General Microbiology* **27** : 509 – 520.

Mosse B, Hepper CM. 1975. Vesicular-arbuscular mycorrhizal infections in root organ cultures. *Physiology and Plant Pathology* **5**: 215-223.

Mosse B, Phillips JM. 1971. The influence of phosphate and other nutrients on the development of vesicular-arbuscular mycorrhiza in culture. *Journal of General Microbiology* **69** : 157-166.

Nair MG, Safir GR, Siqueira JO. 1991. Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. *Applied and Environmental Microbiology* **57**: 434-439.

Norman JR, Atkinson D, Hooker JE. 1996. Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae*. *Plant and Soil* **185**: 191-198.

Paula MA, Siqueira JO. 1990. Stimulation of hyphal growth of the VA mycorrhizal fungus *Gigaspora margarita* by suspension-cultured *Pueraria phaseoloides* cells and cell products. *New Phytologist* **115**: 69-75.

Pawlowska TE, Douds Jr. DD, Charvat I. 1999. *In vitro* propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. *Mycological Research* **103**: 1549-1556.

Persad-Chinnery SB, Chinnery LE; Dales RBG. 1992. Enhancement of in vitro spore germination of *Gigaspora rosea* by cellulase preparation. *Mycological Research* **8**: 626-628.

Safir GR, Coley SC, Siqueira JO, Carlson PS. 1990. Improvement and synchronisation of VA mycorrhizal fungal spore germination by short-term cold storage. *Soil Biology & Biochemistry* **22**: 109-111.

Setiadi Y. 1995. The practical application of arbuscular mycorrhizal fungi for reforestation in Indonesia. PhD thesis, University of Kent at Canterbury, Kent, England.

Siqueira JO, Sylvia DM, Gibson J, Hubbell DH. 1985. Spores, germination, and germ tubes of vesicular-arbuscular mycorrhizal fungi. *Canadian Journal of Microbiology* **31**: 965-972.

Smith SE, Read DJ. 1997. Mycorrhizal Symbiosis. 2nd edition. London: Academic Press.

Somasegaran P, Hoben HJ. 1994. Handbook for rhizobia: methods in legume-rhizobium technology. Springer-Verlag, New York.

Sylvia DM, Schenck NC. 1983. Germination of clamydospores of three *Glomus* species as affected by soil matric potential and fungal contamination. *Mycologia* **75**: 30-35.

Tommerup IC. 1983. Spore dormancy in vesicular-arbuscular mycorrhizal fungi. *Transaction of British Mycological Society* **81**: 37-45.

Tommerup IC. 1984. Persistence of infectivity by germinated spores of vesicular-arbuscular mycorrhizal fungi in soil. *Transaction of British Mycological Society* **82**: 275-282.

Vierheilig H, Ocampo JA. 1990. Effect of isothiocyanates on germination of spores of *G. mosseae*. *Soil Biology & Biochemistry* **22**: 1161-1162.

Vosatka M, Dodd JC. 1998. The role of different arbuscular mycorrhizal fungi in the growth of *Calamagrotis villosa* and *Deschampsia flexuosa*, in experiments with simulated acid rain. *Plant and Soil* **200**: 251-263.

Vilariño A, Frey B, Schüepp H. 1997. MES [2-(N-morpholine)-ethane sulphonic acid] buffer promotes the growth of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices* in an alkaline sand. *Biology and Fertility of Soil* **25**: 79-81.

Wang CL, Tschen JSM. 1994. Production of mycorrhizal inoculum by soilless culture. *Journal of Agricultural Association of China* **167** : 50 – 60.

Watrud LS, Heithaus JJ III, Jaworski EG. 1978. Geotropism in the endomycorrhizal fungus *Gigaspora margarita. Mycologia* **70**: 449-452.

VI. Conclusions

The studies described in this thesis have revealed three important findings:

1. Rhizobial isolates nodulating *Acacia mangium* and *Paraserianthes falcataria* (two tree species indigenous to Indonesia) were phenotypically diverse, but were closely related to *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum*. These rhizobial isolates were ubiquitous throughout the Indonesian archipelago. PCR-RFLP-SSCP was the principal method for establishing relationships and is a rapid, reliable, reproducible and relatively cost-effective technique.
2. Functional diversity (measured by plant growth and N-fixation) of rhizobia nodulating *A. mangium* and *P. falcataria* increased in the presence of arbuscular mycorrhizal fungi (AMF). Relationships between AMF and rhizobial isolates in promoting plant growth and nutrient acquisition of *P. falcataria* were unique, and there was no species of AMF which was best for all rhizobial isolates, and *vice versa*. Under the conditions used, *A. mangium* was more selective in its AMF partner and rhizobial symbionts, compared with *P. falcataria*, showing optimal compatibility only with *Gi. rosea* BEG111 and *B. elkanii* isolates.
3. The Petri-dish microcosm (PDM) is a novel non-agar based system which is useful for studying the establishment of AMF species from different genera without destructive sampling. The species of AMF from the different genera exhibited different patterns for the development of their extra-radical structures, which may reflect their ecological strategies and their requirements to complete their life-cycles.

1. Phenotypic and genotypic characteristics of rhizobia nodulating *A. mangium* and *P. falcataria* provenances from Indonesia

This study showed that rhizobia nodulating Indonesian provenances of *A.mangium* and *P. falcataria* isolated from different locations across the Indonesian archipelago were phenotypically diverse. However, in general, these isolates had typical characteristics of the genus *Bradyrhizobium* (Chapter 2). These confirm previous studies on the phenotypic characterisation of rhizobia nodulating these tree species (Souvannavong & Galiana, 1991; Turk & Keyser, 1992; Moreira *et al.*, 1993). Further characterisation of these isolates using molecular techniques (PCR-RFLP-SSCP and sequence analysis of the 16S rRNA gene) confirmed that these isolates were *Bradyrhizobium* and could be separated into two

distinct groups: *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* (Chapter 3). The data support the results of earlier work on the genetic characterisation of rhizobia nodulating *A. mangium* (Nuswantara et al., 1997) and *P. falcataria* (Moreira *et al.*, 1998) where these trees were nodulated by *B. elkanii*. In addition, *A. mangium* was also nodulated by rhizobial isolates closely related to *Mesorhizobium loti*. Data from the present study also showed that some rhizobia nodulating *A. mangium* shared identical genetic characteristics with some of those of *P. falcataria* as indicated by PCR-RFLP-SSCP analysis, and this was confirmed by sequence analysis, of the 16S rRNA gene.

The data highlight the need to characterise large numbers of isolates of rhizobia from diverse geographical areas to reveal the true diversity and species richness of rhizobial species nodulating a particular leguminous tree. The use of PCR-RFLP-SSCP, as demonstrated here, enabled the identification of a large number of rhizobial isolates relatively quickly and economically compared with other currently available molecular techniques (such as PCR-RFLP, Rep-PCR, and PCR-RAPD) with a sensitivity comparable with DNA sequencing (Chapter 3). The fact that rhizobia nodulating *A. mangium* and *P. falcataria* were closely related to soybean bradyrhizobia (*B. elkanii* and *B. japonicum*) following molecular characterisation, did not suggest that these isolates could automatically nodulate soybeans. This was checked in host-range experiment (Chapter 2), which showed that not all of the *B. elkanii* and *B. japonicum* isolates from *A. mangium* and *P. falcataria* were capable of nodulating soybeans. This shows that some of rhizobia from these tree species may differ from those of soybeans at the biovar level. The presence of rhizobial isolates from other host plants other than soybeans, but genetically close to *B. elkanii* and *B. japonicum* has been noted by Young (1996). He suggested the need to define species within *Bradyrhizobium* according to overall phenotypic and genotypic similarity, and independently from the ability to nodulate soybean.

2. Distribution of rhizobial isolates in Indonesia nodulating *A. mangium* and *P. falcataria*

The study shows that rhizobial isolates nodulating *A. mangium* and *P. falcataria* were ubiquitous across the Indonesian archipelago (Chapter 2, 3). They were found in all sampling sites, except in Lampung and Irian Jaya, where only one isolate (*B. elkanii*) was found in each location. A repeated sampling in these two locations will be necessary to confirm the absence or presence of *B. japonicum* isolates. Reports from other studies

show that these species could also be found in the Eastern North America (Parker 1999), South-Western Australia (Marsudi *et al.* 1999), and Brazil (Moreira *et al.* 1998), nodulating diverse species of legumes, such as *Apios americana*, *Desmodium glutinosum*, *Acacia saligna*, *Glycine* spp., as well as *A. mangium* and *P. falcataria*. This provides further evidence that these species are ubiquitous. Since *A. mangium* and *P. falcataria* are not native to all but one (Irian Jaya) of the sampling locations, it would be interesting to determine which are the alternative host plants for rhizobia in the area. This would provide useful information for a management and *in situ* conservation strategy of these rhizobial isolates. In a wider application, the presence of these plants could be used as an indicator for the suitability of a particular location for the establishment of new plantations of *A. mangium* and *P. falcataria*.

3. Interactions between AMF and rhizobia on growth of *A. mangium* and *P. falcataria*

The study of the interactions between AMF and rhizobia on *A. mangium* and *P. falcataria* clearly show that both synergistic and antagonistic relationships existed, as determined by the effects on plant growth . The strategy to select efficient combinations of the microsymbionts could be different for *A. mangium* and *P. falcataria*. For *A. mangium*, early identification of the rhizobial isolate to species level could reduce the number of isolates included in the screening for the effectiveness of the isolates to promote plant growth, since this species forms an effective symbiosis only with isolates of *B. elkanii* (Chapter 4). In contrast, it is hard to predict the outcome of interactions between different AMF and rhizobial isolates on *P. falcataria*. This latter tree species can be nodulated and fix-N effectively when nodulated by isolates of both *B. elkanii* and *B. japonicum*, but the effectiveness varied considerably among isolates. These efficiencies, however, could change substantially in the presence of AMF in the plant roots. Therefore, selection for efficient combinations of AMF and rhizobia for *P. falcataria* by screening the microsymbionts individually may produce misleading data. Future work needs to look at multiple interactions between several apparently effective AMF and rhizobial isolates together.

The data also showed that the symbioses between AMF, rhizobia and leguminous trees became crucial when some environmental factors were limiting. Contrasting results were demonstrated in this study, when two species of leguminous trees which have different

light intensity demands, were compared. *Acacia mangium* has a high demand for light (shade intolerant species), and did not start growing until 12 weeks after inoculation, as indicated by the height growth data. This growth coincided with a period when both light intensity and daylength increased, i.e. at the beginning of spring despite supplementary lighting (data not shown). In contrast, *P. falcataria* was more shade-tolerant than *A. mangium* and started growing 7 weeks after inoculation (during a period in winter). The implication of these differences is that carbon availability is needed for growth of all three partners in the symbiosis. The outcome of the tripartite symbiosis for plant growth is the result of competition between the differing nutrient sinks occurring within the tripartite symbiosis (Fig. 1). When the flow of resources (C, N, and P) from the plant photosynthate is positive, the outcome of the symbiosis will result in an improved plant growth and nutrient status. This could be demonstrated in the synergistic symbiosis between *Glomus manihotis* BEG112, PFJB1040 and *P. falcataria* which enhanced plant growth more than inoculation with either single microsymbiont alone, or in an uninoculated control (Chapter 4). In contrast, if there is an incompatible relationship this could result in the reduction of plant growth, e.g. the interaction between *Sc. heterogama* BEG40, *B. japonicum* isolates and *A. mangium* which caused plant growth depressions compared with inoculation with the effective rhizobial isolate alone. The possibility is that *A. mangium*, a shade intolerant species, could not manufacture and release sufficient C under low winter light intensities, to counterbalance the drain to the microsymbionts resulting in plant growth depression. Studies of photosynthate partitioning in nodulated plants have been reported and reviewed by Pate (1996). It was estimated that 27% (*Vigna unguiculata*) to 47% (*Lupinus albus*) of photosynthate from shoot was allocated to the root and nodules. Jakobsen & Rosendahl (1990) reported that 20% of the photosynthate from cucumber seedlings was allocated to the AMF when plants were inoculated with an isolate of *Glomus fasciculatum*. However, no such figures are available for photosynthate distribution in tripartite symbioses and this needs further investigation.

