Mycelial colonization by bradyrhizobia and azorhizobia

GAMINI SENEVIRATNE* and H S JAYASINGHEARACHCHI

Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

**Corresponding author (Fax, 94-8-232131; Email, gaminis@ifs.ac.lk)*

This study examines mycelial colonization of common soil fungi by bradyrhizobia and an azorhizobial strain, resulting in the forming of biofilms. The effects of the fungal exudates on a bradyrhizobial strain have also been investigated. Bradyrhizobia gradually colonized the mycelia for about 18 days, after which the biofilm structures collapsed with the release of the rhizobial cell clusters to the culture medium. The azorhizobial strain showed differential colonization of the mycelia. In general, there were no considerable mycotoxin effects of the fungal exudates on the bradyrhizobial strain used, instead the rhizobial strain utilized the exudates as a source of nutrition. This study indicates that the present microbial association with biofilm formation has important implications in the survival of rhizobia under adverse soil conditions devoid of vegetation. Further, it could have developed an as yet unidentified nitrogen fixing system that could have contributed to the nitrogen economy of soils.

[Seneviratne G and Jayasinghearachchi H S 2003 Mycelial colonization by bradyrhizobia and azorhizobia; *J. Biosci*. **28** 243–247]

1. Introduction

Bacteria and unicellular eukaryotes, such as yeast and filamentous fungi, are found together in myriad environments and exhibit synergistic and antagonistic interactions (Garbaye 1994). Examples of synergism are interactions between arbuscular mycorrhizal (AM) fungi and *Rhizobium* spp. (Xavier and Germida 2002); while antagonism of *Bacillus* spp. towards *Curvularia lunata* and *Fusarium oxysporum* towards indigenous soil bacteria have been reported by Mezzalamaa *et al* (1998) and Basha and Ulaganathan (2002) respectively. In many ecosystems, it is common to find assemblages of microorganisms adherent to each other and embedded in a matrix of polymers (Morris *et al* 1997), leading to metabolic cooperation (Davey and O'toole 2000). In the soil-plant system, physical adhesion to AM fungal structures has been observed *in vitro* for *Azosprillum*, *Rhizobium* and *Pseudomonas* strains, the interactions ranging from apparently simple association through attachment, to intimate and obligatory symbioses (Perotto *et al* 1998). Through attachment, the bacteria not only position themselves on a surface but also form communities (biofilms) and obtain the additional benefit of the phenotypic versatility of their neighbours (Davey and O'toole 2000). These biofilms provide an ideal environment for the establishment of syntropic relationships. These relationships have been well studied with regard to methanogenic degradation (Davey and O'toole 2000). Biofilms also form in many extreme environments in response to stress factors (Edwards *et al* 2000; O'toole *et al* 2000). They conduct a variety of biological processes, such as photosynthesis, nitrogen fixation and fermentation (Davey and O'toole 2000). Studies on common soil fungi-rhizobial interactions have been limited and confined to investigations on the possibility of co-culturing of the two microbes in peat inoculants (Rice *et al* 1995). These interactions may be important in the survival of rhizobia under stressed soil conditions devoid of vegetation. Therefore present study reports on the first *in vitro* observations of the

Keywords. *Azorhizobium*; biofilms; *Bradyrhizobium*; soil fungi

interactions between the soil fungi, and bradyrhizobia and azorhizobia. The interactions are also assessed for effects of the fungal exudation.

