

GUS REPORTER GENE IN THE STUDY OF BACTERIA AND LEGUME INTERACTION

CONSTANCIO A ASIS JR¹, VLADIMIR K CHEBTAR², UI-GUM KANG³ & SHOICHIRO AKAO⁴

¹Supervising Science Research Specialist (asis_tony@yahoo.com), Philippine Rice Research Institute, Maligaya, Science City of Muñoz, 3119 Nueva Ecija, Philippines. ²Head, Laboratory of Microbial Technology, All-Russia Research Institute for Agricultural Microbiology, Shosse Podbelskogo 3, St. Petersburg – Pushkin 8, 189620 Russia. ³Plant Environment Division, National Yeongnam Agricultural Research Institute, P.O. Box 6, Milyang 627-130 South Korea. ⁴Professor, Department of Biochemistry and Applied BioScience, Faculty of Agriculture, Miyazaki University, Miyazaki, 889-2192 Japan

Knowledge about the colonization behavior of microbial inoculants is a prerequisite to their effective use in agriculture. In this study, we used GUS reporter gene to observe the behavior of symbiotic nitrogen-fixing and plant growth-promoting bacteria in alfalfa, white clover, and soybean. The gusA gene from Escherichia coli S17-1 lambda-pir with plasmid mTn5SSgusA20 was inserted into the genome of the recipient strains by triparental mating. There was no significant difference in the acetylene reduction activity (ARA) and nodulation of alfalfa when inoculated with either gusA-marked Rhizobium meliloti or parent strain, indicating that GUS marking did not affect the nitrogen-fixing properties of the transconjugants. Co-inoculation of Rhizobium leguminosarum bv. trifolii and gusA-marked Azospirillum lipoferum increased the ARA and nodulation of white clover by 2 - 3 times from 5 to 20 days after inoculation (DAI) and ARA by 2.3 - 2.7 times at 20 DAI. The abundant colonization of A. lipoferum on the roots, root hairs, and sites near or on the nodules suggests that the formation of additional infection site by A. lipoferum may be the mechanism that enhances the ARA and nodulation of white clover. Co-inoculation of Bradyrhizobium japonicum and gusA-marked Pseudomonas fluorescens also increased the ARA and nodule number of soybean at 10 and 20 DAI and stimulated the growth and colonization of B. japonicum on soybean roots. The results indicate that the enhanced ARA and nodulation of soybean are due to the high colonization of P. fluorescens on soybean roots and the production of substances that stimulate the growth of B. japonicum.

biological nitrogen fixation, GUS reporter gene, legumes, plant growth promoting bacteria, soybean, white clover

INTRODUCTION

Microbial inoculation of soil is required for a number of applications, such as inhibition of plant pathogens, biodegradation of toxic compounds, improvement of soil structure, microbial leaching of metals, and promotion of plant growth. Plant growth-promoting bacterial (PGPB) inoculants include a broad spectrum of prokaryotes that have beneficial effects on the host plants owing to their biological control traits, induction of systemic resistance to the host plant, promotion of plant growth, and biological nitrogen fixation (BNF) (Tomashow & Weller 1995).

Legume-rhizobia symbiosis is the major source of BNF in agricultural production. The bacterial symbionts are Gram-negative and N-fixing

prokaryotes (diazotrophs) belonging to the Rhizobiaceae family either of genus *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, or *Sinorhizobium*. The use of competitive and effective rhizobial inoculant is an important factor in increasing the contribution of BNF to the plant's nutrition in terms of nitrogen. The inoculant rhizobia must survive in an atypical environment until the seedlings are established.

The use of symbiotic and associative diazotrophs or nondiazotrophic rhizosphere PGPB (mixed inoculants) is another way of enhancing nitrogen fixation. Some microbial mixtures allow the bacteria to interact with each other synergistically – by providing nutrients, removing inhibitory products, and stimulating each other through physical or

biochemical processes (Holguin & Bashan 1996). Oliveira et al (1997) reported that inoculation of a mixture of *Azospirillum brasilense* Sp7 and *Rhizobium* sp. increased the ARA, nodulation, and shoot dry weight of clover. The nodulation of pea was increased when co-inoculated with *Rhizobium leguminosarum* and *Pseudomonas fluorescens* (Andrade et al 1998).