4. Establishment of arbuscular mycorrhizas from four different genera of AMF

The PDM is a new system to study the development of AMF and has been shown to be suitable to see the establishment of *Acaulospora tuberculata* BEG41, *Glomus manihotis* BEG112, *Gigaspora rosea* BEG111, and *Scutellospora heterogama* BEG40 on plant roots

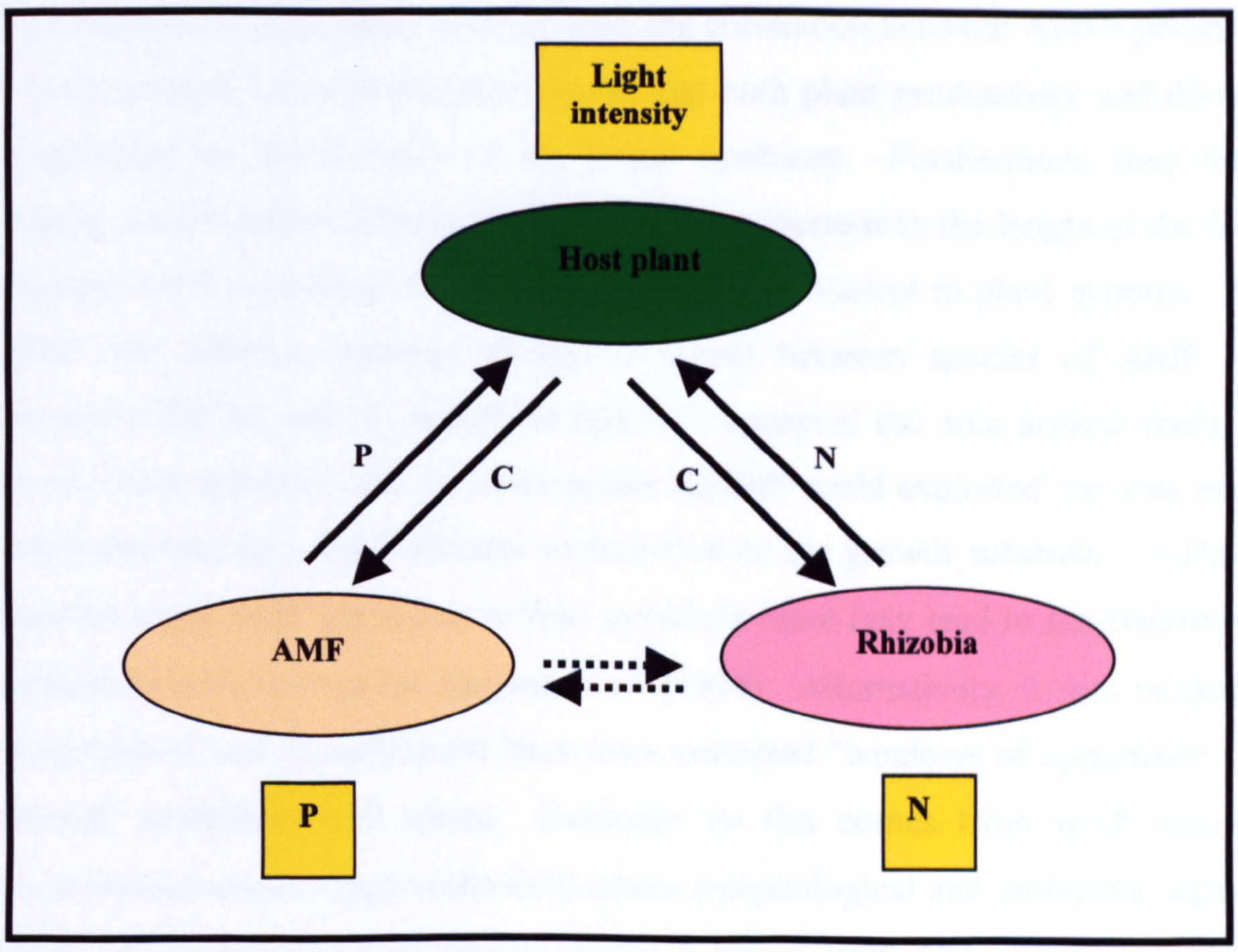


Figure 1. A diagram showing the relationships between AMF, rhizobia and the host plant in C, N and P demands and supply to and from each symbiont. When environmental factors, such as light intensity, N and P (yellow boxes) are limiting, the effectiveness of the symbiont which is responsible for providing the resources (closest to the corresponding yellow boxes) will determine the overall outcome of the symbioses. Arrows with broken line connecting AMF and rhizobia indicate that direct transfer of resources between these microsymbionts has not been shown.

(Chapter 5). The non-agar based system could be easily used to observe the whole course of development of fungal-root interactions as well as the extra-radical structures of AMF without destructive sampling. It was found that these AMF species were unique with regard to spore germination, root colonisation, the architecture of the extra-radical mycelium (ERM), the spread of the ERM in the media, and sporulation. Van der Heijden *et al.* (1998) in a recent study to investigate the correlation between AMF-species richness and plant productivity and diversity, found that both plant productivity and diversity can be dependent on the diversity of the fungal symbiont. Furthermore, they found that increased AMF-species richness led to a significant increase in the length of the ERM, to a decreased soil P concentration, and to an increased P content in plant material. Our data showed that different patterns of hyphal spread between species of AMF (e.g. *Ac. tuberculata* BEG41 and *G. manihotis* BEG112 occupied the area around roots, co-exist with *Gi. rosea* BEG111 and *Sc. heterogama* BEG40 could exploited the area away from roots) could lead to a more efficient exploitation of the growth substrate. Differences in the period when AMF are active in their symbiotic state may lead to the stability of plant communities seen by Van der Heijden *et al.* (1998). Alternatively, it may be that species of *Acaulospora* and *Scutellospora* have more restricted "windows of opportunity" to form functional symbioses with plants. Evidence for this comes from work with bluebell (*Hyacinthoides non-scripta*) in the field where morphological and molecular signatures of AMF in roots were compared (Merryweather & Fitter, 1995; Helgason *et al.*, 1999). The frequency of occurrence of *Scutellospora* sp. signatures peaked in December compared with July for *Acaulospora* spp. There is no evidence that all AMF behave similarly in their symbiotic states, e.g. efficient P-uptake and transfer to the plant, or protection from a pathogen (Newsham *et al.*, 1995). Those in situ studies remain to be done.

The result of AMF-plant interactions in the presence of other symbionts, e.g. rhizobia, as demonstrated in the interaction experiment (Chapter 4) can be explained partly by the life-cycles of the AMF species observed in the PDM. The completion of the life-cycle (the formation of spores) in species of AMF from the Gigasporaceae takes longer than those from species of *Glomus* and their spores are much larger. Spore formation also requires carbohydrate from the plant photosynthate. Dodd *et al.* (2000) suggested that the Gigasporaceae regulate phosphorus transfer to the plant in order to receive greater amounts of carbon for spore formation over a longer period of time. This is supported by evidence that the ERM of *Gi. rosea* BEG111 accumulated polyphosphate, but none was observed in the ERM of *G. manihotis* BEG112 using 4',6-diaminido-2-phenylindole

(DAPI) staining technique (Boddington & Dodd, 1999). Similar observations of polyphosphate accumulation were noted in *Ac. tuberculata* BEG41 and *Sc. heterogama* BEG40 (Vosatka & Dodd, unpublished data). However, polyphosphate was never detected in auxiliary cells or in older mycelium supporting mature spores of *Gigaspora* and *Scutellospora* spp., and the decline in polyphosphate accumulation was followed by the increase of alkaline phosphatase activity in the auxiliary cells (Boddington & Dodd, 1999). This might indicate a greater P transfer activity from the AMF to the host after spores were formed. Therefore, *Sc. heterogama* BEG40 may be depressing growth of *A. mangium* compared to *Gi. rosea* BEG111 (both belong to the Gigasporaceae) by draining C from the host over a longer period until spores form. Boddington & Dodd (1999) noted that spore formation by *Gi. rosea* BEG111 was immediately after the first flush of auxiliary cell formation (7 week). In this and other studies we noted that spore formation takes much longer in some species of *Scutellospora*. Pearson & Schweiger (1994), in studies with an isolate of *Scutellospora calospora* growing on *Trifolium subteraneum*, showed that this delay in spore production was not observed and sporulation occurred also after 7 weeks. This indicates that there maybe considerable interspecific variation as well as intergeneric. From the PDM studies it was found that *Sc. heterogama* BEG40 sporulated later and had significantly larger spores compared with *Gi. rosea* BEG111. As a result, *Sc. heterogama* BEG40 required carbohydrate more than the latter species over an extended time period, thus increasing carbon drain and possible causing plant growth depression if C is limiting.

5. Application of AMF and rhizobia in Indonesian forestry

A significant role of legumes in maintaining the productivity of polycultures (a mixture of plants in the same area) has been reported recently by Hector *et al.* (1999). They investigated the relationships between plant diversity and productivity in grasslands across 7 countries in Europe, and noted that productivity (accumulated net annual aboveground biomass, estimated from one or two harvest in each season around the time of peak biomass) of grasslands declines with the loss of plant diversity. In particular they found that the omission of an N-fixing *Trifolium pratense* markedly reduced productivity of the grassland by 360 g m^{-2}. This shows the importance of leguminous species as N suppliers for the ecosystem. In this present study, it is clear that the productivity of

leguminous trees was determined by the effectiveness of AMF and rhizobia associated with their roots. Considering the high diversity of rhizobia nodulating *A. mangium* and *P. falcataria* in Indonesia, in terms of phenotypic and genotypic characteristics, and especially their effectiveness in improving plant growth and nutrient acquisition, screening and inoculation of rhizobia in forestry is highly recommended. The fact that AMF could alter the effectiveness of rhizobia, it is essential that rhizobia should be screened on mycorrhizal plants.

The application of dual inoculants, AMF and rhizobia, in forestry will significantly reduce the cost of fertilisers, especially N and P, which are commonly difficult to obtain in isolated forest regions. In contrast, AMF inoculum could be produced on site and applied at a low-cost, such as that demonstrated by Setiadi (1995) in Indonesia. Rhizobial inoculation is usually economical, since a small amount of inoculum can be mixed with a large amount of seed prior to sowing (Young & Burns, 1993). However, before the best combinations of AMF and rhizobia obtained in our study can be used for large-scale application, several field experiments need to be done to investigate the stability of the combinations to improve plant growth and nutrient acquisition in the presence of indigenous species in forest nurseries under various environmental conditions. This should become the a priority for the future studies. With the advent of molecular techniques, detection of the survival and competitiveness of inoculum of rhizobia and AMF in the field with indigenous isolates should become easier. Techniques for the detection of rhizobia released in the environment have been summarised by Wilson (1995), including PCR-fingerprinting and marker genes. Several techniques are also available to detect AMF in roots, such as mycorrhiza-specific isoenzymes (Tisserant *et al.* 1998), and PCR-based techniques, i.e. selective enrichment of amplified DNA (SEAD, Clapp *et al.*, 1995), the used of specific-primers for particular species of AMF (Di Bonito *et al.*, 1995; Lanfranco *et al.*, 1995), and more recently using 25S rDNA-targeted nested PCR (Van Tuinen *et al.*, 1998). It is imperative that these and newer techniques are used in the field to yield information on the ecology of these microsymbionts and their importance in stabilising plant communities in the tropics.

References

Boddington CL, Dodd JC. 1999. Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life-cycle strategies. *New Phytologist* **142**: 531-538.

Clapp JP, Young JPW, Merryweather JW, Fitter AH. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist* **130**: 259-265.

Di Bonito R, Elliott ML, Des Jardin EA. 1995. Detection of an arbuscular mycorrhizal fungus in roots of different plant species with the PCR. *Applied and Environmental Microbiology* **61**: 2809-2810.

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I. 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant and Soil.* (in press)

Hector A, Schmid B, Beierkuhnlein C, *et al.* 1999. Plant diversity and productivity experiments in European Grasslands. *Science* **286**: 1123-1127.

Lanfranco L, Wyss P, Marzachi C, Bonfante P. 1995. Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. *Molecular Ecology* **4**: 61-68.

Marsudi NDS, Glenn AR, Dilworth MJ. 1999. Identification and characterisation of fast- and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. *Soil Biology Biochemistry* **31**: 1229-1238.

Merryweather J, Fitter AH. 1995. Phosphorus and carbon budgets: mycorrhizal contribution in Hyacinthoides non-scripta (L.) Chouard ex Rothm. under natural conditions. *New Phytologist* **129**: 619-627.

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. 1993. Characterisation of rhizobia isolated from different divergence groups of tropical leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology* **16**: 135-146.

Moreira FMS, Haukka K, Young JPW. 1998. Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. *Molecular Ecology* **7**: 889-895.

Newsham KK, Fitter AH, Watkinson AR. 1995. Arbuscular mycorrhizas protect an annual grass from root pathogenic fungi in the field. *Journal of Ecology* **83**: 991-1000.

Nuswantara S, Fujie M, Sukiman HI, Yamashita M, Yamada T. 1997. Phylogeny of bacterial symbionts of the leguminous tree *Acacia mangium*. *Journal of Fermentation and Bioengineering* **84**: 511-518.

Parker MA. 1999. Relationships of bradyrhizobia from the legumes *Apios americana* and *Desmodium glutinosum*. *Applied and Environmental Microbiology* **65**: 4914-4920.

Pate JS. 1996. Photoassimilate partitioning and consumption in nitrogen-fixing crop legumes. In Zamski E, Schaffer AA. eds. *Photoassimilate distribution in Plants and Crops*: source-sink relationships. Marcel Dekker, Inc. Basel. 467-477.

Pearson JN, Schweiger P. 1994. Scutellospora calospora (Nicol. & Gerd.) Walker & Sanders asscociated with subteranean clover produces non-infective hyphae during sporulation. *New Phytologist* **127**: 697-701.

Setiadi Y. 1995. The practical application of arbuscular mycorrhizal fungi for reforestation in Indonesia. PhD thesis. University of Kent at Canterbury. England.

