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Use of *lacZ* and *gusA* Reporter Genes to Trace the Infection Process of Nitrogen-Fixing Bacteria

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Abstract

To determine whether the *gusA* gene, which encodes β-glucuronidase (GUS) and *lacZ* gene, which encodes β-galactosidase are suitable for tracing nitrogen-fixing bacteria in the infection process, *Bradyrhizobium japonicum* strains labelled with each gene were constructed. Both introduced genes were expressed in rhizobia, but it was difficult to specify the sites where lacZ-labelled bacteria were present, since endogenous β-galactosidase levels were high in soybean root tissues. On the other hand, endogenous β-glucuronidase activity has not been detected in soybean root tissues. The *gusA-marked Bradyhizobium, Rhizobium* and *Azospirillum* strains were constructed for assessing the use of their GUS-marked bacteria to trace the presence of introduced bacteria manifested by colonization on the root surface, as well as infection sites, invasion modes and nodulation competitiveness between bacteria. *Bradyrhizobium japonicum* inoculated to soybean colonized in the form of spots on the root surface. In the spots, curling roots with infection threads were observed. In this report, we describe only the *gusA*-marked (*Brady*)*rhizobium* and Azospirillum strains which we constructed.

Discipline: Biotechnology
Additional key words: β-glucuronidase, β-galactosidase, Azospirillum, nitrogen fixation

Introduction

Studies for practical use of the biological nitrogen-fixing (BNF) system are being increasingly important, since BNF contributes significantly to the maintenance and promotion of sustainable agricultural production. Ecological studies of the nitrogen-fixing bacteria in soil, root surface, intercellular spaces and nodules of host plants are essential to increase the contribution of nitrogen-fixing bacteria. Antibiotic-resistant markers and immunofluorescence have been used for these objectives. The strains marked with antibiotic resistance cannot be distinguished by light microscopy from the wild strain. The immunofluorescence method enables the detection of specific bacteria *in situ*, especially in tissues, but it requires a high technological level due to the interference from the background of host tissues. Recently, reporter genes such as *lacZ* and *gusA* have been used to facilitate the detection of individual marked strains of bacteria. Many plants and bacteria of economic importance do not contain indigenous glucuronidase (GUS) and do not show background GUS activity[20].

The (*Brady*)*rhizobium* strain encoded with the *gusA* reporter gene can be identified very clearly on the root surface or in infection threads by its blue color, because the marked bacteria turn blue when treated with the substrate. This finding may enable to trace the colonization and movement of marked

bacteria and thus the mode of early stages of infection. Moreover, studies on the competition for nodule occupancy, fate of introduced bacteria in the plant-soil ecosystem, and also on the interaction with plants could be very efficiently carried out with the use of the GUS reporter gene.

Evaluation of the advantages and disadvantages of various reporter genes for practical application may enable to use this new technique for future research work in ecological and molecular studies of microorganisms and plant-microbe interaction. Here in this report attempts were made to evaluate the use of reporter genes for tracing introduced bacteria, and the infection mode of rhizobia in the non-nodulating soybean mutant T201 after treatment with synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D).

Materials and methods

1) Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Bradyrhizobium* and *Rhizobium* were grown at 28-30°C in yeast-mannitol (YM) medium for 3-6 days. The antibiotics used in the media for culturing *gusA*-marked *Bradyrhizobium* (*B. japonicum* MA172, MA173, USDA61A124a and *B. elkanii* MA31), *Rhizobium* (*R. meliloti* YAO3M2 and *R. leguminosarum* bv. *trifolii* ANU843 (Tp3) and *B. japonicum* NA-A1017 carrying plasmid pRKRT290 were spectinomycin (50 μg mL^{-1}) and kanamycin (200 μg mL^{-1}), respectively. *Escherichia coli* was grown overnight at 36°C in LB medium[13] or in HM medium[14].