The success in inoculating beneficial bacteria, however, depends on the colonization potential of the introduced strains. The colonization of plant roots by the introduced bacteria is an important step in establishing an effective plant-bacteria interaction (Shippers et al 1987). The main limiting factor in the assessment and identification of competitive and efficient strains under field conditions is the lack of suitable methodology to screen the success of an individually isolated strain in competing for nodule occupancy in different legume cultivars and under different agronomic conditions.

The common methods applied for rhizobial competition studies involve the use of antibiotic resistance, serological properties, and transposon Tn5 insertion (Kang et al 1991). The strains with antibiotic resistance, however, cannot be distinguished from the wild strains using light microscopy. The immunofluorescence method enables detection of specific bacteria *in situ* especially in tissues, but requires a high technological level to deal successfully with the interference from the background of host tissues. Recently, Wilson et al (1995) introduced a new technique that can easily differentiate the strain, occupying the nodule from other competing strains using the β -glucuronidase (GUS) transposon. The GUS transposon employs an exogenous molecular marker and provides simple color assays even at the early stage of root colonization and infection.

The objectives of this study were to observe the nodule occupancy of *gusA*-marked *S. meliloti* in alfalfa, clarify the co-inoculation effect of *Rhizobium* and *Azospirillum* in white clover, and evaluate the effect of nondiazotrophic rhizosphere PGPB (*Pseudomonas fluorescens* and *Azomonas agilis*) on the nodulation and growth of soybean.

MATERIALS & METHODS

The study involved 3 experiments:

Expt 1: Nodulation competitiveness of *gusA*-marked *S. meliloti* in alfalfa

Expt 2. Co-inoculation of white clover with *Rhizobium* & *Azospirillum*

Expt 3. Co-inoculation of soybean with *Bradyrhizobium* & nondiazotrophic rhizosphere plant growth promoting bacteria.

Nodulation competitiveness of *gusA*-marked *S. meliloti* in alfalfa

Bacterial strains & transposon mutagenesis

Sinorhizobium meliloti strains used in this experiment were recipient strain YAO3 (Kang et al 1991), *gusA*-marked mutants strains YAOM2 and YAOM6 (this study), helper strain *Escherichia coli* HB101 with plasmid pRK2013, and donor strain *E. coli* S17 λ pir with plasmid pmTn5SsgusA20 (Wilson et al 1995). The recipient strain was cultured in low concentration of organic nutrient (LON) broth at 30°C for 1 day, the donor and helper strains in Luria Broth (LB) medium with 25 mg/mL spectinomycin overnight. Plate matings were carried out as described by Wilson et al (1995) on LON medium (Dreyfus et al 1983). Transconjugants were selected from LON agar containing 100 μ g/mL of spectinomycin and then tested for their GUS activity in LON agar plates with 100 μ g/mL spectinomycin and 50 μ g/mL of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide cyclohexylammonium salt (X-Gluc) at 30°C.

Plant culture & inoculation

Seeds of alfalfa cv. 'Vernal' were surface-sterilized using 70% EtOH for 20 min, followed by 3% H₂O₂ for 5 min, rinsed thoroughly with sterile water, and germinated in 2% agar plates. Seedlings were transferred onto sterilized Seed Pack Growth Pouch (American Scientific Products, Minneapolis, Minnesota) containing N-free nutrient solution (Akao & Kouchi 1989) at 2 seedlings/pouch with 3 replications.

Inoculants of *S. meliloti* strains YAO3, YAO3M2, and YAO3M6 were prepared by growing them separately in yeast mannitol (YM) medium. At log phase, each strain was centrifuged at 7,000 rpm at 4°C for 15 min. Pellets were washed twice with sterile saline and cell density was adjusted to 10⁸ cells/mL. For the nodulation competitiveness test, wild and mutant strains were combined at 1:1 ratio and 1 mL of the cell suspension was inoculated at 5 days after planting (DAT). Plants were grown in the greenhouse at 28/23°C day/night time and 14/10 photoperiod.