Souvannavong O, Galiana A. 1991. *Acacia mangium-Rhizobium* symbiosis: selection and propagation of the host plant and the microorganism. In Taylor DA, MacDicken

KG. eds. *Research on multipurpose tree species in Asia.* Winrock International. Bangkok, Thailand. 216-222

Tisserant B, Brenac V, Requena N, Jeffries P, Dodd JD. 1998. The detection of *Glomus* spp. (arbuscular mycorrhizal fungi) forming mycorrhizal in three plants, at different stages of seedling development, using mycorrhiza-specific isozymes. *New Phytologist* **138**: 225-239.

Turk D, Keyser HH. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Canadian Journal of Microbiology* **38**: 451-460.

Van der Heijden M G A, Klironimos J N, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders I R. 1998 Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69-72.

Van Tuinen, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V. 1998. Characterisation of root colonisation profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targetted nested PCR. *Molecular Ecology* 7: 879-887.

Wilson KJ. 1995. Molecular Techniques for the study of rhizobial ecology in the field. *Soil Biology and Biochemistry* **27**: 501-514.

Young JPW. 1996. Phylogeny and taxonomy of rhizobia. *Plant and Soil* **186**: 45-52.

Young CS, Burns RG. 1993. Detection, survival and activity of bacteria added to soil. In Bollag J-M, Stotzky G. eds. *Soil Biochemistry*, Vol. 8, Marcel Dekker, Inc. Basel. 1-63.

Publications

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I. 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant and Soil.* (in press)

Abstract

It is often assumed that all species of arbuscular mycorrhizal fungi (AMF) have the same function because of the ubiquity of the arbuscular mycorrhizal symbiosis and the fact that all AMF occupy the same plant/soil niche. Despite apparent differences in the timing of evolutionary divergence and the morphological characteristics of AMF from the different genera, the majority of studies on these fungi use only species of *Glomus*. There is increasing evidence, however, that the mechanisms involved in the establishment of a mycorrhiza may differ for species/genera of AMF and influence their subsequent function. The aim of this paper is to highlight the diversity in the formation and function of AMF from different genera, knowledge of which is vital in understanding their ecological roles. The potential use of biochemical and molecular approaches to detect AMF *in planta* and *ex planta* is also discussed.

Poster session

International Conference on Mycorrhiza: Mycorrhiza in sustainable tropical agriculture and forest ecosystems. Bogor, Indonesia, 17-30 October 1997.

Mansur I, Dodd JC, Jeffries P, Setiadi Y. 1997. Dual inoculation effects of rhizobial and AMF isolates on early growth and nitrogen fixation of *Paraserianthes falcataria.*

Abstract

Arbuscular mycorrhizal fungi (AMF) were inoculated with rhizobia on *Paraserianthes falcataria* to study the contribution of the microsymbionts singly and in combinations to the early growth (6 weeks after planting) and N-fixation of the plant. A factorial in completely randomised block design was employed in this study, with three AMF and five rhizobial treatments. Mycorrhiza inoculation played important roles in the early growth of *P. falcataria*. Plants inoculated with *Glomus manihotis* INDO-1 generally performed better that those inoculated with *Gigaspora rosea* EC-3 or controls. Inoculation with rhizobia enhanced the amount of N-fixed , and root colonisation by AMF. Combination of AMF and rhizobial isolates significantly affected the height of the seedlings with combination of *G. manihotis* INDO-1 and rhizobial isolate PFP225 gave the greatest height growth. The results also suggested that careful selection of compatible AMF and rhizobial isolates was necessary to get a better result, in term of plant growth improvement.

Appendix I

Phenotypic diversity of root-nodule bacteria isolated from tropical leguminous tree species *Acacia mangium* and *Paraserianthes falcataria* growing in Indonesia

A. N-free nutrient solution (Broughton & Dilworth, 1971).

Stock solution	Chemical	g l^{-1}
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

All stock solutions were sterilised by autoclaving at 121°C for 20 min. To feed *A. mangium* and *P. falcataria*, 10 ml of each stock solution were added to 5 l sterile dH2O, mixed, and then made up to 10 l with sterile dH2O. To make N-free nutrient agar, 10 g of agar (Oxoid no. 2) was added per litre N-free nutrient solution. The N and P-free nutrient solution was prepared as for N-free nutrient solution except that stock solution no. 2 was omitted.

B. Yeast extract mannitol agar (YMA) (Vincent, 1970).

Chemical	g l^{-1}
Mannitol	10.0
K2HPO4	0.5
MgSO4.7H2O	0.2
NaCl	0.1
Yeast extract	0.5
Agar (Oxoid no. 2)	15.0

The pH of the medium was adjusted to 6.8 then was autoclaved at 121°C for 20 min. To make YMB medium, the agar was omitted from the preparation. To prepare YMA+congo red, 10 ml of congo red stock solution (250 mg congo red in 100 ml dH2O) was added to 1 l of YMA. Whilst YEMA+bromthymol blue was prepared by adding 5 ml of bromthymol blue stock solution (0.5 g of bromthymol blue in 100 ml of ethanol) to 1 l of YEMA.

C. Tryptone-Yeast (TY) medium (Beringer, 1974).

Chemical	g l^{-1}
Tryptone	5.0
Yeast extract	3.0
CaCl2.H2O	0.87
Agar (Oxoid no. 2)	15.0

The pH of the medium was adjusted to 6.8 then was autoclave at 121°C for 20 mins. To make TY broth, the agar was omitted from the preparation.

D. MOPS-salt (MS) medium (Jordan, 1984).

Chemical	g l^{-1}
Morpholinopropane-sulfonic acid (MOPS buffer)	8.37
KOH	1.12
NH_4Cl	1.07
$MgSO_4$	0.48
K_2HPO_4	0.21
KH_2PO_4	0.05
NaCl	0.005
$CaCO_3$	0.020
H_3BO_3	0.001
$ZnSO_4.7H_2O$	0.001
$CuSO_4.5H_2O$	0.0005
$MnCl_2.4H_2O$	0.0005
$NaMoO_4.2H_2O$	0.001
Ethylenediamine tetraacetic acid (EDTA)	0.010
Na-Fe-EDTA	0.002

Stock solution of MOPS-KOH (sterilised by filtration) and of MgSO4 (sterilised by autoclaving) were prepared and added aseptically to the autoclaved salts solution so as to provide the correct final concentrations. The final pH of the medium should be 7.2. All carbon sources to be added were prepared as stock solutions (sterilised by filtration) and were added to the medium to give a final concentration of 15 mM.

Appendix II

Ribotyping of rhizobia nodulating *Acacia mangium* and *Paraserianthes falcataria* from different geographical areas in Indonesia using PCR-RFLP-SSCP.

A. Sequence alignment of the 16S rRNA genes of *B. elkanii* U35000 and *B. japonicum* U69638 showing the positions of the base differences between the two species of *Bradyrhizobium*.

```
B.elkanii     AGAGTTTGAT CCTGGCTCAG AGCGAACGCT GGCGGCAGGC TTAACACATG  50
B.japonicum   AGAGTTTGAT CCTGGCTCAG AGCGAACGCT GGCGGCAGGC TTAACACATG

B.elkanii     CAAGTCGATC GGGCATAGCA ATATGTCAGC GGCAGACGGG TGAGTAACGC 100
B.japonicum   CAAGTCGAGC GGGCGTAGCA ATACGTCAGC GGCAGACGGG TGAGTAACGC

B.elkanii     GTGGGAACGT ACCTTTTGGT TCGGAACAAC TGAGGGAAAC TTCAGCTAAT 150
B.japonicum   GTGGGAACGT ACCTTTTGGT TCGGAACAAC ACAGGGAAAC TTGTGCTAAT

B.elkanii     ACCGGATAAG CCCTTACGGG GAAAGATTTA TCGCCGAAAG ATCGGCCCGC 200
B.japonicum   ACCGGATAAG CCCTTACGGG GAAAGATTTA TCGCCGAAAG ATCGGCCCGC

B.elkanii     GTCTGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCAGT 250
B.japonicum   GTCTGATTAG CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCAGT

B.elkanii     AGCTGGTCTG AGAGGATGAT CAGCCACATT GGGACTGAGA CACGGCCCAA 300
B.japonicum   AGCTGGTCTG AGAGGATGAT CAGCCACATT GGGACTGAGA CACGGCCCAA

B.elkanii     ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG GCGCAAGCCT 350
B.japonicum   ACTCCTACGG GAGGCAGCAG TGGGGAATAT TGGACAATGG GGCAACCCT

B.elkanii     GATCCAGCCA TGCCGCGTGA GTGATGAAGG CCCTAGGGTT GTAAAGCTCT 400
B.japonicum   GATCCAGCCA TGCCGCGTGA GTGATGAAGG CCCTAGGGTT GTAAAGCTCT

B.elkanii     TTTGTGCGGG AAGATAATGA CGGTACCGCA AGAATAAGCC CCGGCTAACT 450
B.japonicum   TTTGTGCGGG AAGATAATGA CGGTACCGCA AGAATAAGCC CCGGCTAACT

B.elkanii     TCGTGCCAGC AGCCGCGGTA ATACGAAGGG GGCTAGCGTT GCTCGGAATC 500
B.japonicum   TCGTGCCAGC AGCCGCGGTA ATACGAAGGG GGCTAGCGTT GCTCGGAATC

B.elkanii     ACTGGGCGTA AAGGGTGCGT AGGCGGGTCT TTAAGTCAGG GGTGAAATCC 550
B.japonicum   ACTGGGCGTA AAGGGTGCGT AGGCGGGTCT TTAAGTCAGG GGTGAAATCC

B.elkanii     TGGAGGTCAA CTCCAGAACT GCCTTTGATA CTGAAGATCT TGAGTTCGGG 600
B.japonicum   TGGAGCTCAA CTCCAGAACT GCCTTTGATA CTGAGGATCT TGAGTTCGGG

B.elkanii     AGAGGTGAGT GGAACTGCGA GTGTAGAGGT GAAATTCGTA GATATTCGCA 650
B.japonicum   AGAGGTGAGT GGAACTGCGA GTGTAGAGGT GAAATTCGTA GATATTCGCA

B.elkanii     AGAACACCAG TGGCGAAGGC GGCTCACTGG CCCGATACTG ACGCTGAGGC 700
B.japonicum   AGAACACCAG TGGCGAAGGC GGCTCACTGG CCCGATACTG ACGCTGAGGC

B.elkanii     ACGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACGCCG 750
B.japonicum   ACGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACGCCG

B.elkanii     TAAACGATGA ATGCCAGCCG TTAGTGGGTT TACTCACTAG TGGCGCAGCT 800
B.japonicum   TAAACGATGA ATGCCAGCCG TTAGTGGGTT TACTCACTAG TGGCGCAGCT
```

```
B.elkanii     AACGCTTTAA GCATTCCGCC TGGGGAGTAC GGTCGCAAGA TTAAAACTCA 850
B.japonicum   AACGCTTTAA GCATTCCGCC TGGGGAGTAC GGTCGCAAGA TTAAAACTCA

B.elkanii     AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG 900
B.japonicum   AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
B.elkanii     ACGCAACGCG CAGAACCTTA CCAGCCCTTG ACATCCCGGT CGCGGACTCC 950
B.japonicum   ACGCAACGCG CAGAACCTTA CCAGCCCTTG ACATGTCCAG GACCGGTCGC

B.elkanii     AGAGACGGAG TTCTTCAGTT CGGCTGGACC GGAG-ACAGG TGCTGCATGG 999
B.japonicum   AGAGATGT-G ACCTTCTCTT CGG--AGCCT GGAGCACAGG TGCTGCATGG 997

B.elkanii     CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG 1049
B.japonicum   CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG 1047

B.elkanii     CAACCCCCGT CCTTAGTTGC TACCATTTAG TTGAGCACTC TAAGGAGACT 1099
B.japonicum   CAACCCCCGT CCTTAGTTGC TACCATTTAG TTGAGCACTC TAAGGAGACT 1097

B.elkanii     GCCGGTGATA AGCCGCGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC 1149
B.japonicum   GCCGGTGATA AGCCGCGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC 1147

B.elkanii     CCTTACGGGC TGGGCTACAC ACGTGCTACA ATGGCGGTGA CAATGGGATG 1199
B.japonicum   CCTTACGGGC TGGGCTACAC ACGTGCTACA ATGGCGGTGA CAATGGGATG 1197

B.elkanii     CTAAGGGGCG ACCCTTCGCA AATCTCAAAA ATCCGTCTCA GTTCGGATTG 1249
B.japonicum   CTAAGGGGCG ACCCTTCGCA AATCTCAAAA AGCCGTCTCA GTTCGGATTG 1247

B.elkanii     GGCTCTGCAA CTCGAGCCCA TGAAGTTGGA ATCGCTAGTA ATCGTGGATC 1299
B.japonicum   GGCTCTGCAA CTCGAGCCCA TGAAGTTGGA ATCGCTAGTA ATCGTGGATC 1297

B.elkanii     AGCACGCCAC GGTGAATACG TTCCCGGGCC TTGTACACAC CGCCCGTCAC 1349
B.japonicum   AGCACGCCAC GGTGAATACG TTCCCGGGCC TTGTACACAC CGCCCGTCAC 1347

B.elkanii     ACCATGGGAG TTGGTTTTAC CTGAAGACGG TGCGCTAACC -GAAAGGGGG 1398
B.japonicum   ACCATGGGAG TTGGTTTTAC CTGAAGACGG TGCGCTAACC CGCAAGGGAG 1397

B.elkanii     G-AGCCGGCC ACGGTAGGGT CAGCGACTGG GGTGAAGTCG TAACAAGGTA 1447
B.japonicum   GCAGCCGGCC ACGGTAGGGT CAGCGACTGG GGTGAAGTCG TAACAAGGTA 1447

B.elkanii     GCC 1450
B.japonicum   GCC 1450
```

B. Sequence alignment of fragments produced by the 16S rRNA gene of *B. elkanii* U35000 and *B. japonicum* U69638 digested with a restriction enzyme *MSp*I.