2. Materials and methods

Six species of common soil fungi [*Aspergillus niger*, *A. nidulans*, *A. terrus*, and *Aspergillus* spp. (on czapek dox agar, colonies are typically brownish-green in colour with white cleistothecia developing within and upon the conidial layer, an unidentified species), *Penicillium* spp. and *Mucor* spp.], three bradyrhizobial strains (*Bradyrhizobium japonicum* TAL 102, TAL 620 and *B. elkanii* SEMIA 5019) and *Azorhizobium caulinodans* ORS 571 were used in this study. Two methods of inoculation and incubation were employed and four sets of cultures were maintained. In first two sets, both rhizobia and fungi were inoculated simultaneously. They were separately incubated under static and shake culture conditions at 30°C. The other two sets were inoculated with fungi, 24 h after rhizobial inoculation, and were incubated separately as above. While culturing of bradyrhizobia and fungi was done in yeast mannitol broth (YMB) – without agar, *A. caulinodans* was grown with fungi in yeast peptone broth (YPB) – without agar. To examine the rhizobial and fungal interactions, observations using a phase contrast microscope under oil immersion lens (magnification: 20 × 100) were made everyday till 21 days. Lacto phenol cotton blue stain was used to visualize fungi and rhizobia (Collins *et al* 1989). To determine the effect of fungal exudates on rhizobia, *B. elkanii* was used. It was inoculated to YMB without mannitol and without agar, to which fungal exudates of seven day-old fungi yeast broth of the four *Aspergillus* spp. and *Mucor* spp. were added. To prepare fungal exudates, the mycelia were initially crushed in the broth and then it was centrifuged at 5000 rpm for 10 min. Supernatant was filter-sterilized using 0.2 µm membrane filters. A rhizobial culture without the fungal exudates, but with mannitol served as a control. After six days of incubation at 28°C, 1 ml of the rhizobial broth of exudate-treated and control cultures was taken and serially diluted in 9 ml of autoclaved distilled water, and plated on yeast mannitol agar with congo red (CRYMA). Number of viable cells was recorded by counting colonies after 6 days of incubation at 28°C.

3. Results

All the cultures, whether inoculated simultaneously or sequentially, did not show any difference in their pattern of growth, and/or attachment of the rhizobia and the mycelia. Up to 4 days after inoculation of all rhizobial strains, few cells were observed to attach to the mycelia of all fungi in both static and dynamic cultures, with large number of planktonic cells (figure 1a). After 5 days, all bradyrhizobial strains attached with high cell densities along the filaments of all six species of fungi, in a similar manner forming biofilms (figure 1b,c). Heavy colonization sustained for 18 days after which, rhizobial cell clusters with broken mycelia were observed in liquid phase,