Nodulation & acetylene reduction assay

Plants were harvested two weeks after inoculation to examine the nodule occupancy, nodule number, and ARA of the *gusA*-marked and wild strains. For nodule occupancy, nodulated roots of the plants inoculated with combined wild and mutant strains were immersed in test tubes with the GUS assay solution (5 mL 0.1M sodium phosphate buffer at pH 7.0, 20 μ L 2% X-Gluc, and 50 μ L 10% sodium dodecyl sulfate (SDS) in vacuum for 15 min, incubated at 30°C, washed with distilled water, and observed under stereoscopic microscope. The percentage of blue-stained nodules to the total number of nodules observed was recorded as the percentage of nodule occupancy of the *gusA*-marked strain. Moreover, nodulated roots of the plants inoculated with either the wild or mutant strain were assayed for nitrogenase activity following the procedure of Francisco et al (1992). Then, the nodule number was obtained by counting the number of globular structures measuring more than 1 mm in diameter. Analysis of variance was computed and treatment means were compared based on least significant difference (LSD) test using the software IRRISTAT for Windows, Version 4.0 (IRRI, Philippines).

Co-inoculation of *Rhizobium* & *Azospirillum* in white clover

Plant culture & inoculation

The bacteria used in this experiment were *A. lipoferum*137, *gusA*-marked *A. lipoferum* T137, *R. leguminosarum* bv. *trifolii* ANU 843, and *gusA*-marked *R.l.* bv. *trifolii* ANU 843 [Tp3]. Surface sterilized seeds of white clover (*Trifolium repens*) cv. 'California Ladino' were germinated on petri plates with 2% agar in the dark at 28°C. Seedlings were transplanted at 3 seedlings to each Sterile Seed Pack Growth Pouch. Sterile water and half strength N-free nutrient solution (Akao & Kouchi 1989) were supplied alternately to each pouch. Seedlings were kept in the greenhouse with a day/night temperature of 28/23°C day/night time and 14/10-hour photoperiod.

For inoculation tests, *A. lipoferum* strains were grown overnight on Döbereiner agar medium with and without 20 μ g/mL spectinomycin (Vassiyuk et al 1990) at 28°C while the *R.l.* bv. *trifolii* ANU 843 strains were grown on yeast mannitol agar (YMA) plates with 50 μ g/mL spectinomycin. Each strain was centrifuged at 10,000 rpm, 4°C for 10 min. Pellets were washed twice with sterile saline solution, and cell density was

adjusted to 4.0×10^7 cells/mL with sterile deionized water. Single or dual (1:1 cell ratio) inoculation of *Rhizobium* spp. and *Azospirillum* spp. at 1 mL/pouch was done 3 days after transplanting white clover seedlings.

Nodulation & acetylene reduction assay

The nodulation and colonization patterns of white clover roots by *Azospirillum* spp. and *Rhizobium* spp. were observed at 5, 10, and 20 DAI. Five replications were used for each observation. Exhumed roots with or without nodules were placed in test tubes with a GUS assay done according to the procedure above. Analysis of variance was computed and treatment means were compared with each other based on the least significant difference (LSD) test using IRRISTAT.

Co-inoculation of *Bradyrhizobium* & *nondiazotrophic* bacteria in soybean

Plant culture & inoculation

The bacteria used in this experiment were kasugamycin (1 mg) resistant strain of *B. japonicum* A1017kas^r, *B. japonicum* A1017 as well as *gusA*-marked strain of *Pseudomonas fluorescens* 2137, *Pseudomonas fluorescens* WCS365 and *Azomonas agilis* 125. Soybean cv. 'Enrei' seeds were surface-sterilized with 70% alcohol for 5 min, followed by 5% H₂O₂ for 5 min, then washed 5 times with sterile deionized distilled water at 2 min interval. The surface-sterilized seeds were sown in sterile glass jars with vermiculite and allowed to germinate under dark conditions at 28°C. Seedlings were transplanted onto growth pouches at one seedling per pouch. Sterile water and half-strength N-free nutrient solution (Akao & Kouchi 1989) were alternately supplied to each pouch daily. Seedlings were kept in an environmentally controlled growth chamber at day/night temperature of 28/23°C and a photosynthetic photon flux of approximately 500 μ mol/m²s provided by halide lamps for 14 hours daily.