- E= *B. elkanii*
- J = *B. japonicum*
- Number following E or J indicating the fragment number

```
E1/J1
Total length= 152 bp
Differences= 7 bp, position 59,65,74,131,132,143,144

E1  AGAGTTTGAT CCTGGCTCAG AGCGAACGCT GGCGGCAGGC TTAACACATG CAAGTCGATC GGGCATAGCA ATATGTCAGC
J1  AGAGTTTGAT CCTGGCTCAG AGCGAACGCT GGCGGCAGGC TTAACACATG CAAGTCGAGC GGGCGTAGCA ATACGTCAGC

E1  GGCAGACGGG TGAGTAACGC GTGGGAACGT ACCTTTTGGT TCGGAACAAC TGAGGGAAAC TTCAGCTAAT AC
J1  GGCAGACGGG TGAGTAACGC GTGGGAACGT ACCTTTTGGT TCGGAACAAC ACAGGGAAAC TTGTGCTAAT AC
```

E2/J2
Total length= 289 bp
Differences= 2 bp, position 190, 195 (342,347)

E3/J3
Total length= E=495 bp/J=502 bp
Differences= 4 bp, position 115,144,494,495 (556,585,935,936) + J 5 bp position 497-501 (938-942)

E4+E5 (Total length= 43+123 bp=166 bp)/J4 (Total length= 156 bp+3 bp deletion)
E4, differences= 21 bp, position 2-6,8,10-13,20,22,23,25,26,31,32,38-40,42 (938-942,944,946-949,956,958,959,961,962,967,967,974-976,978)+ E5, differences= 2 bp, position 1,6'deletion' (980,985)

E6/J5
Total length= 222 bp
Differences= 1 bp, position 130 (1232)

E7 (Total length= 79)/J6 (Total length= 81)

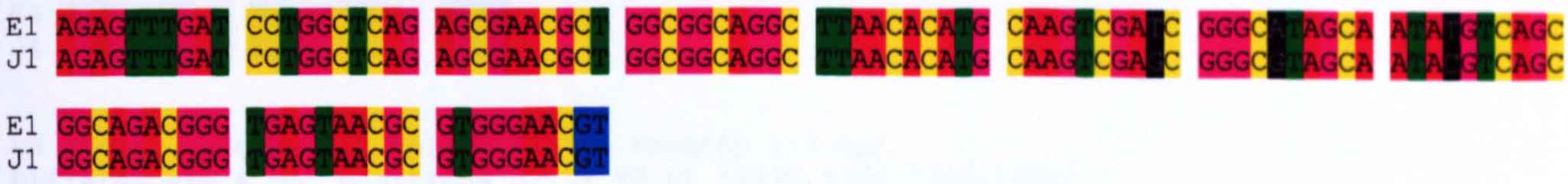
Differences= 4 bp, position 67,69,75,78 (1391,1393,1399,1402)

E8/J7
Total length= 48 bp
Differences= 0 bp

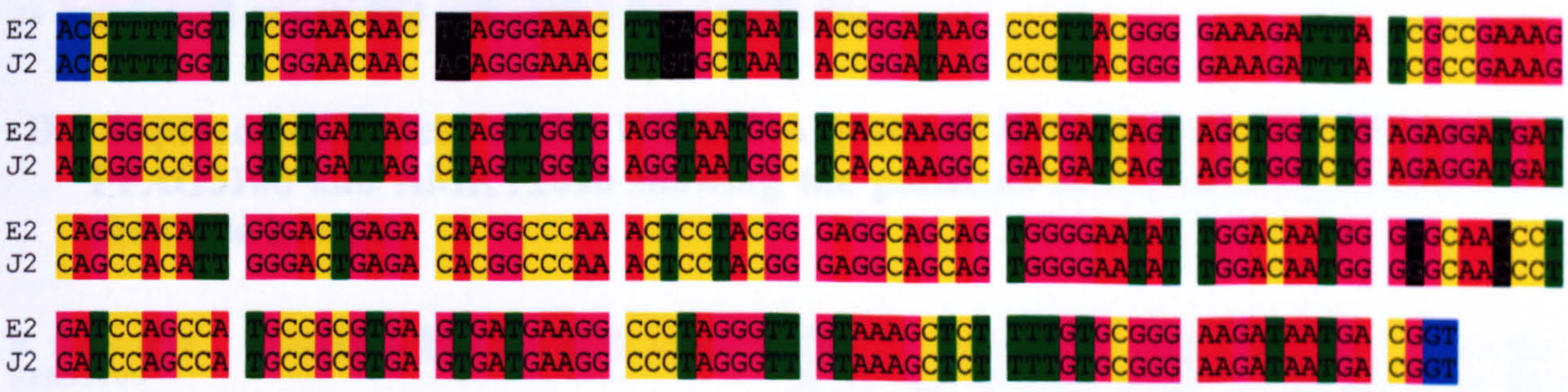
C. Sequence alignment of fragments produced by the 16S rRNA gene of *B. elkanii* U35000 and *B. japonicum* U69638 digested with a restriction enzyme *RSa*I.

- E= *B. elkanii*
- J = *B. japonicum*
- Number following E or J indicating the fragment number

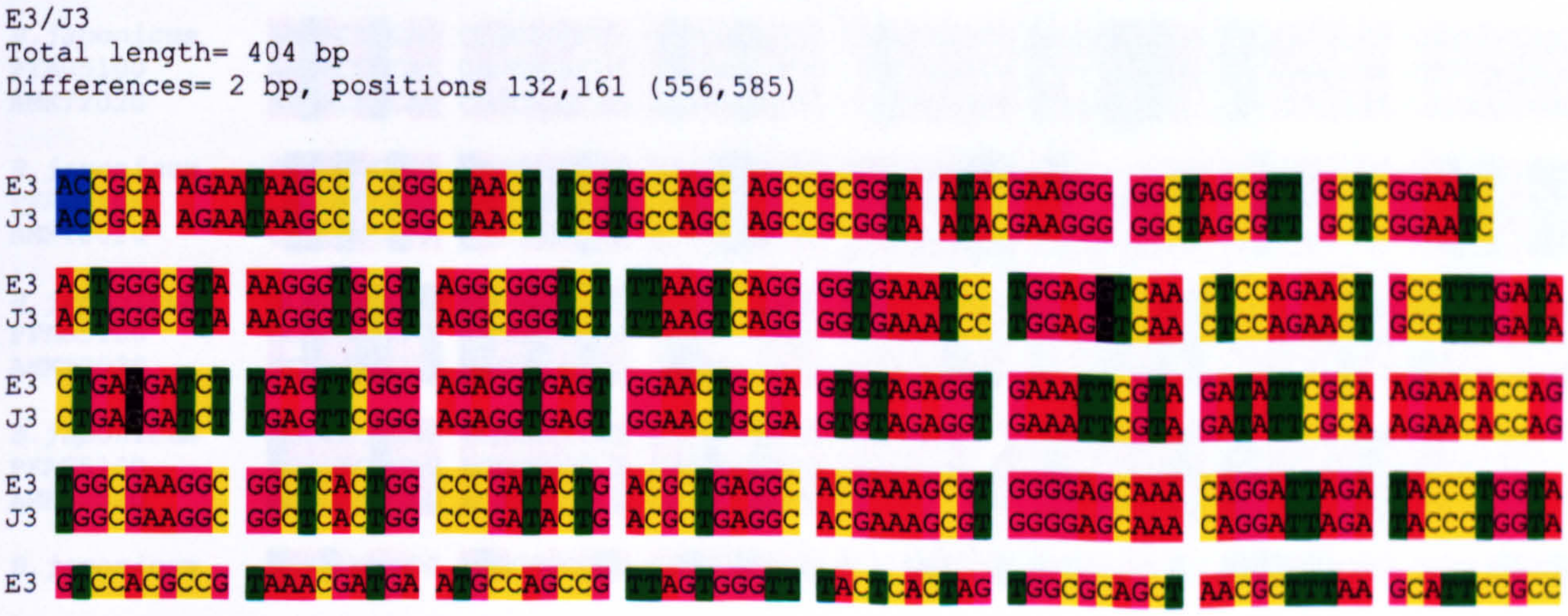
E1/J1
Total length= 110 bp
Differences= 3 bp, positions 59,65,74

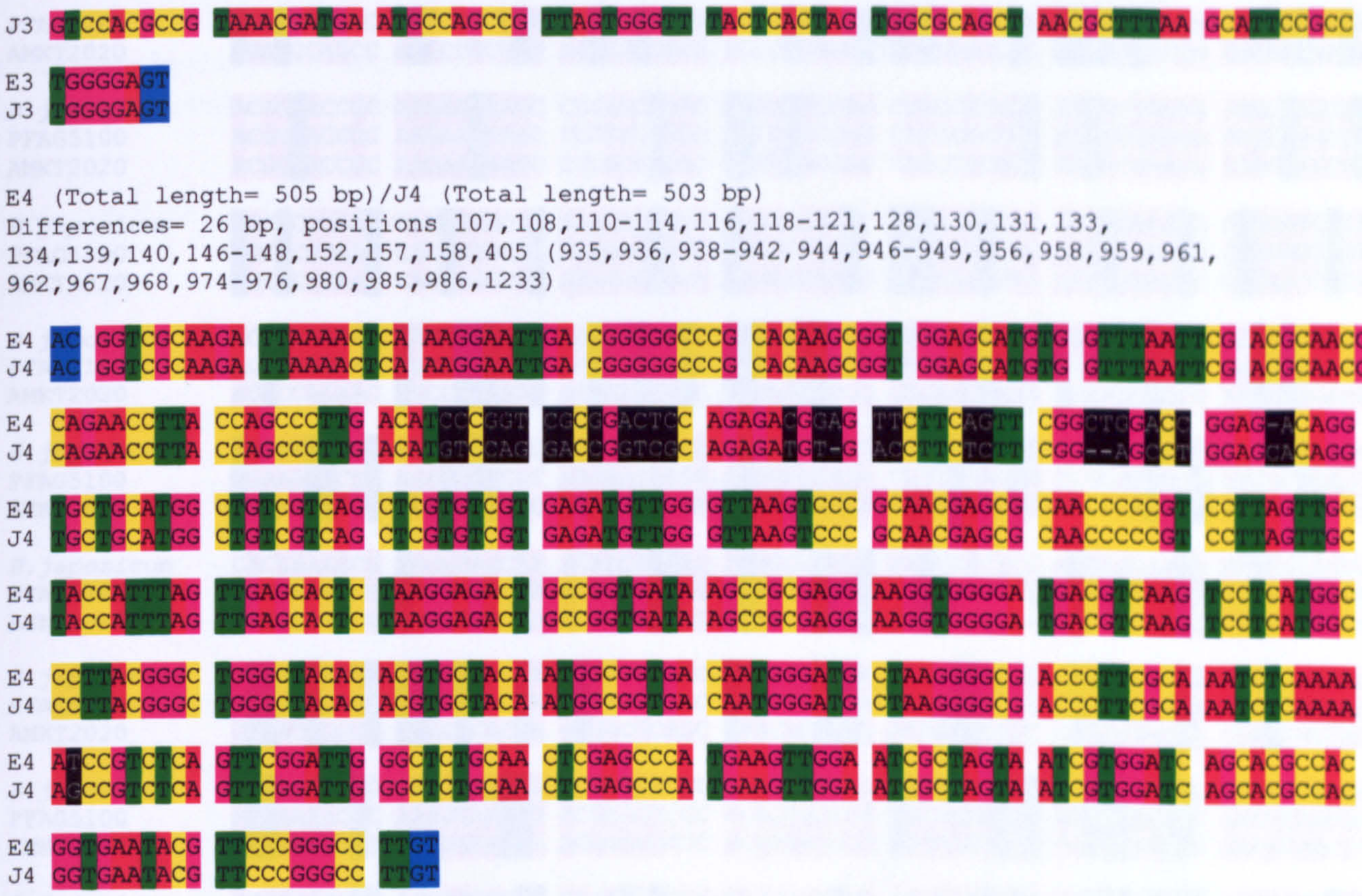


E2/J2
Total length= 314 bp
Differences= 6 bp, positions 21,22,33,34,232,237 (131,132,143,144,342,347)

