Can mushrooms fix atmospheric nitrogen?

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It is generally reported that fungi like *Pleurotus* spp. can fix nitrogen (N_2). The way they do it is still not clear. The present study hypothesized that only associations of fungi and diazotrophs can fix N_2. This was tested *in vitro*. *Pleurotus ostreatus* was inoculated with a bradyrhizobial strain nodulating soybean and *P. ostreatus* with no inoculation was maintained as a control. At maximum mycelial colonization by the bradyrhizobial strain and biofilm formation, the cultures were subjected to acetylene reduction assay (ARA). Another set of the cultures was evaluated for growth and nitrogen accumulation. Nitrogenase activity was present in the biofilm, but not when the fungus or the bradyrhizobial strain was alone. A significant reduction in mycelial dry weight and a significant increase in nitrogen concentration were observed in the inoculated cultures compared to the controls. The mycelial weight reduction could be attributed to C transfer from the fungus to the bradyrhizobial strain, because of high C cost of biological N_2 fixation. This needs further investigations using ^{14}C isotopic tracers. It is clear from the present study that mushrooms alone cannot fix atmospheric N_2. But when they are in association with diazotrophs, nitrogenase activity is detected because of the diazotrophic N_2 fixation. It is not the fungus that fixes N_2 as reported earlier. Effective N_2 fixing systems, such as the present one, may be used to increase protein content of mushrooms. Our study has implications for future identification of as yet unidentified N_2 systems occurring in the environment.

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1. Introduction

Rhizobium is a genus of symbiotic N_2-fixing soil bacteria that induce the formation of root nodules of leguminous plants. Microbial biofilms are communities of microorganisms attached to biotic or abiotic surfaces in the environment and differentiated to from a complex structure, and to conduct a range of functions. Formation of the biofilms by rhizobia with common soil fungi through mycelial colonization has been observed recently (Seneviratne and Jayasinghearachchi 2003). Further, nitrogenase activity of a developed *Penicillium* spp.-bradyrhizobial biofilm was detected using acetylene reduction assay (Jayasinghearachchi and Seneviratne 2004). Here, the *Penicillium* spp. alone did not show the nitrogenase activity. In general, it is reported using N balance studies that fungi such as *Pleurotus* spp. can fix N_2 biologically on natural substrates and that this might be quite commonly found among them (Ginterová and Maxianová 1975; Rangaswami *et al* 1975). However, it is still not clear how they fix N_2. In the present study, it is hypothesized that the formation of associations of the fungi with diazotrophs may be a possible means of N_2 fixation. This *in vitro* study evaluates the possibility of biofilm formation and N_2 fixation of an association of *P. ostreatus* and a bradyrhizobial strain nodulating soybean.

2. Materials and methods

2.1 *Culturing bradyrhizobia*

Bradyrhizobium elkanii SEMIA 5019 developed for soybean was used for the study. Cultures were maintained in yeast manitol broth (YMB) (Somasegaran and Hoben 1994), but without agar. They were incubated on a rotary shaker at 28°C for 6 days.

Keywords. Biofilms; mushroom; N_2 fixation; *Pleurotus*; *Rhizobium*

Abbreviations used: ARA, Acetylene reduction assay; He, helium; YMA, yeast manitol agar; YMB, yeast manitol broth.

2.2 *Development of a bradyrhizobial*-Pleurotus ostreatus *biofilm and evaluating for nitrogenase activity*