For the inoculation test, *B. japonicum* A1017kas^r was grown for 48 hours on YM medium with 1 mg/mL kasugamycin. *P. fluorescens* 2137_{gus} was grown for 48 hours on LON medium supplemented with 20 μ g/mL spectinomycin while *P. fluorescens* WCS365_{gus} and *Azomonas agilis* 125_{gus} were grown for 48 hours on YM with 20 μ g/mL spectinomycin. The incubation temperature was 28°C. Each strain was centrifuged for 10 min at 10,000 rpm and 4°C. Pellets were

washed twice with sterile saline solution and cell density was adjusted to 2.4×10^7 cells/mL for rhizobacteria and 1.2×10^7 cells/mL for *B. japonicum* A1017kas^r using sterile deionized water. Single and dual (1:1 cell ratio) inoculation of *B. japonicum* A1017kas^r with either *P. fluorescens* 2137gus, or *P. fluorescens* WCS365gus were done at 1 mL/pouch of inoculum 7 days after transplanting.

Root colonization, nodulation, & acetylene reduction assay

Soybean plants were harvested at 5, 10, and 20 DAI to observe the colonization patterns of the introduced strains and determine their colonization rate as well as their effects on the nodulation and

and all colonies on the YM plates with kasugamycin were computed, respectively. The total number of bacteria was calculated per 1 gram of fresh roots. For nodulation and colonization pattern assay, soybean roots with or without nodules were placed in test tubes and assayed for GUS activity as described above. Nodules were observed by naked eye and considered as globular structure when the diameter was ≥ 0.5 mm. Roots were observed under a light microscope (Nikon Optiphot) equipped with a camera. Acetylene reduction was determined at 10 and 20 DAI following the procedure of Francisco et al (1992).

To determine the effect of co-inoculation of *P. fluorescens* 2137gus on the growth of *B. japonicum* A1017kas^r, 2-day old liquid YM medium-grown *P.*

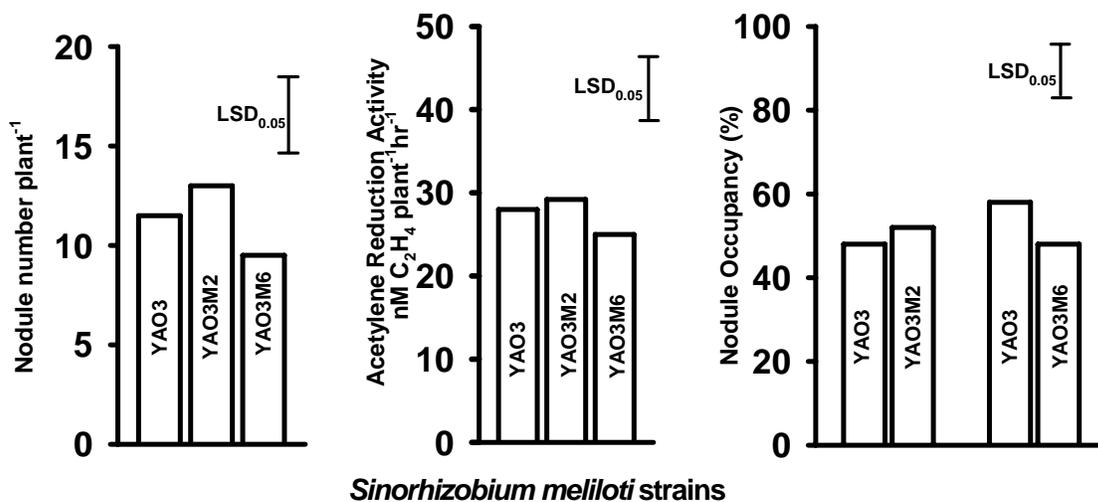


Figure 1. Nodulation (A), nitrogen fixation (B), and nodule occupancy (C) of wild (YAO3) and *gusA*-marked (YAOM2 and YAOM6) strains of *S. meliloti* on alfalfa 2 weeks after inoculation. Values are means of 3 replications.