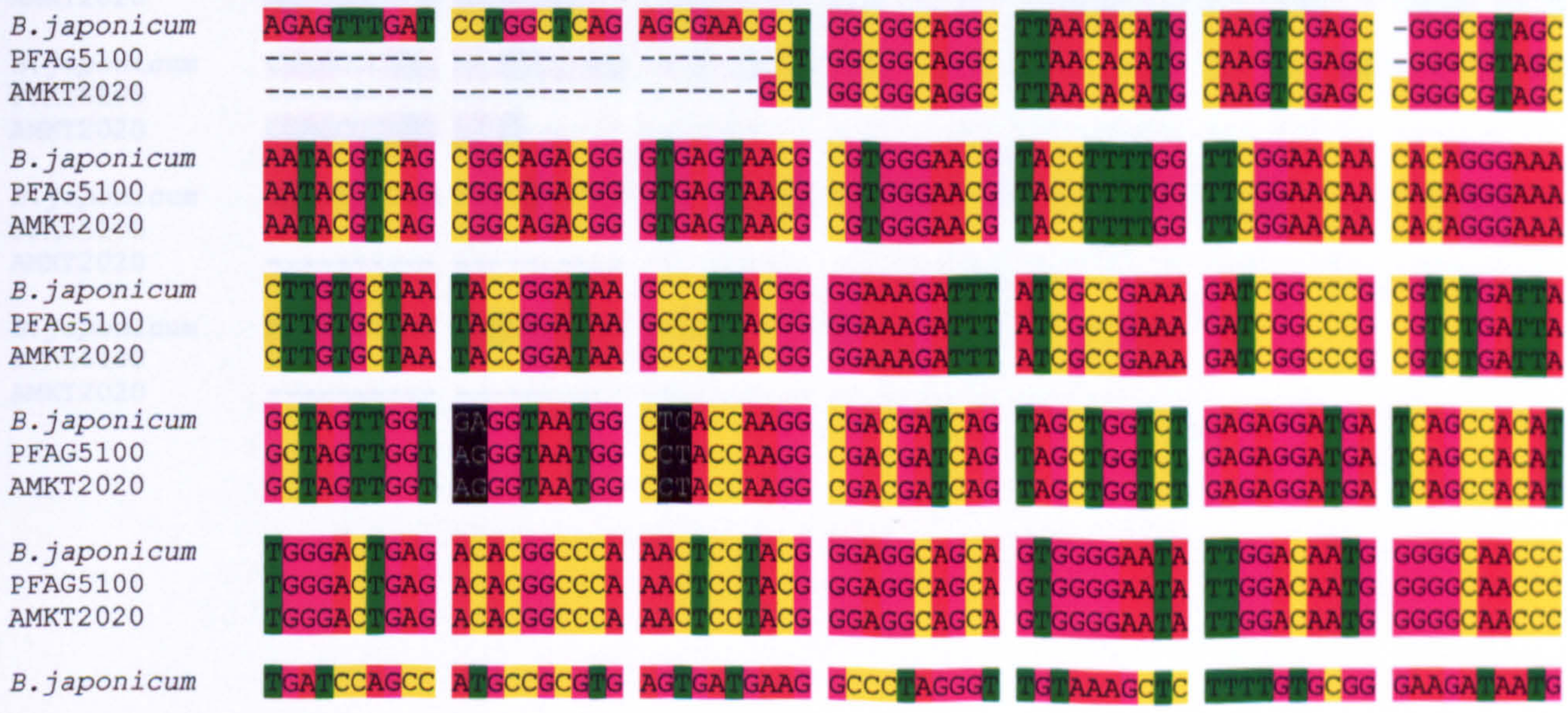


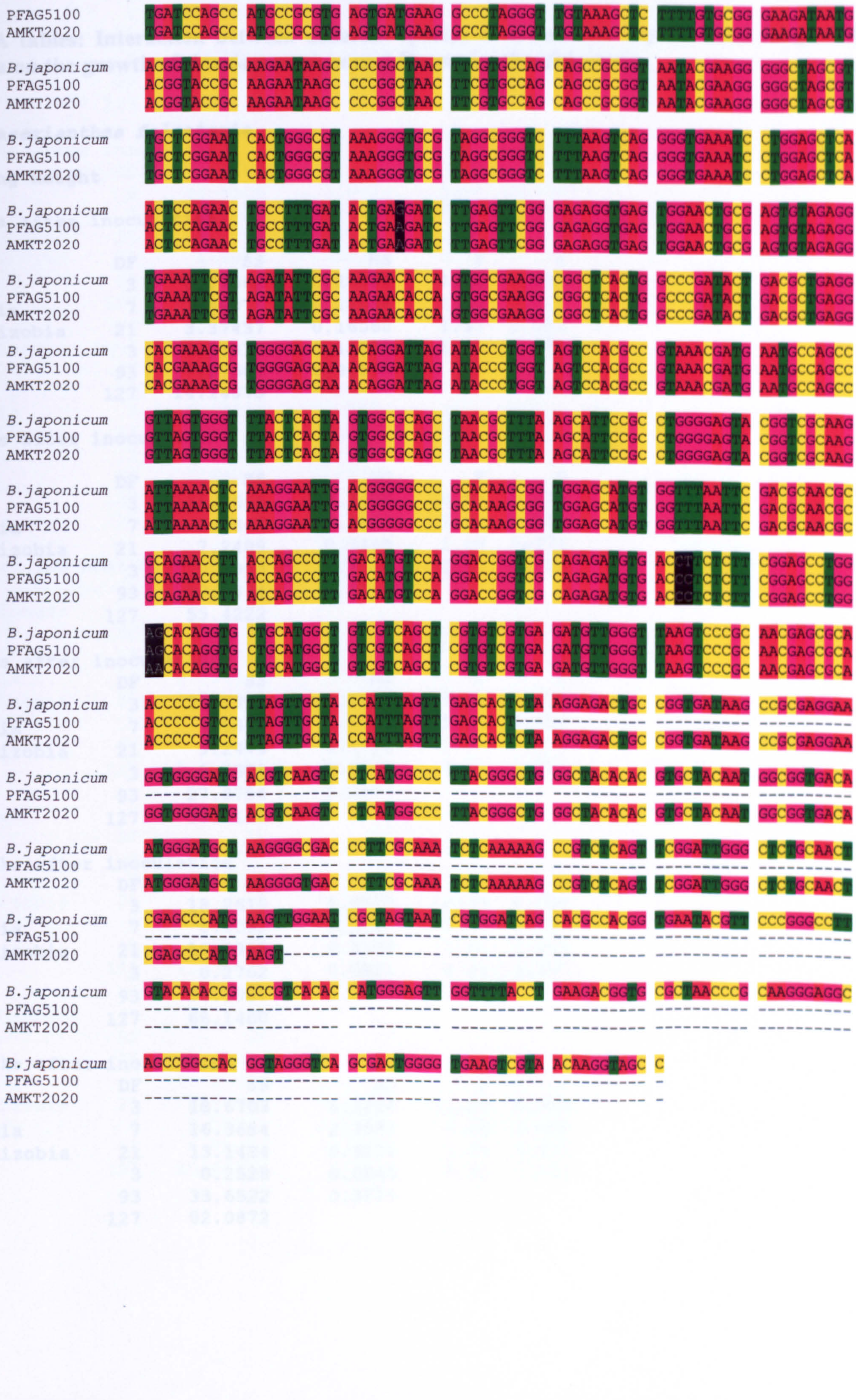
E3/J3
Total length= 404 bp
Differences= 2 bp, positions 132,161 (556,585)





D. Sequence alignment of the 16S rRNA genes of *B. japonicum* U69638, and isolates PFAG5100 and AMKT2020 showing the positions of the base differences between the three isolates.




PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020
B.japonicum
PFAG5100
AMKT2020

Appendix III

ANOVA tables: Interaction between different species of arbuscular mycorrhizal fungi and rhizobia on the growth of *Acacia mangium* and *Paraserianthes falcataria*

A. Paraserianthes falcataria

Seedling Height

2 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	0.77562	0.25854	3.01	0.034
Rhizobia	7	1.68375	0.24054	2.80	0.011
AMF*Rhizobia	21	3.37437	0.16068	1.87	0.022
Block	3	0.33813	0.11271	1.31	0.275
Error	93	7.97687	0.08577		
Total	127	14.14875			

7 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	15.6666	5.2222	16.53	0.000
Rhizobia	7	2.7347	0.3907	1.24	0.291
AMF*Rhizobia	21	7.2409	0.3448	1.09	0.371
Block	3	0.3966	0.1322	0.42	0.740
Error	93	29.3834	0.3160		
Total	127	55.4222			

9 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	15.7752	5.2584	17.55	0.000
Rhizobia	7	4.8437	0.6920	2.31	0.032
AMF*Rhizobia	21	9.2479	0.4404	1.47	0.108
Block	3	0.2821	0.0940	0.31	0.815
Error	93	27.8704	0.2997		
Total	127	58.0193			

11 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	18.2619	6.0873	18.75	0.000
Rhizobia	7	8.5062	1.2152	3.74	0.001
AMF*Rhizobia	21	10.8969	0.5189	1.60	0.066
Block	3	0.2762	0.0921	0.28	0.837
Error	93	30.1987	0.3247		
Total	127	68.1400			

13 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	18.6703	6.2234	17.20	0.000
Rhizobia	7	16.3684	2.3383	6.46	0.000
AMF*Rhizobia	21	13.1434	0.6259	1.73	0.039
Block	3	0.2528	0.0843	0.23	0.873
Error	93	33.6522	0.3619		
Total	127	82.0872			

15 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	20.7509	6.9170	13.61	0.000
Rhizobia	7	33.5049	4.7864	9.42	0.000
AMF*Rhizobia	21	16.3823	0.7801	1.53	0.085
Block	3	0.2615	0.0872	0.17	0.915
Error	93	47.2760	0.5083		
Total	127	118.1755			

17 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	20.8506	6.9502	9.91	0.000
Rhizobia	7	73.9062	10.5580	15.06	0.000
AMF*Rhizobia	21	23.8769	1.1370	1.62	0.061
Block	3	1.8956	0.6319	0.90	0.444
Error	93	65.2194	0.7013		
Total	127	185.7488			

Stem diameter (mm)

Source	DF	SS	MS	F	P
AMF	3	0.91086	0.30362	3.60	0.016
Rhizobia	7	6.69430	0.95633	11.34	0.000
AMF*Rhizobia	21	2.31227	0.11011	1.31	0.192
Block	3	1.26523	0.42174	5.00	0.003
Error	93	7.84227	0.08433		
Total	127	19.02492			

2. Shoot and root biomass, leaf areas and root length

Root biomass

Source	DF	SS	MS	F	P
AMF	3	0.016919	0.005640	1.21	0.309
Rhizobia	7	0.254530	0.036361	7.82	0.000
AMF*Rhizobia	21	0.089748	0.004274	0.92	0.567
Block	3	0.161475	0.053825	11.58	0.000
Error	93	0.432186	0.004647		
Total	127	0.954859			

Shootdwt

Source	DF	SS	MS	F	P
AMF	3	0.16284	0.05428	1.19	0.320
Rhizobia	7	7.43612	1.06230	23.20	0.000
AMF*Rhizobia	21	1.72343	0.08207	1.79	0.031
Block	3	0.25295	0.08432	1.84	0.145
Error	93	4.25917	0.04580		
Total	127	13.83452			

Total biomass

Source	DF	SS	MS	F	P
AMF	3	0.27299	0.09100	1.26	0.294
Rhizobia	7	10.26220	1.46603	20.26	0.000
AMF*Rhizobia	21	2.45237	0.11678	1.61	0.063
Block	3	0.75135	0.25045	3.46	0.019
Error	93	6.73029	0.07237		
Total	127	20.46920			

Leaf areas

Source	DF	SS	MS	F	P
AMF	3	3045	1015	0.88	0.454
Rhizobia	7	162334	23191	20.13	0.000
AMF*Rhizobia	21	45048	2145	1.86	0.023
Block	3	8889	2963	2.57	0.059
Error	93	107156	1152		
Total	127	326471			

Total root length

Source	DF	SS	MS	F	P
AMF	3	759597	253199	5.21	0.002
Rhizobia	7	1294559	184937	3.81	0.001
AMF*Rhizobia	21	659274	31394	0.65	0.873
Block	3	219848	73283	1.51	0.217
Error	93	4515484	48554		
Total	127	7448761			

Shoot/root ratio

Source	DF	SS	MS	F	P
AMF	3	0.1384	0.0461	0.13	0.940
Rhizobia	7	47.2118	6.7445	19.45	0.000
AMF*Rhizobia	21	10.4468	0.4975	1.43	0.123
Block	3	9.0884	3.0295	8.74	0.000
Error	93	32.2444	0.3467		
Total	127	99.1297			

3. AMF colonisation and root nodule formation

%root colonisation by AMF

Source	DF	SS	MS	F	P
AMF	3	100814.2	33604.7	230.68	0.000
Rhizobia	7	1709.1	244.2	1.68	0.124
AMF*Rhizobia	21	4242.7	202.0	1.39	0.145
Block	3	289.8	96.6	0.66	0.577
Error	93	13547.8	145.7		
Total	127	120603.7			

Root length colonised by AMF

Source	DF	SS	MS	F	P
AMF	3	2893984	964661	72.57	0.000
Rhizobia	7	111037	15862	1.19	0.314
AMF*Rhizobia	21	255374	12161	0.91	0.573
Block	3	102820	34273	2.58	0.058
Error	93	1236235	13293		
Total	127	4599450			