2) Plants

Soybean (*Glycine max* (L.) Merr.) cultivar Enrei, T202 and its non-nodulating isoline T201, wild type soybean (*Glycine soja*), siratro (*Macroptilium atropurpureum*), alfalfa (*Medicago sativa* L. cv. Vernal) and clover (*Trifolium repens* L. cv. California ladino) were used. Seeds of soybean cultivars (Enrei T202 and T201) were kindly provided by Dr. Kokubun (National Agriculture Research Center) and the wild type soybean seeds were provided by Dr. Harada (National Institute of Agrobiological Resources),

Table 1. Bacterial strains and plasmids used in this study

Designation	Source or reference
Strains	
Escherichia coli	
S17-1 λ-*pir*	Wilson (1995)[21]
HB101	Boyer et al. (1969)[3]
(Brady)rhizobium	
B. japonicum A1017	Takahashi[a]
B. japonicum MA172	Yuhashi et al. (1997)[23]
B. japonicum MA173	Yuhashi et al. (1997)[23]
B. japonicum USDA61A124a	Gresshoff[b]
B. japonicum USDA110	Keyser[c]
B. elkanii MA31	This study
R. meliloti YAO3	Kang et al. (1997)[12]
R. meliloti YAO3M2	Kang et al. (1997)[12]
R. leguminosarum bv. *trifolii* ANU843 (Tp3)	The CAMBIA Molecular Genetic Service, Australia
Azospirillum	
A. lipoferum 137	Vassyuk[d]
A. lipoferum T137-1	Tchebotar (1998)[19]
Plasmids	
pmTn*5*SS*gusA20*	Wilson (1995)[21]
pMC1403	Casadaban et al. (1980)[4]
pRK290	Ditta et al. (1980)[8]
pRK2013	Figurski and Helinski (1979)[9]
pRKRT290	This study

a): Agricultural Research Institute, Tokachi Federation of Agricultural Cooperatives.
b): The University of Tennessee, USA.
c): United States Department of Agriculture, Beltsville, MD.
d): Research Institute for Agricultural Microbiology, Russia.

while siratro, alfalfa and clover seeds were obtained from Yukijirushi Co., Japan.

3) Plant culture and inoculation

The seeds were surface-sterilized by immersion in 70% ethanol for 20 min, followed by soaking in 3% hydrogen peroxide for 5–10 min and then washed 5 times with sterile deionized water. Sterilized seeds were germinated in 2% agar plates or sterile vermiculite placed in 500 mL glass jars. Seedlings were transferred to sterile growth pouches (MEGA International of Minneapolis, USA) supplied with 30 mL of half-strength N-free nutrient solution[1] or to sterile vermiculite placed in 500 mL glass jars supplied with 90 mL of N-free nutrient solution.

Cultures of rhizobial strains as described above were centrifuged at 7,000 rpm, 4°C for 15 min. Cells were washed twice with sterile saline and resuspended in sterile deionized water. Cell density of the suspension was measured using a Thoma's haemocytometer and the suspension was diluted with sterile water to 10^7-10^8 cells mL^{-1}. For the co-inoculation test, the number of viable cells in the bacterial suspension was confirmed by the plate counting method using 10-fold serial dilution.

4) Conjugal transfer of pRKRT290 through mating

Transfer of the plasmid pRKRT290 carrying *lacZ* gene to *B. japonicum* A1017 was achieved by triparental mating. Culture of the donor *E. coli* HB101 (pRKRT290), HB101 harboring the helper plasmid pRK2013 and the recipient rhizobia from the mid-log phase were mixed on nitrocellulose filters (pore size 0.45 μm), grown on YM agar, and incubated at 28°C for 3 days. The colonies were suspended in 5 mL of sterile physiological saline solution, and the transconjugants were selected by plating onto HM medium[7] containing 200 $\mu g\ mL^{-1}$ of kanamycin. Transconjugants were again restreaked on selective medium to obtain single colony isolates. The galactosidase activity of the isolates was checked by the presence of the blue color after growth on YM medium supplied with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as substrate for the blue color staining.

5) Conjugal transfer of transposons through mating

Introduction of transposon into *B. elkanii* USDA31 was achieved by biparental mating. Donor *E. coli* S17-1 λ-pir with plasmid pmTn*5*SS*gusA20* and recipient rhizobia were mixed on nitrocellulose filter, and incubated. Cells on the filters were washed and resuspended with sterile saline. The suspension was spread on HM plates containing 100 $\mu g\ mL^{-1}$ of spectinomycin, and then tested for their GUS activity in stab-culture of HM medium containing 100 $\mu g\ mL^{-1}$ of spectinomycin and 50 $\mu g\ mL^{-1}$ of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc) at 28°C for 6 days.