nitrogen fixation in soybean roots. Three replications were used for each observation. To determine the colonization rate of the bacteria, soybean roots were weighed and macerated in mortar with 20 mL of sterile deionized water by sterile pestle. Ten-fold serial dilution was prepared and the 2nd, 4th, and 6th dilutions were used for 3 replications, plating on YM agar medium with 20 μ g/mL spectinomycin and 10 μ g of X-Gluc/mL for *gusA*-marked bacteria or 1,000 μ g/mL kasugamycin for *B. japonicum*. Petri plates were incubated at 28°C for 5 days. Blue-stained colonies on YM plates with spectinomycin and X-Gluc

fluorescens 2137gus was centrifuged at 10,000 rpm for 10 min. The supernatant was passed through a 0.45 μ m sterile membrane filter and 10 mL of sterile supernatant was diluted with 100 mL of YMB medium containing 50 μ g/mL spectinomycin in 300 mL Erlenmeyer flask. Moreover, 10 mL of sterile YMB medium was added to another flask as a control. One mL of *B. japonicum* A1017kas^r cell suspension ($OD_{610nm} = 2.5$) was added in each flask and flasks were placed in a rotary shaker with 200 rpm at 28°C for 5 days. The bacterial cell density was determined every 24 hours by plate counting.

RESULTS & DISCUSSION

Nodulation competitiveness of gusA-marked S. meliloti in alfalfa

The nodulation ability, ARA, and nodule occupancy of *gusA*-marked mutants were compared with those of

report supports the claim of Wilson et al (1995b) that transposon of *gusA* does not have an intrinsic effect on the competitive ability of the rhizobia. This study also showed that transposable plasmid mTn5SSgusA20 was carefully inserted into the genome of *R. meliloti* YAO3 without causing any damage on the genes

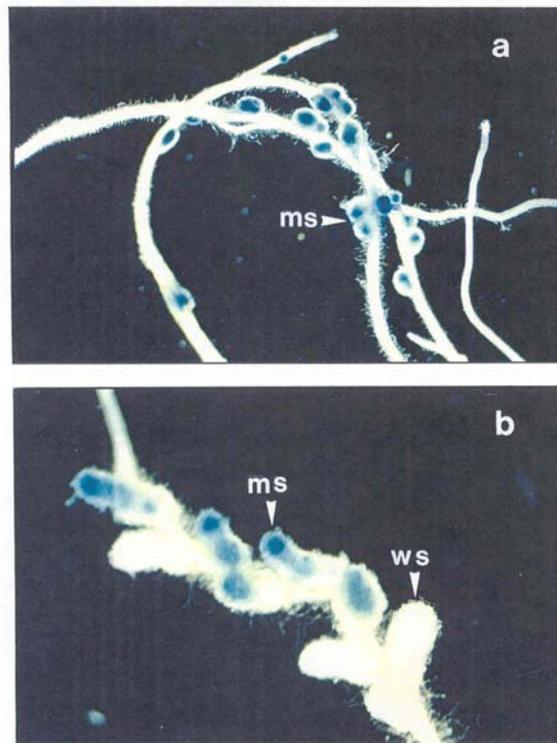


Figure 2. Alfalfa root nodules formed two weeks after inoculation with wild and *gusA*-marked *S. meliloti* strains: (a) *gusA*-marked *S. meliloti* YAOM2 (ms), (b) ms+wild *S. meliloti* YA3 (ws)

the wild-type parent in symbiotic association with alfalfa to determine if plasmid insertion changes the characteristics of the bacteria. The results showed that alfalfa plants inoculated with *gusA*-marked *R. meliloti* YAO3M2 or *R. meliloti* YAO3M6 had comparable nodule number, ARA, and both mutants had similar abilities to compete for nodule occupancy with the parent strain (Figure 1). These suggest that plasmid insertion does not create any changes on the nodulation and symbiotic traits of the bacteria. This

responsible for nodulation and competitiveness. Thus, both *gusA*-marked mutant strains can be used to characterize the nodulation and competitiveness of *R. meliloti* YAO3.