Root nodules diam. <2 mm

Source	DF	SS	MS	F	P
AMF	3	384.52	128.17	1.37	0.257
Rhizobia	7	1103.43	157.63	1.68	0.122
AMF*Rhizobia	21	2886.04	137.43	1.47	0.108
Block	3	131.96	43.99	0.47	0.704
Error	93	8701.29	93.56		
Total	127	13207.24			

Root nodules diam. >2 mm

Source	DF	SS	MS	F	P
AMF	3	212.31	70.77	0.98	0.405
Rhizobia	7	7642.75	1091.82	15.13	0.000
AMF*Rhizobia	21	1356.94	64.62	0.90	0.597
Block	3	1821.38	607.13	8.41	0.000
Error	93	6712.62	72.18		
Total	127	17746.00			

Total number of root nodules

Source	DF	SS	MS	F	P
AMF	3	823.9	274.6	1.41	0.243
Rhizobia	7	7988.1	1141.2	5.88	0.000
AMF*Rhizobia	21	6574.0	313.0	1.61	0.063
Block	3	2041.9	680.6	3.51	0.018
Error	93	18052.9	194.1		
Total	127	35480.7			

Nodule dry weight

Source	DF	SS	MS	F	P
AMF	3	3382	1127	1.09	0.357
Rhizobia	7	125129	17876	17.30	0.000
AMF*Rhizobia	21	33797	1609	1.56	0.078
Block	3	25410	8470	8.20	0.000
Error	93	96113	1033		
Total	127	283832			

4. Seedling N and P level, and Ndfa

N concentrations

Source	DF	SS	MS	F	P
AMF	3	0.2027	0.0676	0.67	0.571
Rhizobia	7	14.8815	2.1259	21.24	0.000
AMF*Rhizobia	21	1.7201	0.0819	0.82	0.688
Block	2	0.6581	0.3291	3.29	0.044
Error	62	6.2063	0.1001		
Total	95	23.6688			

Root N contents

Source	DF	SS	MS	F	P
AMF	3	7.430	2.477	0.65	0.583
Rhizobia	7	401.342	57.335	15.15	0.000
AMF*Rhizobia	21	103.440	4.926	1.30	0.210
Block	2	12.033	6.016	1.59	0.212
Error	62	234.595	3.784		
Total	95	758.840			

Shoot N contents

Source	DF	SS	MS	F	P
AMF	3	59.12	19.71	0.62	0.606
Rhizobia	7	5868.25	838.32	26.29	0.000
AMF*Rhizobia	21	1568.84	74.71	2.34	0.005
Block	2	98.77	49.38	1.55	0.221
Error	62	1976.95	31.89		
Total	95	9571.93			

Total N contents

Source	DF	SS	MS	F	P
AMF	3	102.50	34.17	0.65	0.584
Rhizobia	7	9249.87	1321.41	25.24	0.000
AMF*Rhizobia	21	2382.28	113.44	2.17	0.010
Block	2	179.59	89.80	1.71	0.188
Error	62	3246.33	52.36		
Total	95	15160.58			

%Ndfa

Source	DF	SS	MS	F	P
AMF	3	348.5	116.2	0.97	0.415
Rhizobia	7	61981.7	8854.5	73.61	0.000
AMF*Rhizobia	21	3114.7	148.3	1.23	0.257
Block	2	439.1	219.6	1.83	0.170
Error	62	7458.3	120.3		
Total	95	73342.3			

Root Ndfa

Source	DF	SS	MS	F	P
AMF	3	10.796	3.599	1.04	0.379
Rhizobia	7	545.518	77.931	22.62	0.000
AMF*Rhizobia	21	102.487	4.880	1.42	0.146
Block	2	12.202	6.101	1.77	0.179
Error	62	213.631	3.446		
Total	95	884.635			

Shoot Ndfa

Source	DF	SS	MS	F	P
AMF	3	84.88	28.29	0.93	0.430
Rhizobia	7	5318.66	759.81	25.05	0.000
AMF*Rhizobia	21	1390.30	66.20	2.18	0.009
Block	2	74.49	37.24	1.23	0.300
Error	62	1880.63	30.33		
Total	95	8748.95			

Total Ndfa

Source	DF	SS	MS	F	P
AMF	3	153.18	51.06	1.00	0.400
Rhizobia	7	9206.75	1315.25	25.72	0.000
AMF*Rhizobia	21	2181.84	103.90	2.03	0.016
Block	2	146.98	73.49	1.44	0.245
Error	62	3170.35	51.13		
Total	95	14859.10			

p concentrations (%P)

Source	DF	SS	MS	F	P
AMF	3	1.6239	0.5413	0.93	0.432
Rhizobia	7	9.0188	1.2884	2.21	0.045
AMF*Rhizobia	21	14.0556	0.6693	1.15	0.327
Block	2	0.9692	0.4846	0.83	0.440
Error	62	36.1511	0.5831		
Total	95	61.8186			

Root P contents

Source	DF	SS	MS	F	P
AMF	3	1.1153	0.3718	1.11	0.352
Rhizobia	7	9.6247	1.3750	4.10	0.001
AMF*Rhizobia	21	5.7082	0.2718	0.81	0.696
Block	2	0.1924	0.0962	0.29	0.751
Error	62	20.7781	0.3351		
Total	95	37.4188			

Shoot P contents

Source	DF	SS	MS	F	P
AMF	3	8.813	2.938	1.13	0.344
Rhizobia	7	283.944	40.563	15.62	0.000
AMF*Rhizobia	21	101.175	4.818	1.85	0.032
Block	2	1.628	0.814	0.31	0.732
Error	62	161.041	2.597		
Total	95	556.601			

Total P contents

Source	DF	SS	MS	F	P
AMF	3	19.693	6.564	1.94	0.133
Rhizobia	7	378.943	54.135	15.97	0.000
AMF*Rhizobia	21	102.628	4.887	1.44	0.134
Block	2	21.591	10.795	3.19	0.048
Error	62	210.116	3.389		
Total	95	732.970			

B. Acacia mangium

1. Height and stem diameter

10 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	2.6825	0.8942	3.31	0.025
Rhizobia	5	1.0563	0.2113	0.78	0.566
AMF*Rhizobia	15	6.6963	0.4464	1.65	0.082
Block	3	4.0125	1.3375	4.95	0.004
Error	69	18.6375	0.2701		
Total	95	33.0850			

12 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	4.3825	1.4608	2.79	0.047
Rhizobia	5	0.5458	0.1092	0.21	0.958
AMF*Rhizobia	15	8.3550	0.5570	1.06	0.405
Block	3	6.0225	2.0075	3.84	0.013
Error	69	36.1125	0.5234		
Total	95	55.4183			

14 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	7.391	2.464	2.24	0.091
Rhizobia	5	0.803	0.161	0.15	0.981
AMF*Rhizobia	15	16.108	1.074	0.98	0.489
Block	3	5.598	1.866	1.70	0.176
Error	69	75.940	1.101		
Total	95	105.840			

16 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	7.567	2.522	1.43	0.243
Rhizobia	5	2.827	0.565	0.32	0.900
AMF*Rhizobia	15	27.536	1.836	1.04	0.429
Block	3	2.063	0.688	0.39	0.761
Error	69	122.030	1.769		
Total	95	162.022			

18 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	6.759	2.253	0.88	0.458
Rhizobia	5	22.108	4.422	1.72	0.142
AMF*Rhizobia	15	34.200	2.280	0.89	0.582
Block	3	0.546	0.182	0.07	0.975
Error	69	177.531	2.573		
Total	95	241.145			

20 weeks after inoculation

Source	DF	SS	MS	F	P
AMF	3	7.063	2.354	0.60	0.615
Rhizobia	5	64.052	12.810	3.28	0.010
AMF*Rhizobia	15	46.373	3.092	0.79	0.682
Block	3	0.007	0.002	0.00	1.000
Error	69	269.496	3.906		
Total	95	386.990			

Stem diameter

Source	DF	SS	MS	F	P
AMF	3	0.18708	0.06236	0.67	0.572
Rhizobia	5	2.72208	0.54442	5.87	0.000
AMF*Rhizobia	15	0.58042	0.03869	0.42	0.969
Block	3	0.14542	0.04847	0.52	0.668
Error	69	6.39458	0.09268		
Total	95	10.02958			

2. Seedling biomass, leaf areas and root length

Root biomass

Source	DF	SS	MS	F	P
AMF	3	0.037991	0.012664	2.69	0.053
Rhizobia	5	0.047375	0.009475	2.01	0.088
AMF*Rhizobia	15	0.077414	0.005161	1.10	0.377
Block	3	0.019708	0.006569	1.39	0.252
Error	69	0.324944	0.004709		
Total	95	0.507433			

Shoot biomass

Source	DF	SS	MS	F	P
AMF	3	0.39897	0.13299	2.54	0.064
Rhizobia	5	2.87534	0.57507	10.98	0.000
AMF*Rhizobia	15	0.90204	0.06014	1.15	0.333
Block	3	0.02398	0.00799	0.15	0.928
Error	69	3.61530	0.05240		
Total	95	7.81562			

Total biomass

Source	DF	SS	MS	F	P
AMF	3	0.66764	0.22255	2.62	0.058
Rhizobia	5	3.62275	0.72455	8.53	0.000
AMF*Rhizobia	15	1.45272	0.09685	1.14	0.339
Block	3	0.08165	0.02722	0.32	0.811
Error	69	5.85959	0.08492		
Total	95	11.68435			

Leaf areas

Source	DF	SS	MS	F	P
AMF	3	7321.6	2440.5	3.29	0.026
Rhizobia	5	38128.2	7625.6	10.29	0.000
AMF*Rhizobia	15	12523.1	834.9	1.13	0.351
Block	3	396.6	132.2	0.18	0.911
Error	69	51138.3	741.1		
Total	95	109507.8			

Total root length

Source	DF	SS	MS	F	P
AMF	3	3270115	1090038	6.76	0.000
Rhizobia	5	1730449	346090	2.15	0.070
AMF*Rhizobia	15	1742121	116141	0.72	0.756
Block	3	269305	89768	0.56	0.645
Error	69	11129297	161294		
Total	95	18141288			

Shoot/root ratio

Source	DF	SS	MS	F	P
AMF	3	1.5801	0.5267	1.49	0.225
Rhizobia	5	34.2694	6.8539	19.37	0.000
AMF*Rhizobia	15	4.6451	0.3097	0.88	0.593
Block	3	1.0565	0.3522	1.00	0.400
Error	69	24.4111	0.3538		
Total	95	65.9623			

3. AMF colonisation and root nodule formation

%AMF colonisation

Source	DF	SS	MS	F	P
AMF	3	71950.8	23983.6	501.24	0.000
Rhizobia	5	650.9	130.2	2.72	0.027
AMF*Rhizobia	15	1679.7	112.0	2.34	0.009
Block	3	259.9	86.6	1.81	0.153
Error	69	3301.6	47.8		
Total	95	77842.9			

Root length colonisation

Source	DF	SS	MS	F	P
AMF	3	5706675	1902225	76.52	0.000
Rhizobia	5	213799	42760	1.72	0.142
AMF*Rhizobia	15	353072	23538	0.95	0.519
Block	3	42995	14332	0.58	0.632
Error	69	1715311	24860		
Total	95	8031852			

Numbers of root nodule diam. <1 mm

Source	DF	SS	MS	F	P
AMF	3	11319	3773	0.16	0.920
Rhizobia	5	2735924	547185	23.82	0.000
AMF*Rhizobia	15	321502	21433	0.93	0.533
Block	3	74218	24739	1.08	0.365
Error	69	1585280	22975		
Total	95	4728242			

Numbers of root nodule diam. 1-2 mm

Source	DF	SS	MS	F	P
AMF	3	7245	2415	1.69	0.178
Rhizobia	5	39678	7936	5.54	0.000
AMF*Rhizobia	15	23614	1574	1.10	0.373
Block	3	1794	598	0.42	0.741
Error	69	98771	1431		
Total	95	171101			

Numbers of root nodules diam. >2 mm

Source	DF	SS	MS	F	P
AMF	3	919.87	306.62	4.02	0.011
Rhizobia	5	14757.33	2951.47	38.66	0.000
AMF*Rhizobia	15	1758.25	117.22	1.54	0.117
Block	3	264.38	88.12	1.15	0.334
Error	69	5268.13	76.35		
Total	95	22967.96			

Total numbers root nodule

Source	DF	SS	MS	F	P
AMF	3	45112	15037	0.54	0.655
Rhizobia	5	2425139	485028	17.47	0.000
AMF*Rhizobia	15	452654	30177	1.09	0.384
Block	3	71136	23712	0.85	0.469
Error	69	1915508	27761		
Total	95	4909549			

Root nodule dry weight

Source	DF	SS	MS	F	P
AMF	3	5367.2	1789.1	3.12	0.031
Rhizobia	5	123267.0	24653.4	43.01	0.000
AMF*Rhizobia	15	11581.5	772.1	1.35	0.199
Block	3	696.5	232.2	0.41	0.750
Error	69	39548.2	573.2		
Total	95	180460.4			

4. Seedling N and P levels, and Ndfa

N concentrations (%N)

Source	DF	SS	MS	F	P
AMF	3	0.03252	0.01084	0.28	0.838
Rhizobia	5	5.46611	1.09322	28.42	0.000
AMF*Rhizobia	15	1.20949	0.08063	2.10	0.028
Block	2	0.01773	0.00887	0.23	0.795
Error	46	1.76973	0.03847		
Total	71	8.49559			