6) X-Gal staining

The staining was performed by the method of Bovin et al.[2]. Primary root segments 3 cm long were placed in 10 mL test tubes containing 0.5% glutaraldehyde buffer with 0.2 M sodium cacodylate, pH 7.2, under vacuum for 60 min and cut into three 1 cm segments. One cm segments including nodules were embedded in 5% agar medium and transversely sectioned (0.1 mm thick) with a microslicer. The sections of root with nodules were immersed in the solution containing 800 μL of 0.2 M sodium cacodylate pH 7.2, 50 μL of 50 mM $K_3Fe(CN)_6$ and 50 mM $K_4Fe(CN)_6$, and 40 μL of 2% X-Gal in N, N-dimethylformamide overnight.

7) X-Gluc staining

For the detection of GUS-marked rhizobia in the plant tissues, transversal sections (0.1 mm thick) of root with nodules were used, as well as nodulated root segments (about 3 cm) for nodule occupancy. The samples were immersed in a test tube containing the GUS assay solution (5 mL 0.1 M sodium phosphate buffer at pH 7.0, 20 μL 2% X-Gluc, and 50 μL 10% SDS), *in vacuo* for 15–60 min, and incubated overnight.

Results and discussion

1) Tracing microbes in plant tissues

Soybean inoculated with *Bradyrhizobium japonicum* A1017 harboring the *lacZ* gene shows a deep blue color inside the nodules (infection zone) and some other parts of the plant tissues when stained with the substrate X-Gal (Fig. 1A). The plant tissues contain indigenous galactosidase, which reacts with the substrate and forms a deep blue color. As a result, it is difficult to trace the infection zone. Hence, to suppress the indigenous galactosidase, root with nodules was treated with glutaraldehyde buffer containing a high concentration (200 mM) of potassium ferri- and ferrocyanide. However, some parts showing a blue color remained along the vascular bundles, and most parts of the rhizobial infection zone filled with bacteroids did not stain. The former