Photographs of the colonization and occupancy of *gusA*-marked and parent strains of *Rhizobium meliloti* in the nodule of alfalfa are shown in Figure 2. Nodules occupied by *gusA*-marked inoculum strains were identified by the development of a blue color after incubation of the nodulated roots in the GUS substrate,

X-Gluc. Those occupied by the unmarked parent strain were recognized by the unstained bacteroids in the nodules. Thus, nodules with blue and unstained bacteroids were considered as co-occupied by wild and *gusA*-marked strains. These portray the advantage of using the GUS system compared with other methods

reporter gene could contribute significantly to the improvement in rhizobial strain selection and ecology. Compared with the products of other reporter genes, GUS is a very stable gene product. This enzyme accumulates over time, and minute quantities of GUS activity can be accurately measured (Jefferson et al

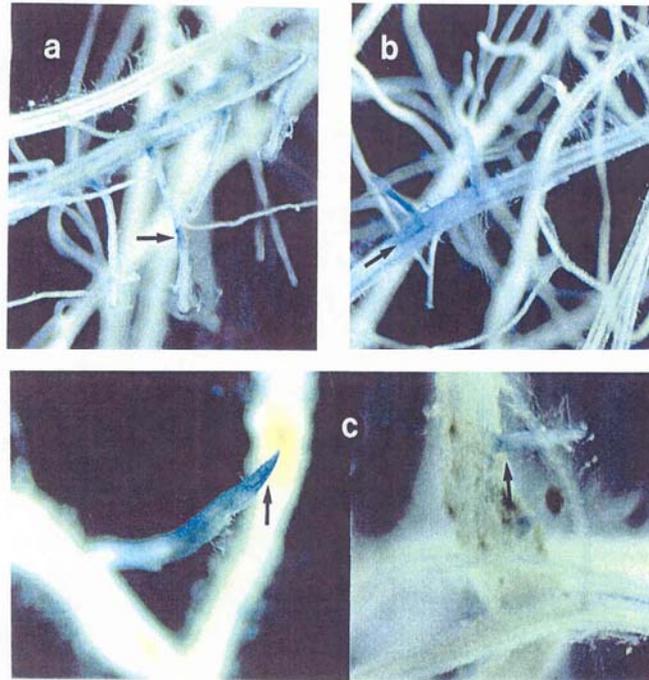


Figure 3. Colonization of clover roots by *gusA*-marked *A. lipoferum* T1371 at 5, 10, and 20 DAI. Blue staining (arrow) indicates GUS (β -D-glucuronidase) activity of the bacteria. The colonization of the main roots, secondary roots, and portion of the root hairs is sparse at 5 DAI (a) and 10 DAI (b) but a strong colonization of the root tips and root hairs is observed at 20 DAI (c).

in studying the competitiveness of an inoculant strain. It can differentiate the marked strain occupying the nodule from other competing strains due to the production of a visible blue precipitate, which is an indoxyl derivative from cleavage of the GUS enzyme into the substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (Wilson et al 1995). Thus, this method answers the problem of assessing the competitiveness and efficiency of an inoculant strain. Moreover, this technique is not only simple and reliable but also cost-effective because it takes only a short time to finish. Consequently, it is believed that the use of the GUS

1987). A key advantage of GUS is the generalized absence of this activity within the taxon Eubacteria (Wilson et al 1992), with the exception of *Escherichia coli* and some species of *Shigella*, *Streptococcus*, *Staphylococcus*, *Corynebacteria*, and *Clostridium*. Moreover, there is little or no detectable β -glucuronidase activity in almost any higher plant (www.biologie.fuberlin.d/lampart/gp03/GUS_assay.html). The absence of GUS activity both in the plant tissue and rhizobia and the stable integration of *gusA* gene in the bacterial genome, make the GUS system a powerful tool in studying the behavior of bacteria.