Root N contents

Source	DF	SS	MS	F	P
AMF	3	10.9003	3.6334	4.17	0.011
Rhizobia	5	80.5763	16.1153	18.49	0.000
AMF*Rhizobia	15	23.8796	1.5920	1.83	0.060
Block	2	1.2157	0.6078	0.70	0.503
Error	46	40.1010	0.8718		
Total	71	156.6729			

Shoot N contents

Source	DF	SS	MS	F	P
AMF	3	124.33	41.44	3.86	0.015
Rhizobia	5	1553.44	310.69	28.94	0.000
AMF*Rhizobia	15	258.87	17.26	1.61	0.109
Block	2	5.03	2.51	0.23	0.792
Error	46	493.79	10.73		
Total	71	2435.46			

Total N contents

Source	DF	SS	MS	F	P
AMF	3	207.33	69.11	4.13	0.011
Rhizobia	5	2331.11	466.22	27.89	0.000
AMF*Rhizobia	15	427.98	28.53	1.71	0.083
Block	2	11.18	5.59	0.33	0.718
Error	46	769.04	16.72		
Total	71	3746.64			

%Ndfa

Source	DF	SS	MS	F	P
AMF	3	4338.0	1446.0	4.74	0.006
Rhizobia	5	67060.0	13412.0	43.94	0.000
AMF*Rhizobia	15	6177.3	411.8	1.35	0.213
Block	2	525.5	262.7	0.86	0.430
Error	46	14039.8	305.2		
Total	71	92140.7			

Root Ndfa

Source	DF	SS	MS	F	P
AMF	3	11.855	3.952	3.89	0.015
Rhizobia	5	154.862	30.972	30.50	0.000
AMF*Rhizobia	15	27.865	1.858	1.83	0.059
Block	2	1.089	0.544	0.54	0.589
Error	46	46.718	1.016		
Total	71	242.389			

Shoot Ndfa

Source	DF	SS	MS	F	P
AMF	3	105.78	35.26	3.51	0.023
Rhizobia	5	1581.08	316.22	31.46	0.000
AMF*Rhizobia	15	228.89	15.26	1.52	0.138
Block	2	6.26	3.13	0.31	0.734
Error	46	462.29	10.05		
Total	71	2384.30			

Total Ndfa

Source	DF	SS	MS	F	P
AMF	3	188.11	62.70	3.71	0.018
Rhizobia	5	2721.04	544.21	32.21	0.000
AMF*Rhizobia	15	408.83	27.26	1.61	0.107
Block	2	11.23	5.62	0.33	0.719
Error	46	777.22	16.90		
Total	71	4106.43			

P concentrations (%P)

Source	DF	SS	MS	F	P
AMF	3	1.8257	0.6086	2.81	0.050
Rhizobia	5	26.6701	5.3340	24.61	0.000
AMF*Rhizobia	15	10.8684	0.7246	3.34	0.001
Block	2	0.5972	0.2986	1.38	0.262
Error	46	9.9719	0.2168		
Total	71	49.9333			

Root P contents

Source	DF	SS	MS	F	P
AMF	3	2.9817	0.9939	4.85	0.005
Rhizobia	5	7.9770	1.5954	7.78	0.000
AMF*Rhizobia	15	5.7832	0.3855	1.88	0.051
Block	2	0.1032	0.0516	0.25	0.779
Error	46	9.4352	0.2051		
Total	71	26.2803			

Shoot P contents

Source	DF	SS	MS	F	P
AMF	3	33.005	11.002	5.42	0.003
Rhizobia	5	237.916	47.583	23.42	0.000
AMF*Rhizobia	15	57.844	3.856	1.90	0.049
Block	2	0.572	0.286	0.14	0.869
Error	46	93.443	2.031		
Total	71	422.780			

Total P contents

Source	DF	SS	MS	F	P
AMF	3	55.236	18.412	5.73	0.002
Rhizobia	5	316.921	63.384	19.72	0.000
AMF*Rhizobia	15	95.715	6.381	1.99	0.038
Block	2	1.119	0.559	0.17	0.841
Error	46	147.852	3.214		
Total	71	616.843			

C. Data for cluster analysis of combination treatments for *P. falcataria*

(1)

Treatment	Biomass (g)	Root dry weight (g)	Root length (cm)	Shoot dry weight (g)	Leaflet number	Leaf area (cm2)
no-AMF+no-rhizobium	0.42	0.16	461.1	0.26	220	37.7
no-AMF+PFAG5070	0.94	0.23	684.1	0.7	481	98
no-AMF+PFAG5040	1.14	0.33	718.1	0.81	466	115.1
no-AMF+PFAG6040	0.94	0.24	673.1	0.7	492	92.6
no-AMF+PFIR3040	0.68	0.24	497.1	0.44	251	59.4
no-AMF+PFAG6030	1.19	0.32	877.7	0.88	552	124.7
no-AMF+PFJB1040	1.28	0.29	771.7	0.99	621	137.7
no-AMF+PFAG5030	0.82	0.23	627.4	0.6	286	76.9
Glm+no-rhizobium	0.34	0.16	312	0.18	155	23.9
Glm+PFAG5070	0.67	0.18	598.8	0.49	311	71.2
Glm+PFAG5040	1.03	0.26	520.2	0.77	501	113.3
Glm+PFAG6040	1.36	0.34	622.9	1.02	531	148.8
Glm+PFIR3040	0.87	0.24	526.8	0.63	479	91.3
Glm+PFAG6030	0.96	0.27	693.4	0.7	396	99.8
Glm+PFJB1040	1.64	0.34	526.3	1.29	789	202.5
Glm+PFAG5030	0.92	0.25	522.6	0.67	359	89.4
Gir+no-rhizobium	0.44	0.19	459.7	0.25	174	32.9
Gir+PFAG5070	0.9	0.23	634.4	0.67	355	90.2
Gir+PFAG5040	1.45	0.36	772.7	1.09	535	166.3
Gir+PFAG6040	1.34	0.33	1005.4	1.01	659	131.1
Gir+PFIR3040	0.77	0.27	720.4	0.49	339	70.2
Gir+PFAG6030	1.22	0.3	872.4	0.92	489	108
Gir+PFJB1040	1.31	0.32	814.7	0.99	579	152.4
Gir+PFAG5030	0.88	0.23	636.6	0.65	380	84
Sch+no-rhizobium	0.43	0.21	565.4	0.23	164	25
Sch+PFAG5070	1.42	0.31	804.8	1.11	553	151.4
Sch+PFAG5040	1.08	0.29	640.8	0.79	382	104.6
Sch+PFAG6040	1.15	0.3	835	0.85	410	110.5
Sch+PFIR3040	0.82	0.25	799.3	0.57	310	71.8
Sch+PFAG6030	1.05	0.26	649.4	0.79	441	100.2
Sch+PFJB1040	1.34	0.32	800.1	1.02	552	135.4
Sch+PFAG5030	0.99	0.27	636.4	0.72	527	101.7

(2)

Treatment	Shot/root	Diameter (mm)	Height 17 weeks (cm)	%P	P content (mg)		
					Total	Root	Shoot
no-AMF+no-rhizobium	2	1.6	1.93	7.2	27.1	11.3	15.8
no-AMF+PFAG5070	3	2.1	3.3	7.1	75	18.3	56.7
no-AMF+PFAG5040	2.6	2.1	4.18	7.2	83.9	24.9	59.3
no-AMF+PFAG6040	3.2	2.2	3.28	6.9	67.1	17.2	50
no-AMF+PFIR3040	1.8	1.8	2.4	7.3	39.2	19.4	34.7
no-AMF+PFAG6030	2.8	2.1	3.7	6.5	83.2	21.6	61.6
no-AMF+PFJB1040	3.7	2.2	3.65	6.8	82.7	22.9	75.1
no-AMF+PFAG5030	2.7	2.1	2.8	6.8	56.4	17.4	45.9
Glm+no-rhizobium	1.2	1.4	2.15	8	27.2	12.9	14.3
Glm+PFAG5070	2.6	1.6	3.18	7.2	50.1	10.6	24.8
Glm+PFAG5040	2.9	2.1	4.1	6.9	68.3	19.4	54.3
Glm+PFAG6040	3	2.3	4.45	6.7	100.3	24.7	75.6
Glm+PFIR3040	2.7	2	2.75	7.1	65.2	18.7	47
Glm+PFAG6030	2.5	2.1	3.43	7.5	83.7	21.7	61.9
Glm+PFJB1040	3.8	2.4	5.18	7	109.2	25.8	92.6
Glm+PFAG5030	2.7	2.1	3.6	8.4	77	22.5	65
Gir+no-rhizobium	1.4	1.6	1.85	8.9	37.3	16	19
Gir+PFAG5070	2.9	2	2.75	7.1	66.6	16.8	43.2
Gir+PFAG5040	3.2	2.5	4.42	6.9	95.6	26.7	73.2
Gir+PFAG6040	3.1	2.4	4.55	7.2	97.8	25.9	74.5
Gir+PFIR3040	1.6	2	1.23	7.5	39.8	22.4	44.5
Gir+PFAG6030	3.2	2.4	3.6	6.4	85.9	19.1	61.2
Gir+PFJB1040	3.1	2.5	3.88	6.9	91.4	23.5	67.8
Gir+PFAG5030	2.7	2	2.93	7.3	77.9	19.2	58.7
Sch+no-rhizobium	1.1	1.7	2.9	7.8	36.8	16.5	18.1
Sch+PFAG5070	3.6	2.5	5.05	7.7	105.1	24.4	91.3
Sch+PFAG5040	2.7	2.4	4.08	7.4	93.9	23	55.7
Sch+PFAG6040	2.8	2.3	4.33	7.4	87	24.3	68.2
Sch+PFIR3040	2.4	2.1	3.78	6.7	54.2	19.5	39.9
Sch+PFAG6030	3.1	2.2	4.4	7.4	78.2	18.9	65.5
Sch+PFJB1040	3.3	2.4	5.15	7	93.8	25.5	73.4
Sch+PFAG5030	2.8	2	3.38	6.5	61.6	21.5	54.7

(3)

Treatment	%N	N content (mg)			%Ndfa	Ndfa (mg)		
		Total	Root	Shoot		Total	Root	Shoot
no-AMF+no-rhizobium	1.8	6.6	2.5	4.1	6.8	0.6	0.1	0.5
no-AMF+PFAG5070	2.77	29.3	7.2	22.1	74.4	21.8	5.3	16.5
no-AMF+PFAG5040	2.51	29.1	8.5	20.6	75.8	22.2	6.4	15.8
no-AMF+PFAG6040	2.58	24.9	6.3	18.6	76.6	19.1	4.8	14.3
no-AMF+PFIR3040	2.06	16.1	5.4	10.6	48.9	10.5	3.1	7.3
no-AMF+PFAG6030	2.88	37.1	9.7	27.5	81.5	30.3	7.9	22.4
no-AMF+PFJB1040	2.49	35.7	8.3	27.4	86.2	30.8	7.2	23.6
no-AMF+PFAG5030	2.53	23.9	6.4	17.5	68.5	16.9	4.4	12.5
Glm+no-rhizobium	1.5	5.1	2.4	2.7	2.7	0.1	0.1	0.1
Glm+PFAG5070	2.68	12.8	3.8	9	55.1	7.1	2.1	5
Glm+PFAG5040	2.55	25.9	6.8	19	82.6	21.6	5.7	16
Glm+PFAG6040	2.62	39.2	9.7	29.5	83.7	33.6	8.3	25.3
Glm+PFIR3040	2.59	23.5	6.8	16.7	76.2	17.8	5.1	12.7
Glm+PFAG6030	2.85	32	8.3	23.6	80.3	25.7	6.7	19
Glm+PFJB1040	2.83	48.9	10.6	38.3	86.5	42.8	9.3	33.5
Glm+PFAG5030	2.74	28.8	7.4	21.4	75.9	22.5	5.7	16.7
Gir+no-rhizobium	1.4	5.5	2.5	3	1.2	0.1	0	0
Gir+PFAG5070	2.52	21.2	5.8	15.3	74.5	15.8	4.3	11.4
Gir+PFAG5040	2.78	40.3	10.8	29.5	86.5	35	9.4	25.6
Gir+PFAG6040	2.68	37.5	9.7	27.7	83.7	31.3	8.1	23.2
Gir+PFIR3040	1.92	18.3	5.9	12.4	53.7	12.6	3.8	8.7
Gir+PFAG6030	2.74	34.9	8.4	26.4	81.7	28.7	6.9	21.8
Gir+PFJB1040	2.84	37.5	9.7	27.8	85.1	31.9	8.3	23.6
Gir+PFAG5030	2.52	26.4	6.6	19.9	79.4	21	5.2	15.8
Sch+no-rhizobium	1.4	6	2.9	3.2	1.5	0.1	0	0
Sch+PFAG5070	2.62	38.8	8.2	30.7	83.8	32.6	6.8	25.7
Sch+PFAG5040	2.61	27	7.9	19.1	78.9	21.4	6.3	15.2
Sch+PFAG6040	2.72	34.8	9.1	25.7	82	29.3	7.6	21.6
Sch+PFIR3040	2.23	19.7	6.5	13.2	70.5	13.8	4.5	9.3
Sch+PFAG6030	2.87	32.7	7.3	25.4	81.8	26.7	5.9	20.7
Sch+PFJB1040	2.44	34.5	8.9	25.7	84.9	29.4	7.5	21.9
Sch+PFAG5030	2.71	30.2	8.4	21.7	78.2	23.8	6.6	17.1