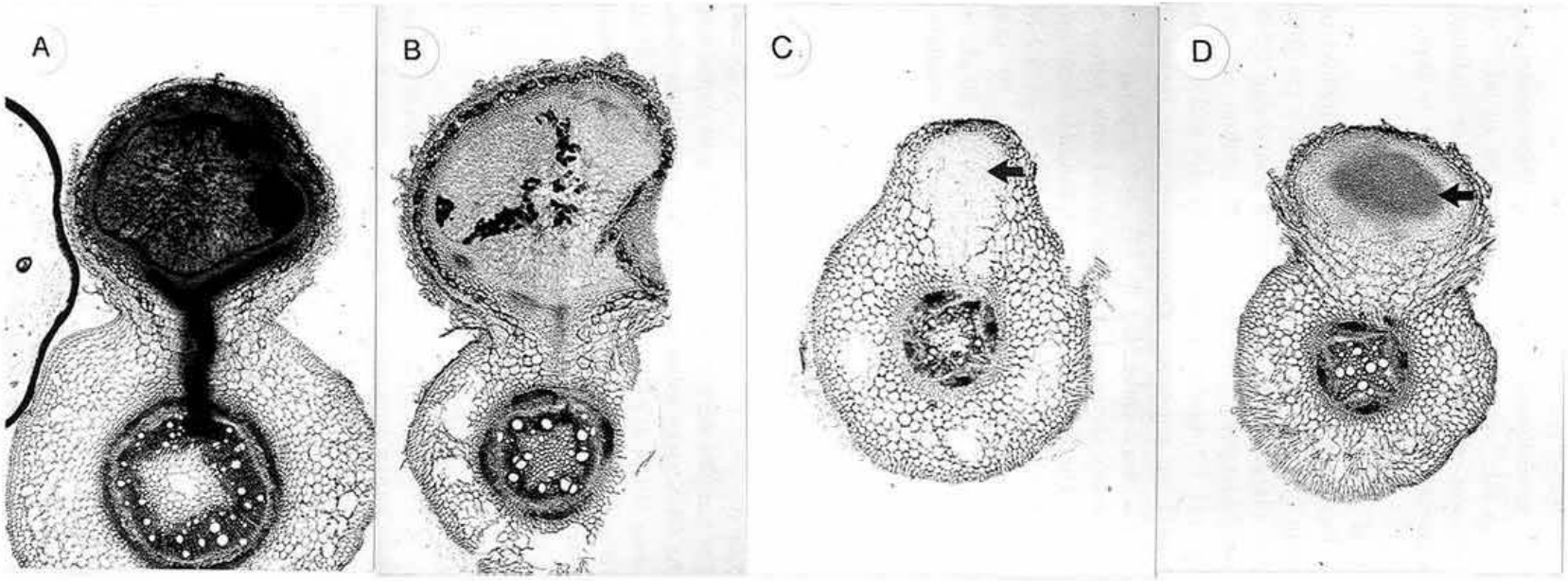

Fig. 1. β-Galactosidase activity in roof nodule inoculated with *lacZ*-marked *B. japonicum*
Addition of 50 mM potassium ferri- and ferrocyanide (A), and 200 mM potassium ferri- and ferrocyanide (B) to *lacZ* detection buffer. β-Glucuronidase (GUS) activity in root nodules. Root nodule induced by *B. japonicum* 61A124a (C), and *gusA*-marked *B. japonicum* USDA110 (D).

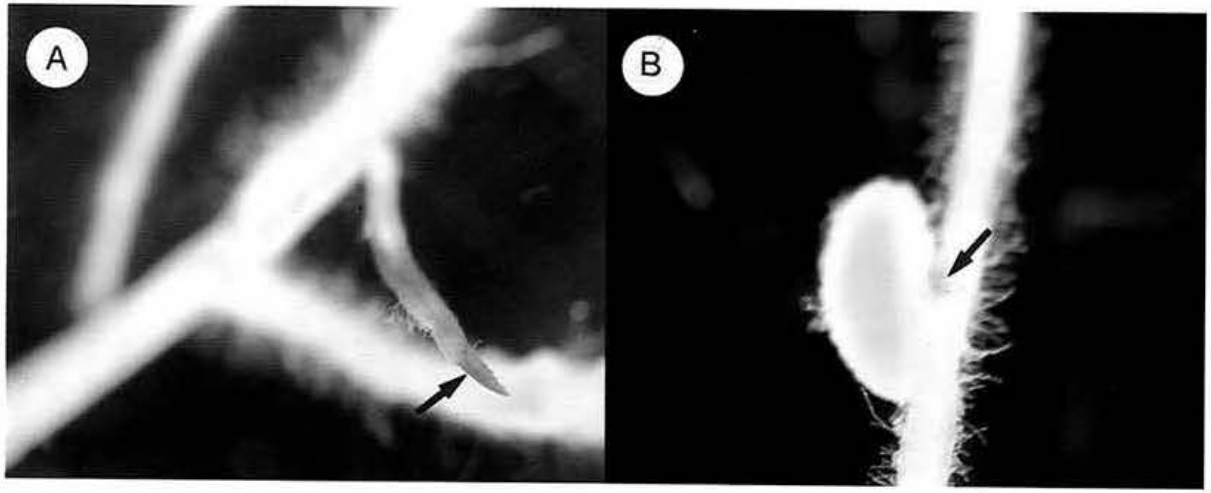

Fig. 3. Colonization and nodulation of white clover at 20 DAI by *gusA*-marked *A. lipoferum* T1371 alone (A), and nodulation by co-inoculation with *R. l.* bv. *trifolii* ANU 843 and *gusA*-marked *A. lipoferum* T1371 (B)
Blue staining (arrows) indicates GUS (β-glucuronidase) activity of the bacteria.

indicates that it is difficult to entirely suppress the indigenous galactosidase, while the latter suggests that the induced plasmid pRKRT290 carrying *lacZ* was lost during rhizobial multiplication (Fig. 1B). As a result, the bacterial infection zone could not be clearly traced by using the *lacZ* gene.