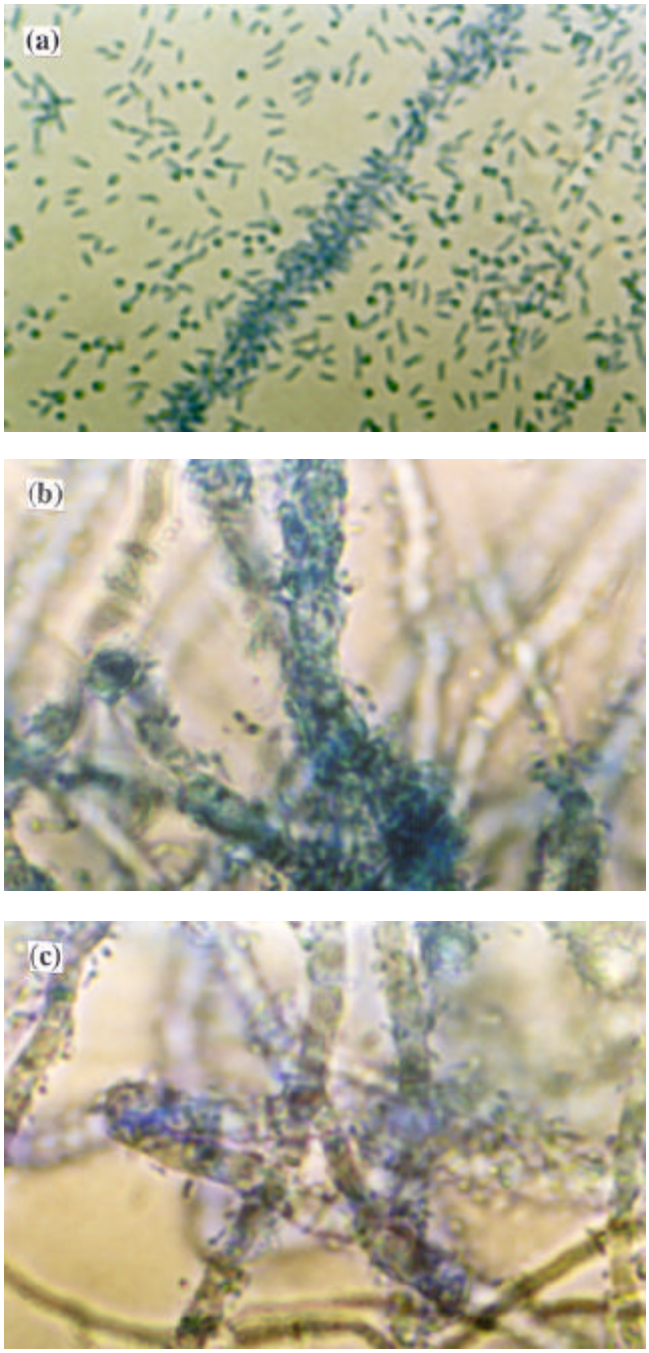

Figure 1a–c. For caption, see page No. 245.