Co-inoculation of white clover with *Rhizobium* & *Azospirillum*

The *gusA*-marked *A. lipoferum* T1371 colonized taproots, secondary roots, and some parts of the root hairs at 5 and 10 DAI. At 20 DAI, strong staining was observed on the root tips and root hairs, suggesting an abundant colonization of the bacteria (Figure 3). When

white clover (Figure 5). The plants produced had 3-fold more nodules at 5 DAI and 2-fold more nodules at 10 and 20 DAI in co-inoculation treatment than those plants inoculated with *R.l. bv. trifolii* ANU 843 (Tp3) alone (control). Moreover, the ARA at 20 DAI was also increased by 2.3-2.7 times more in combined inoculation than that of the control (Figure 6).

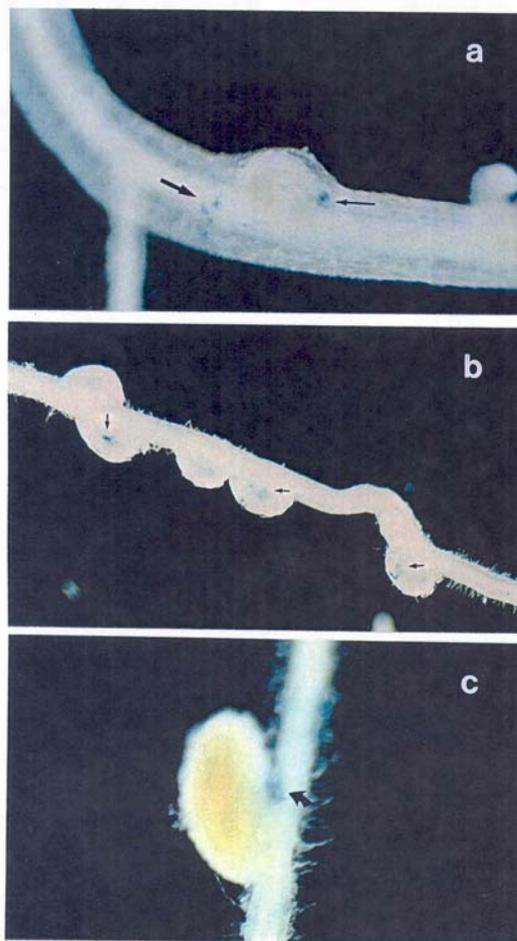


Figure 4. Young root nodules of white clover co-inoculated with *R. leguminosarum* bv. *trifolii* ANU 843 and *gusA*-marked *A. lipoferum* T1371 at 10 DAI (a, b) and 20 DAI (c). Blue stained *A. lipoferum* T1371 (arrow) mostly colonized the surface near the nodule.

co-inoculated with *R.l. bv. trifolii* ANU 843, *A. lipoferum* T1371 colonized the tap root, root hairs, and sites near or on the nodules (Figure 4).

Co-inoculation of *R.l. bv. trifolii* ANU 843 (Tp3) and *A. lipoferum* also increased the nodulation of

Similar colonization patterns were observed in cereal-*Azospirillum* associations (Umali-Garcia et al 1980). Patriquin et al (1983) suggested that *Azospirillum* could invade the intercellular spaces by penetrating the root hairs or regions of branches.

Azospirillum also showed pectinolytic activity in culture that may have facilitated the bacterial attachment to the root surface and its penetration into the root interior. Thus, the enhanced nodule numbers in treatments with combined inoculations could be the result of the formation of more infection sites for *Rhizobium*.

Plazinski & Rolfe (1985) and Yahalom et al (1987) reported that co-inoculation enhanced the number of nodules when *Azospirillum* was applied before or after

of *Rhizobium/Azospirillum* has any effect on the formation of nodules remains to be studied.