Different methods for biofilm formation have been developed depending on diverse objectives (Jackson *et al* 2001; Ceri *et al* 1999; Li and Bowden 1994; Hartley and Schlesinger 2002). In the present study, the biofilms were developed on an agar slab floating on the broth, which allows proper contact of the microbes with acetylene that is important in evaluating nitrogenase activity using the acetylene reduction assay (ARA) (Somasegaran and Hoben 1994). Petridishes (10 cm in diameter) were used for this. To produce biofilm cultures, a 2 × 2 cm yeast manitol agar (YMA) slab was placed in each petridish. A small piece (ca. 1 mm^2) of mycelial mass of *P. ostreatus*, grown from commercially available mushroom spawn on Czapek dox agar (confirmed for the absence of contaminants) was inoculated onto the YMA slab. After 4 days, ten ml of autoclaved YMB were added initially around the agar slab, and subsequently the addition of the YMB was repeated weekly. Four sets of six inoculated plates were prepared. At 5 days of the mycelial growth, one ml of a 6-day-old SEMIA 5019 culture was inoculated to the broth around the slab of each plate, of two sets (biofilm cultures). The other two sets were maintained without the bradyrhizobial inoculation (controls). Here, the use of YMB is essential to get an optimized growth of the bradyrhizobial strain. The cultures were incubated at 28°C with shaking at 4 rpm. One set each of the inoculated and control cultures were stained (lacto-phenol cotton blue) and observed every 3 days under oil immersion lens using a light microscope for maximum mycelial colonization and biofilm formation, when they were subjected to ARA. The other two sets were maintained for one month and used to measure the dry weight of the mycelial mass and N concentration.

Since 16 days old cultures showed maximum mycelial colonization and biofilm formation, they were transferred carefully from the petridishes to 75 ml sterilized-plastic bottles (Nunclon delta surface, NUNC Brand products, Denmark). Rubber stoppers were used to seal the bottles. The air space of the bottles was initially flushed with high purity (99·9995%) helium (He) gas. Then, 5 ml of He was replaced by 5 ml of acetylene (98%) gas. The bottles were incubated for 16 h, because preliminary tests showed that ethylene production of these biofilms peaked at this time (Jayasinghearachchi and Seneviratne 2004). Then, two ml of gas samples from each bottle were analysed using a Shimadzu GC 9 AM gas chromatograph fitted to a fused silica capillary column (Carboxen 1010 Plot, Supelco) equipped with a flame ionization detector. Analyses were done with a programmed column temperature increasing from 140°C at a rate of 24°C/min. Maximum temperature of the column was maintained at 225°C. The injector and the detector temperatures were maintained at 160°C and 180°C, respectively. After the gas samples were taken the fresh weight of the cultures in each bottle was measured.

2.3 *Determination of dry weight and nitrogen concentration*

After one month of incubation, agar in the plates was removed by gentle heating followed by filtration. The mycelial mass in each plate was oven dried at 60°C until it showed a constant weight. Dry weight was then recorded. Total N percentages of the dried mycelial samples were estimated using the Kjeldahl method (Bremner and Tabatabai 1972).

2.4 *Data analysis*

Means and standard errors of the data were calculated, and the means were compared using *T*-test (SAS 1998).

3. Results and discussion

Heavy mycelial colonization by the bradyrhizobial strain was observed in the *Pluerotus* spp.-bradyrhizobial biofilm after 16 days of incubation (figure 1). Nitrogenase activity was present in the biofilm, but not when the fungus or the bradyrhizobial strain was alone (table 1). A similar observation has been made in fungi and bacteria isolated from the soil and flooring of coniferous forests (Kononkov *et al* 1979). Pure fungal cultures have not shown N_2 fixing activity whereas that of pure bacterial cultures has been always lower than that of their associations with the fungi. *Pseudomonas fluorescens*, a diazotroph has been isolated from the surface of *P. ostreatus*, and has been observed to promote the formation of primodia and to enhance the development of basidiome (Cho *et al* 2003). Intracellular bacteria, like *Paenibacillus* spp., have been observed in ectomycorrhizal fungi (Bertaux *et al* 2003), and they possess genes involved in N_2 fixation (Choo *et al* 2003). Thus it is clear that the diazotrophs fix N_2 in association with the fungi. A significant reduction in mycelial dry weight by ca. 50%, while a significant increase in N concentration by ca. 150% was observed in *P. ostreatus* plates inoculated with the bradyrhizobial strain, compared to the no inoculation (table 2). Nitrogen concentration of all *P. ostreatus* cultures with or without inoculation was relatively low compared to when grown on standard media of mushroom (ranging from 2–3%) (Rangaswami *et al* 1975). The mycelial weight reduction and the low N concentration are mainly due to C and nutrient limitations in the present culture medium. Nitrogen in the non-inocu-

lated *P. ostreatus* could be from the fungal biodegradation of organic N in yeast extract of the culture medium. The mycelial weight reduction could be attributed to C transfer from the fungus to the bradyrhizobial strain, because of high C cost of biological N_2 fixation. This needs further investigations using isotopic tracers like ^{14}C-labelled sugars for confirmation.