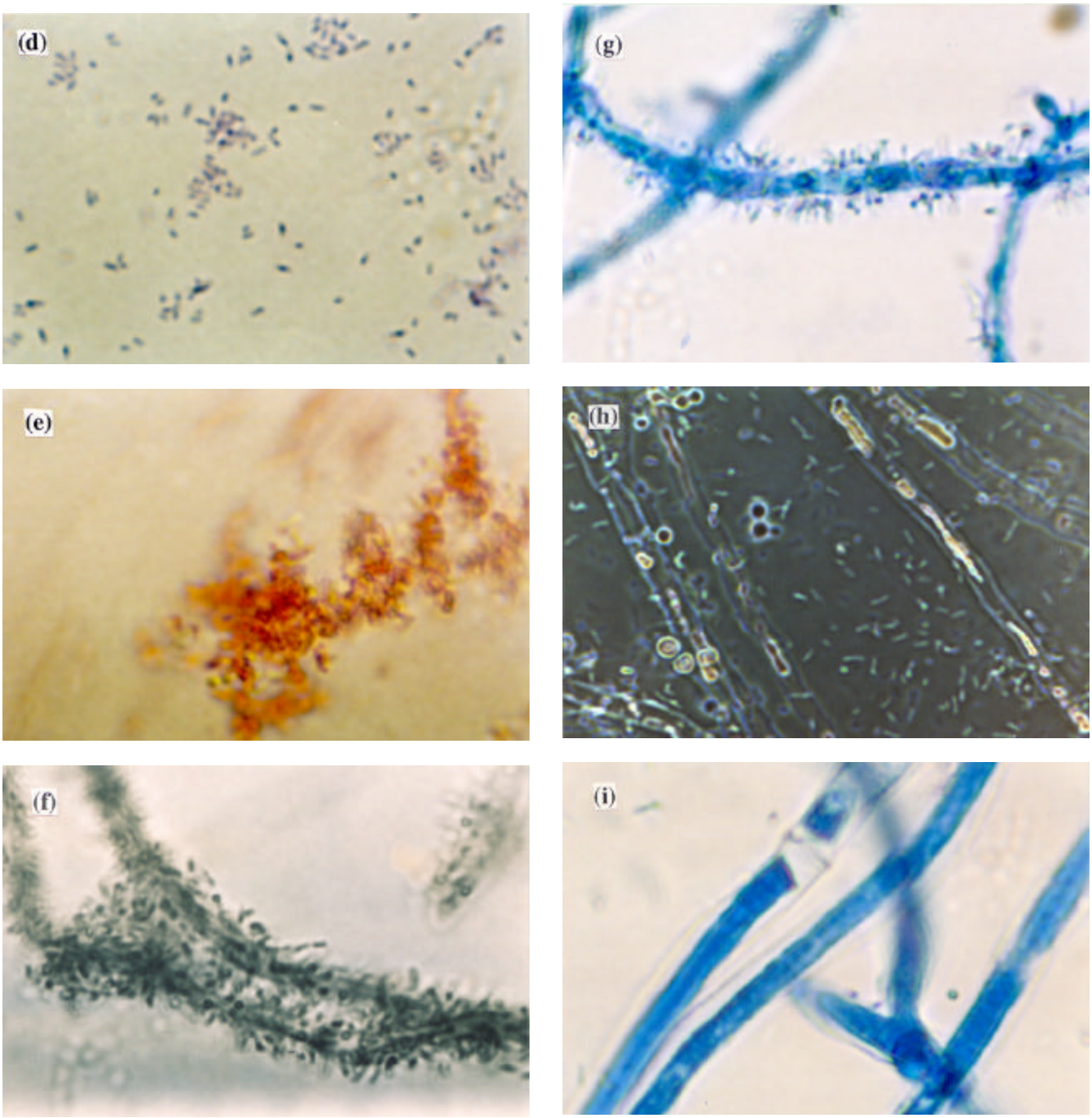

Figure 1. Phase-contrast microscopic examination of the interactions between common soil fungi, and bradyrhizobia and an azorhizobial strain. (**a**) Fungal filament attached with few rhizobial cells, and with a large number of planktonic cells of rhizobia in the medium, at 4 days of co-culturing of *B. elkanii* SEMIA 5019 and *A. niger*. Colonization of *B. elkanii* on *A. niger* (**b**) and *Mucor* spp. (**c**) after 5 days of their co-culturing. Bradyrhizobial cell clusters and individual cells (**d** and **e**), released to the culture medium at 20 days of incubation of the co-cultures of *B. elkanii* and *A. niger*. *A. caulinodans* ORS 571 colonization on *Mucor* spp. (**f**) and *A. niger* (**g** and **h**). (**i**) *A. niger* alone in the absence of rhizobia in the culture medium, a control for a comparison of the biofilm formation.

in both static and dynamic cultures. Eventually, biofilm structures gradually deteriorated towards the end of the observation period of 21 days with the release of the cell clusters and individual cells into the medium (figure 1d,e). *A. caulinodans* colonized well on mycelia of *Mucor* spp. (figure 1f) and *Penicillium* spp., but not on *Aspergillus* spp. (figure 1g, h; figure 1i is only for comparison). A limited number of cells of *A. caulinodans* adhered to the mycelia was observed with *Aspergillus* spp. There was a considerable bradyrhizobial growth (ca. 10^8 cells/ml) in all fungal exudate-treated cultures (table 1). Significantly higher cell growth compared to the control was observed in *Mucor* spp. exudate-treated cultures. However, exudates of *A. terrus* and *Aspergillus* spp. reduced the growth of the bradyrhizobial strain used.

4. Discussion

This study demonstrates that bradyrhizobia successfully colonize on the mycelial surfaces of *Aspergillus* spp., *Penicillium* spp. and *Mucor* spp. Fungal mat provided a site for the adhesion and its exudates served as a source of nutrition for bradyrhizobia, complying with other common associations between fungi and bacteria (Jones 1995). However, reduced growth of the bradyrhizobial strain in exudates of *A. terrus* and *Aspergillus* spp. could be due to a slight mycotoxin effect. The reason for the differential colonization of *A. caulinodans* on different fungi is unclear, which needs further investigation. In general, bacteria experience a certain degree of shelter and homeostasis when residing in a biofilm (Davey and O'toole 2000). In biofilms, residing members facilitate interspecies substrate exchange and the removal or distribution of metabolic products. Rhizobial species do not form spores and hence it is unclear how they survive in the absence of their hosts or under adverse soil conditions devoid of vegetation. Formation of such biofilms is a plausible strategy for their survival during extreme environmental conditions. Biofilms of multi species of bacteria establish new genetic traits by exchanging genetic materials from various origins (Christensen *et al* 1998). Therefore, biofilms when formed with several rhizobial strains could also serve as a source of genetic modification of rhizobia. Present preliminary study implies that the observed microbial cooperation for nutrients, etc. could be either associative or symbiotic. In the soil, this interaction between rhizobia and fungi could have developed an as yet unidentified nitrogen fixing system, which might have important interference in the nitrogen economy of soils. This needs further research to fully understand its effects and potentials.