Then the soybean plants were inoculated with *Bradyrhizobium japonicum* marked with the GUS (β-glucuronidase) reporter gene. When the root with nodules was treated with GUS substrate X-Gluc., a uniform and clear blue color was observed only in the infection zone (Fig. 1C), whereas no color was observed in the nodule (infection zone) inoculated with the unmarked (gus-) *Bradyrhizobium japonicum* strain (Fig. 1D), because the soybean root nodule tissues did not contain indigenous glucuronidase and there was no background activity in plant.

Jefferson et al.[11] reported that the *gusA* gene, encoding GUS, is the most widely used reporter gene in plant molecular biology and the GUS substrate also enables to detect the spatial localization of reporter gene activity. As such *(Brady)rhizobium* encoded with β-glucuronidase (GUS) was found to be highly suitable for bacterial infection studies.

2) Infection mode of nodulating soybean cultivars and non-nodulating mutants with rhizobia

We previously reported that nitrogen-fixing nodules can be induced in a non-nodulating soybean mutant T201, which is defective in nodulation, by 2,4-D treatment and rhizobial inoculation[1]. Nodulating soybeans develop spherical determinate nodules, while the nodules induced in a non-nodulating isoline by 2,4-D treatment are unusual and appear as paired or gourd-like nodules. In the case of the nodulating soybean cultivar Enrei T202, the bacteria colonize the root surface (Fig. 2A), and in the colonized zone, curled root hairs are observed (Fig. 2B). The bacteria penetrate into the root tissues through the curled root hair and infection thread (Fig. 2C), and thus form a nodule (Fig. 2D). While in the case of the non-nodulating soybean mutant T201, the bacteria colonize the root surface and multiply rapidly, causing the swelling of the root (Fig. 2E), where no root hair curling (Fig. 2F) but infection threads are observed (Fig. 2G). The bacteria penetrate into the plant root tissues through the infection thread. Inside the plant tissues, the bacteria multiply rapidly and form a nodule (Fig. 2H). In the distal lobe of the paired nodules, *gusA*-marked bacteria are observed showing a blue color.

The *gusA*-marked bradyrhizobia are very useful for tracing infection sites of nodules in that the bacteria stain blue and can be clearly identified. In the case of nodulating soybean T202, the bacteria penetrated into the root cells through root hair curling and subsequently infection thread formation, while in the non-nodulating soybean T201, there was no evidence of root hair curling and the bacteria invaded through both intercellular spaces and infection threads. Chandler[5] and Chandler et al.[6] reported that *Arachis* and *Stylosanthus* do not form infection threads and rhizobia penetrate into spaces between epidermal cells and invade cortical cells through their cell walls. Thus, root hair curling and formation of the infection thread may be independent phenomena controlled by different factors in the nodulation process. Hence, root hair curling or infection threads are not always necessary for the formation of symbiosis, and the plants may receive rhizobia by various ways which can be studied using GUS reporter genes.

3) Competition between host and bacteria

Many leguminous plants can be nodulated by more than one *(Brady)rhizobium* species. Also some bacteria can form effective nodules in several host plants. *Glycine max* cv. Enrei, *Glycine soja* and *Macroptilium atropurpureum* formed effective nodules by infection with both *Bradyrhizobium japonicum* (USDA110) as well as *Bradyrhizobium elkanii* (MA31). *Bradyrhizobium elkanii* (MA31) is a *gusA*-marked mutant of *Bradyrhizobium elkanii* USDA31. When the above plants were co-inoculated with the bradyrhizobial strains, USDA110 and MA31 at 1:1 ratio, in *Glycine max* cv. Enrei, 81% of the nodules were formed by USDA110 and 19% by MA31, while in *Glycine soja*, 55% of the nodules were formed by USDA110 and 45% by MA31. In *Macroptilium atropurpureum* only 20% of the nodules were formed by USDA110 and 80% by MA31 (Table 2). These findings indicate that USDA110 is more competitive for nodulation in *Glycine max* cv. Enrei, while MA31 is more competitive for nodulation in *Macroptilium atropurpureum*. However, both strains, USDA110 and MA31 are almost equally effective for nodulation in *Glycine soja*.

Furthermore, a large number of nodules were formed in *Glycine max* cv. Enrei (142) by USDA110 and in *Macroptilium atropurpureum* (64) by MA31, while fewer nodules were formed in *Glycine soja* by either strains USDA110 or MA31.