The rhizobial-leguminous symbiosis is host-specific – a particular *Rhizobium* species only nodulates a small, defined range of plants (Price 1999). This is because the host releases specific chemical signal molecules which allow the rhizobia to identify the host. As such, it is interesting to identify the signalling system that occurs between the fungi and such symbiotic bacteria in any future study. This is a cell-to-cell communication between a prokaryote and a lower eukaryote. A similar communication has been reported in the marine ecosystem. Zoospores of a marine alga have been observed to attach to bacterial biofilms through chemical signals produced by the bacteria (Joint *et al* 2002). It is clear from the present study that mushrooms alone cannot fix atmospheric N_2. But when they are in association with diazotrophs, nitrogenase activity is detected because of the diazotrophic N_2 fixation. It is not the fungus that fixes N_2 as reported earlier. Moreover, our study indicates that the present symbiotic bacterium fixes N_2 residing in the biofilm. Effective N_2 fixing systems, such as the one under consideration, may be used to increase protein content of mushrooms. Further, studies should be directed to identify the specificity between the fungi and rhizobia and their effectiveness for improved production of mushrooms. Our study has implications for future identification of as yet unidentified N_2 systems occurring in the environment.

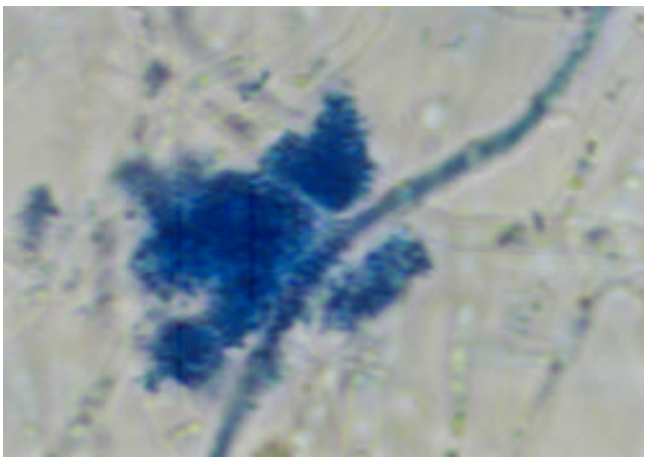

Figure 1. Heavy mycelial colonization of *Pluerotus ostreatus* by *Bradyrhizobium elkanii* SEMIA 5019 in the *Pluerotus* spp.-bradyrhizobial biofilm after 16 days of incubation.

Table 1. Evalution of rates of nitrogenase activity using ARA of: *Bradyrhizobium elkanii* SEMIA 5019; *P. ostreatus*; and *B. elkanii* SEMIA 5019 together with *P. ostreatus*.

Microbe(s)	ARA (nmol C_2H_4/day/g fresh weight)
SEMIA 5019 alone	ND
P. ostreatus alone	ND
SEMIA 5019 + *P. ostreatus* (biofilm)	2929 ± 374

Mean ± SE (n = 6). ND, not detected. Detection limit is 10 nmol C_2H_4.

Table 2. Dry weight and nitrogen concentration of *P. ostreatus* when it was grown on yeast manitol broth with or without *B. elkanii* SEMIA 5019 inoculation.

Treatment	Dry weight (g/plate)	N (%)
With inoculation (biofilm)	$0{\cdot}095^{b} \pm 0{\cdot}009$	$1{\cdot}131^{a} \pm 0{\cdot}017$
Without inoculation (control)	$0{\cdot}186^{a} \pm 0{\cdot}008$	$0{\cdot}458^{b} \pm 0{\cdot}014$

Mean ± SE (n = 6). Means in each column followed by different letters are significantly different at 5% probability level according to *T*-test.

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