Table 1. Growth of *Bradyrhizobium elkanii* SEMIA 5019 in yeast broth with fungal exudates.

Fungal exudate	Number of colonies ($\times 10^8$ cells/ml)
Aspergillus niger	$4{\cdot}12^b$
A. terrus	$3{\cdot}60^c$
Aspergillus spp.	$3{\cdot}57^c$
A. nidulans	$3{\cdot}98^b$
Mucor spp.	$4{\cdot}58^a$
Control†	$4{\cdot}20^b$
LSD (0·05)	0·349
CV (%)	7·33

Values followed by the same letter are not significantly different at 5% probability level, according to Duncan's multiple range test.
†Without the fungal exudate, but with mannitol.

Acknowledgements

K Karunaratne and A Morawakaarachchi of the project, and S Jayaweera of the institute helped in culture maintenance and photography.

References

Basha S and Ulaganathan K 2002 Antagonism of *Bacillus* species (strain BC 121) towards *Curvularia lunata*; *Curr. Sci.* **82** 1457–1463

Christensen B B, Sternberg C, Anderson J B, Eberl L, Moller S, Givskov M and Molin S 1998 Establishment of new genetic traits in a microbial biofilm community; *Appl. Environ. Microbiol.* **64** 2247–2255

Collins C H, Lyne P M and Grange J M 1989 *Collins and Lyne's microbial methods* 6th edition (London: Butterworths) p. 95

Davey M E and O'toole G A 2000 Microbial biofilms: from ecology to molecular genetics; *Microbiol. Mol. Biol. Rev.* **64** 847–867

Edwards K J, Bond P L, Gihring T M and Banfield J F 2000 An archael ion-oxidizing extreme acidophile important in acid mine drainage; *Science* **287** 1796–1799

Garbaye J 1994 Helper bacteria: a new dimension to the mycorrhizal symbiosis; *New Phytol.* **128** 197–210

Jones M V 1995 Fungal biofilms: eradication of a common problem; in *The life and death of a biofilm* (eds) J W T Wimpenny, P Handley, P Gilbert and Lappin Scott (Cardiff: Bioline) pp 157–160

Mezzalamaa M, Ghinia R, Ruffaa P, Ambrosolia R and Garibaldia A 1998 Effects of antagonistic *Fusarium oxysporium* on functional groups of indigenous bacteria in soil; *Appl. Soil. Ecol.* **7** 31–40

Morris C E, Monier J and Jacques M 1997 Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms; *Appl. Envir. Microbiol.* **63** 1570–1576

O'toole G, Kaplan H B and Kolter R 2000 Biofilm formation as microbial development; *Annu. Rev. Microbiol.* **54** 49–79

Perotto S, Bianciotto V, Balestrini R, Corazza C and Bonfante P 1998 Physical interactions of bacterial biofertilizers and biopesticides with arbuscular mycorrhizal fungi; (http://www-icom2.slu.se/ABSTRACTS/Perotto.html)

Rice W A, Olsen P E and Leggett M E 1995 Co-culture of *Rhizobium meliloti* and a phosphorus-solubilizing fungus (*Penicillium bilaii*) in sterile peat; *Soil Biol. Biochem.* **27** 703–705

Xavier L J C and Germida J J 2002 Response of lentil under controlled conditions to co-inoculation with arbascular mycorrhizal fungi and rhizobia varying in efficacy; *Soil Biol. Biochem.* **34** 181–188

MS received 11 November 2002; accepted 18 December 2002

Corresponding editor: VIDYANAND NANJUNDIAH