The GUS transposon was found to be very useful for studying the competition for nodulation in common bean[17,18]. Also, Yuhashi et al.[23] and Wilson

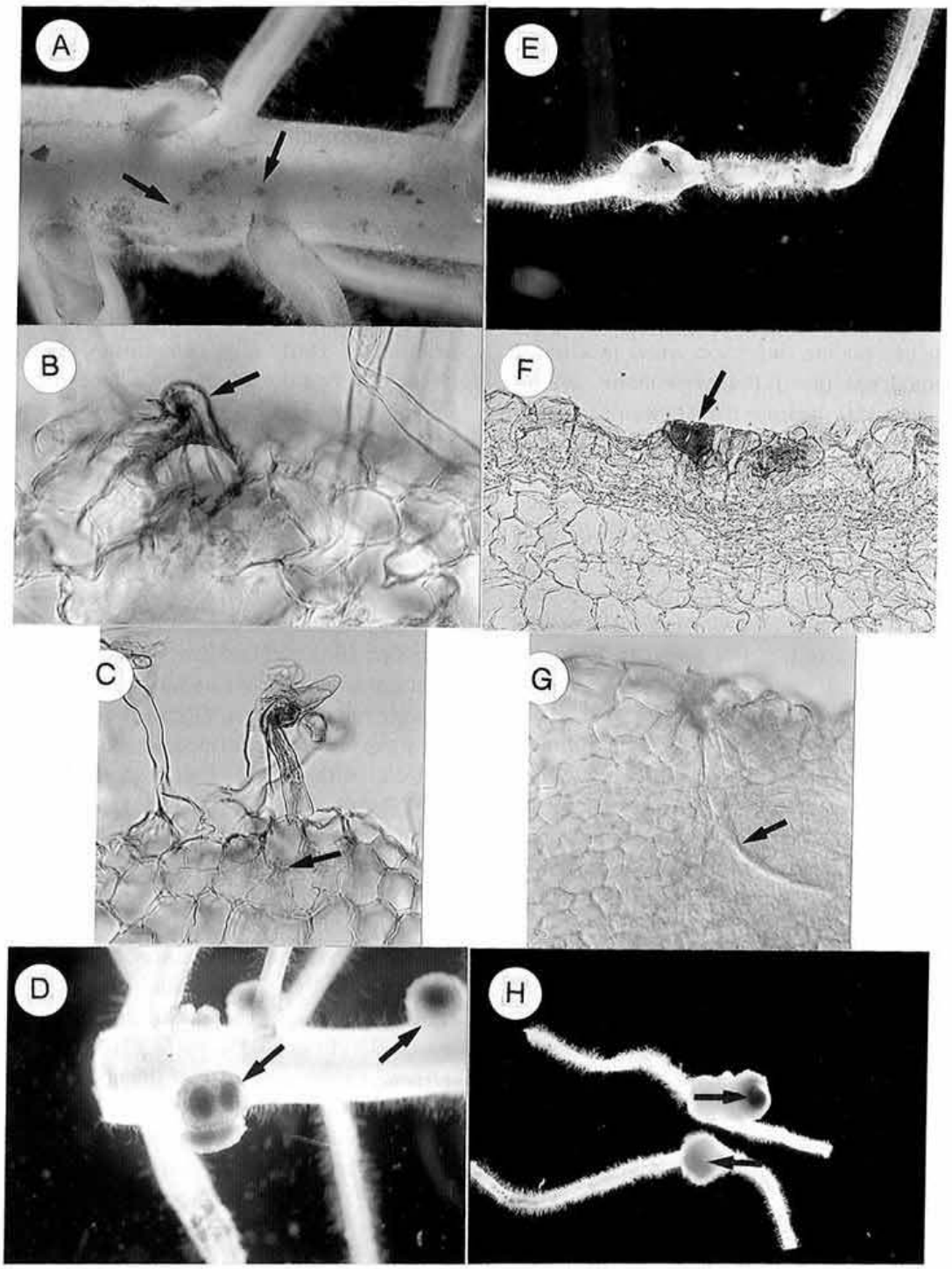

Fig. 2. Infection process of nodulating (A, B, C, D) and non-nodulating (E, F, G, H) soybean
A, bradyrhizobial colonization on root surface and root infection (arrow); B, transverse section of infected root portion showing root hair curling and C, infection thread (arrow); D, invasion of rhizobia inside developing nodule (arrow); E, swelling caused by 2,4-D treatment and formation of nitrogen-fixing nodule; F, transverse section of swelling portion showing bacterial infection but no root hair curling (arrow) and G, invasion of bacterial infection thread (arrow); H, nodules formed by 2,4-D treatment (GUS-marked bacteria are present inside of the distal lobe of the nodule).