(4)

Treatment	Nodule number		Nodule dry weight (mg)	%AMF	Root length colonised (cm)
	> 2 mm	Total			
no-AMF+no-rhizobium	4	27	22.7	0	0
no-AMF+PFAG5070	25	51	93.7	0	0
no-AMF+PFAG5040	22	30	91.9	0	0
no-AMF+PFAG6040	20	28	88.3	0	0
no-AMF+PFIR3040	11	17	70	0	0
no-AMF+PFAG6030	24	30	90.3	0	0
no-AMF+PFJB1040	29	46	102.8	0	0
no-AMF+PFAG5030	17	35	65.3	0	0
Glm+no-rhizobium	0	6	2.4	90	279.1
Glm+PFAG5070	11	16	69.5	86	502
Glm+PFAG5040	23	38	104.9	69	355
Glm+PFAG6040	28	42	104.2	58	362.6
Glm+PFIR3040	10	21	131.7	77	395
Glm+PFAG6030	16	22	83.8	76	515.5
Glm+PFJB1040	24	29	128.6	78	398.2
Glm+PFAG5030	16	33	85.6	72	377
Gir+no-rhizobium	2	16	10	44	204.1
Gir+PFAG5070	17	23	72.8	38	203.5
Gir+PFAG5040	29	35	130	30	230
Gir+PFAG6040	28	49	114.9	28	278.1
Gir+PFIR3040	8	12	63.2	42	309.5
Gir+PFAG6030	23	29	99.8	43	401.5
Gir+PFJB1040	29	35	133.7	45	341
Gir+PFAG5030	20	38	75.2	44	295.3
Sch+no-rhizobium	0	9	3.8	13	71.3
Sch+PFAG5070	27	43	146.4	16	136.2
Sch+PFAG5040	20	30	90.1	26	155.9
Sch+PFAG6040	21	29	103.5	15	145.9
Sch+PFIR3040	15	19	92.5	20	162.1
Sch+PFAG6030	26	41	97.1	9	60.5
Sch+PFJB1040	25	33	116.3	15	133.5
Sch+PFAG5030	16	26	85.5	36	204.3

D. Data for cluster analysis of combination treatments for *A. mangium*

(1)

Treatment	Biomas (g)	Shoot dwt (g)	Root dwt (g)	Root lengt (cm)	Shoot/Root	Diam. (mm)
no-AMF+no-rhizobial isolate	0.45	0.26	0.19	1604.8	1.3	1.4
no-AMF+AMAG3010	0.55	0.34	0.21	1162.7	1.5	1.6
no-AMF+AMJB1010	1.04	0.77	0.28	1429.6	2.8	1.9
no-AMF+AMJB1020	1.2	0.93	0.27	1183.6	3.5	2.1
no-AMF+AMJB3010	0.47	0.3	0.17	1309	1.8	1.6
no-AMF+AMBG2030	0.52	0.35	0.17	1056.7	1.9	1.6
Glm+no-rhizobial isolate	0.45	0.29	0.17	968	1.7	1.5
Glm+AMAG3010	0.56	0.39	0.17	1020.3	2.4	1.7
Glm+AMJB1010	0.82	0.63	0.19	1073.1	3.2	1.9
Glm+AMJB1020	0.76	0.57	0.19	952.4	2.9	1.8
Glm+AMJB3010	0.59	0.4	0.2	933	1.8	1.6
Glm+AMBG2030	0.36	0.22	0.14	660.8	1.6	1.5
Gir+no-rhizobial isolate	0.42	0.24	0.18	1516.9	1.3	1.4
Gir+AMAG3010	0.44	0.28	0.16	922.1	1.8	1.6
Gir+AMJB1010	1	0.77	0.24	1549.9	3.2	1.9
Gir+AMJB1020	1.11	0.84	0.27	1657.9	3.1	2
Gir+AMJB3010	0.9	0.62	0.28	1477.8	2.1	1.9
Gir+AMBG2030	0.55	0.36	0.19	1349.6	1.8	1.6
Sch+no-rhizobial isolate	0.41	0.24	0.17	1335.5	1.4	1.4
Sch+AMAG3010	0.49	0.32	0.18	1000.1	1.8	1.6
Sch+AMJB1010	0.83	0.6	0.23	1348.5	2.6	1.9
Sch+AMJB1020	0.51	0.37	0.14	902.9	2.4	1.8
Sch+AMJB3010	0.4	0.24	0.15	858.1	1.7	1.5
Sch+AMBG2030	0.56	0.36	0.2	1044.2	1.7	1.5

Glm= *G. manihotis* BEG112, Gir= *Gi. rosea* BEG111, and Sch= *Sc. heterogama* BEG40.

(2)

Treatment	Leaf area (cm2)	%AMF	Length colonised (cm)	%P	Total P (mg)	Shoot P (mg)
no-AMF+no-rhizobial isolate	33.1	0	0	9.8	46.5	26.1
no-AMF+AMAG3010	40.9	0	0	8.6	33.8	18.4
no-AMF+AMJB1010	84	0	0	9.2	111.5	81.6
no-AMF+AMJB1020	110.8	0	0	8.4	99.4	76.8
no-AMF+AMJB3010	38.3	0	0	8.7	39.5	24.7
no-AMF+AMBG2030	41.1	0	0	8.9	26.1	15.4
Glm+no-rhizobial isolate	38.1	86	834.4	11.1	50.5	30.6
Glm+AMAG3010	47.1	68.2	662.9	8.9	29.7	19.6
Glm+AMJB1010	76	61.9	644.3	10	86.4	66.1
Glm+AMJB1020	71.5	68.5	668.4	8.1	71.3	53.5
Glm+AMJB3010	50	77	699.6	9	36.1	21
Glm+AMBG2030	29.7	78.8	508.8	9	32.9	19.3
Gir+no-rhizobial isolate	31.6	27.6	431.6	10.5	42.6	24.3
Gir+AMAG3010	32.5	18.1	143.2	8.9	38.7	25.1
Gir+AMJB1010	92.3	13.3	209.3	8.1	83.2	63.6
Gir+AMJB1020	100.8	20.8	332.1	9.2	93.9	70.7
Gir+AMJB3010	78	26.4	391.6	8.4	89	63.6
Gir+AMBG2030	43	18	235.2	9.2	55.6	37
Sch+no-rhizobial isolate	25.9	17	223.7	10.2	40.4	23.4
Sch+AMAG3010	36.1	19.1	178.9	8.7	30.8	19.8
Sch+AMJB1010	68.1	23	316.7	9.4	76.3	56.1
Sch+AMJB1020	40.4	9.3	160.5	8.1	47.1	35
Sch+AMJB3010	30	16.1	126.3	9.3	37.2	21.4
Sch+AMBG2030	37.4	14	148.9	8.8	31.2	18.5

(3)

Treatment	Root P (mg)	%N	N content (mg)			%Ndfa
			total	root	shoot	
no-AMF+no-rhizobial isolate	20.4	1.2	5.4	2.4	3	4.3
no-AMF+AMAG3010	15.4	1.3	5	2.3	2.7	0
no-AMF+AMJB1010	29.9	1.7	20.6	5.5	15.1	78
no-AMF+AMJB1020	22.5	1.9	21.9	5	17	82.7
no-AMF+AMJB3010	14.8	1.3	5.8	2.2	3.6	8
no-AMF+AMBG2030	10.7	1.8	5.2	2	3.2	9
Glm+no-rhizobial isolate	19.9	1.2	5.7	2.2	3.5	1
Glm+AMAG3010	10.1	1.3	4.3	1.5	2.8	27.7
Glm+AMJB1010	20.3	2.2	18.7	4.4	14.3	87
Glm+AMJB1020	17.8	1.9	16.7	4.1	12.7	80.7
Glm+AMJB3010	15.1	1.4	5.4	2.3	3.2	32.7
Glm+AMBG2030	13.6	1.4	5	2.1	3	47.7
Gir+no-rhizobial isolate	18.3	1.2	5	2.2	2.8	1.7
Gir+AMAG3010	13.6	1.3	5.4	1.9	3.5	17.7
Gir+AMJB1010	19.6	1.8	18.2	4.2	13.9	81.3
Gir+AMJB1020	23.2	1.9	19.4	4.8	14.6	79.3
Gir+AMJB3010	25.6	1.6	18.9	5.3	13.6	61.7
Gir+AMBG2030	18.7	1.3	8.2	2.7	5.5	31
Sch+no-rhizobial isolate	17.1	1.2	4.9	2.1	2.8	1
Sch+AMAG3010	11	1.4	4.8	1.7	3.1	1.3
Sch+AMJB1010	20.2	2	16.2	4.3	11.9	73
Sch+AMJB1020	12	2	11.7	3	8.7	69
Sch+AMJB3010	15.8	1.4	5.4	2.3	3.1	34.7
Sch+AMBG2030	12.7	1.3	4.6	1.9	2.7	2.1

(4)

Treatment	Ndfa (mg)			Nodule number		
	Total	Shoot	Root	< 1 mm	1-2 mm	>2 mm
no-AMF+no-rhizobial isolate	0.2	0.1	0.1	0	0	0
no-AMF+AMAG3010	0	0	0	433	36	0
no-AMF+AMJB1010	16.1	11.8	4.3	67	47	34
no-AMF+AMJB1020	18.1	14	4.1	84	88	34
no-AMF+AMJB3010	0.5	0.3	0.2	355	3	0
no-AMF+AMBG2030	0.6	0.4	0.2	284	27	1
Glm+no-rhizobial isolate	0.1	0.03	0	0	0	0
Glm+AMAG3010	1.2	0.8	0.4	342	21	1
Glm+AMJB1010	16.3	12.5	3.8	38	41	22
Glm+AMJB1020	14	10.6	3.4	19	20	18
Glm+AMJB3010	1.8	1	0.7	477	46	4
Glm+AMBG2030	2.4	1.4	1	214	8	0
Gir+no-rhizobial isolate	0.1	0.04	0.04	0	0	0
Gir+AMAG3010	1.3	0.9	0.4	334	7	1
Gir+AMJB1010	15	11.5	3.5	55	71	43
Gir+AMJB1020	15.5	11.6	3.8	72	94	35
Gir+AMJB3010	14.4	10.4	4	549	50	6
Gir+AMBG2030	3.9	2.7	1.2	251	18	3
Sch+no-rhizobial isolate	0	0.03	0.01	0	0	0
Sch+AMAG3010	0.1	0.05	0.02	302	10	3
Sch+AMJB1010	12	8.8	3.2	34	52	19
Sch+AMJB1020	8.6	6.5	2.2	30	27	17
Sch+AMJB3010	2	1.2	0.8	329	6	1
Sch+AMBG2030	0.1	0.05	0.03	467	12	1

(5)

Treatment	Total nodule number	Nodule dry weight (mg)	Height 20 weeks
no-AMF+no-rhizobial isolate	0	0	3
no-AMF+AMAG3010	469	11.7	4.4
no-AMF+AMJB1010	148	85.1	6.4
no-AMF+AMJB1020	206	108.9	6.9
no-AMF+AMJB3010	358	0.7	4.6
no-AMF+AMBG2030	312	12.5	5.6
Glm+no-rhizobial isolate	0	0	4.9
Glm+AMAG3010	364	12	4.9
Glm+AMJB1010	101	74.8	6.2
Glm+AMJB1020	57	64.4	6.4
Glm+AMJB3010	527	19	4.3
Glm+AMBG2030	222	1	3.3
Gir+no-rhizobial isolate	0	0	3.6
Gir+AMAG3010	342	3.7	4.2
Gir+AMJB1010	169	101.4	6.6
Gir+AMJB1020	201	112.2	7
Gir+AMJB3010	605	26.8	6.5
Gir+AMBG2030	272	9.8	6.3
Sch+no-rhizobial isolate	0	0	4.8
Sch+AMAG3010	315	9.3	5.2
Sch+AMJB1010	105	66.8	6
Sch+AMJB1020	74	49.8	5
Sch+AMJB3010	336	2.5	4.6
Sch+AMBG2030	480	7.9	5.2

Appendix IV

ANOVA tables: Establishment of mycorrhizas of four different species of arbuscular mycorrhizal fungi observed using Petri-dish observation chamber (PDOC)

Hyphal diameter

Source	DF	SS	MS	F	P
AMF	3	790.7	263.6	20.13	0.000
Error	196	2566.3	13.1		
Total	199	3357.0			

Hyphal length

Source	DF	SS	MS	F	P
AMF	3	6194	2065	14.02	0.000
Error	16	2356	147		
Total	19	8550			

Spore diameters

Analysis of Variance for diam

Source	DF	SS	MS	F	P
AMF	3	164525	54842	49.20	0.000
Error	96	107014	1115		
Total	99	271539			

KENT
TEMPLEMAN
LIBRARY
UNIVERSITY