Table 2. Relationship between host and rhizobia

Host plant	Nodule numbers			Nodule occupancy (%)	
	USDA110	MA31	Total	USDA110	MA31
G. max cv. Enrei	142	33	175	81	19
G. soja	27	22	49	55	45
M. atropurpureum	16	64	80	20	80

et al.[20] described the advantages of using the *gusA* reporter gene as a marker for rhizobial competition studies.

4) Plant–Azospirillum–Rhizobium *interaction*

Increase in the nodule number and grain yield of various crops has been reported by combined inoculation of *Azospirillum* spp. and *Rhizobium* spp.[16,22]. The role of *Azospirillum* spp. in the infection and nodulation of plants can be clearly examined by using the GUS-reporter gene.

Colonization on the root surface and root tips of white clover was observed when the plant was inoculated with *gusA*-marked *Azospirillum lipoferum* T137-1 alone (Fig. 3A). However, when white clover was inoculated with both *Azospirillum lipoferum* T137-1 and *Rhizobium leguminosarum* bv. *trifolii* ANU843, many nodules and *Azospirillum* colonization of or near the nodules were observed (Fig. 3B). Nodule number and acetylene reduction activity in clover increased by more than 100% at 20 days after inoculation by combined inoculation of *Azospirillum lipoferum* T137-1 and *Rhizobium leguminosarum* bv. *trifolii* ANU843 (Fig. 4).

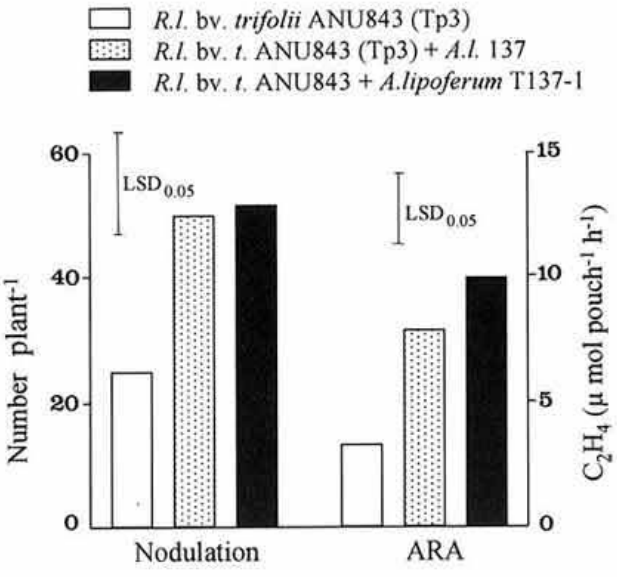

Fig. 4. Nodulation and acetylene reduction activity (ARA) of white clover at 20 DAI as influenced by *Rhizobium* spp. and *Azospirillum* spp. inoculation

Increase in the nodule number and acetylene reduction activity in white clover by dual inoculation with *Azospirillum lipoferum* T137-1 and *Rhizobium leguminosarum* bv. *trifolii* ANU843 may be due to the fact that *Azospirillum* contributes to the development of additional sites for the rhizobia to form more nodules. Plazinski and Rolfe[15] and Yahalom et al.[22] reported that in the case of co-inoculation of *Azospirillum* and *Rhizobium, Azospirillum* stimulated the formation of a larger number of epidermal cells that differentiated into infective root hair cells, or created additional infection sites which were later occupied by the rhizobia. Increase in shoot length, dry weight, number of root hairs and root diameter of alfalfa was also observed by combined inoculation of *Azospirillum* spp. and *Rhizobium* spp.[10].

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