Previous reports have shown that co-inoculation of *Rhizobium* and *Azospirillum* enhanced the nodulation and yield of legumes (Yahalom et al 1987). The mechanism that regulates this phenomenon, however, is not clear. The formation of infection sites on the clover roots is one of the possible modes of action. Hence, it is very important to detect the sites of *Azospirillum* sp. attachment to the root surface when

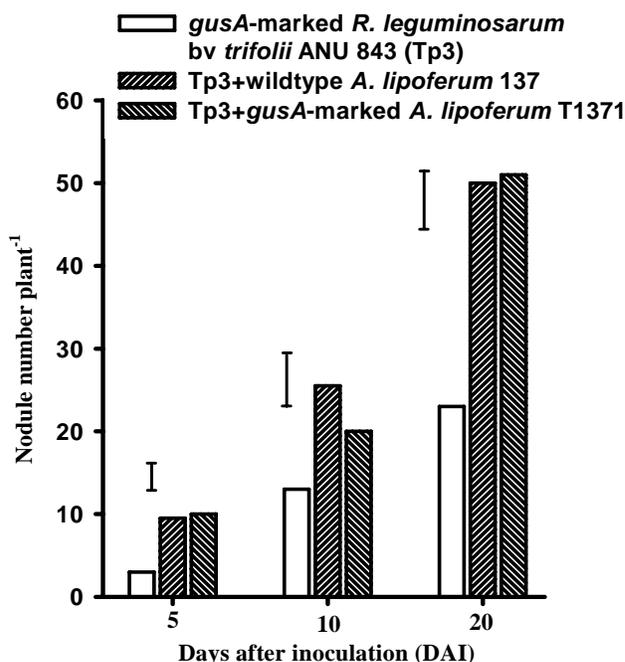


Figure 5. Effect of co-inoculation of *R. leguminosarum* bv *trifolii* and *A. lipoferum* on the nodulation of white clover 5, 10, and 20 days after inoculation. Values are means of five replications. Bars represent $LSD_{0.05}$.

Rhizobium. Nodule formation, however, was inhibited when *Rhizobium* and *Azospirillum* were applied in mixture. In this study, *Rhizobium* and *Azospirillum* was applied in a 1:1 cell ratio and we observed enhancement of nodules at 5, 10, and 20 DAI instead of inhibition. These results could be partly due to strain-cultivar specificity. Similar effects have been observed for combined *Rhizobium-Azospirillum* inoculation in lupine and alfalfa (LF Vassiyuk, personal communication). The question of whether the cell ratio

inoculated alone or in combination with *Rhizobium*. In this study, a *gusA*-marked *A. lipoferum* T1371 that has similar characteristics with that of the wild strain, was used to observe its colonization in white clover roots and evaluate its effect on nodulation.

Co-inoculation of soybean *Bradyrhizobium* & nondiazotrophic growth promoting bacteria

An example of the colonization pattern of *P. fluorescens* 2137 $_{gus}$ in soybean roots taken at 10 and

20 DAI is shown in Figure 7. The bacteria abundantly colonized the root tips or the surface near the root tip. Bacterial cells were detected as blue zone on soybean roots while the roots of uninoculated control plants remained whitish. This result confirmed the lack of endogenous β -glucuronidase activity in soybean roots and provided the specificity of visualization and counting of bacterial colonies.

The number of *gusA*-marked rhizobacteria on soybean roots when inoculated alone or co-inoculated with *B. japonicum* A1017kas^r varied at 20 DAI. The colonization rate ranged from 7.5 to 8.6 log cfu and higher in single inoculation than that of co-inoculation. The population density of *P. fluorescens* 2137gus was highest at 20 DAI but did not differ with *P. fluorescens* WCS 365gus and *A. agilis* 125gus at 10 DAI. The number of bradyrhizobia increased by 18-20% when

A1017kas^r treatment did not increase the nodule number. The *B. japonicum* A1017kas^r + *P. fluorescens* WCS365 treatment, however, had lower nodule number than in single inoculation of *B. japonicum* A1017kas^r at 20 DAI (Figure 8). Moreover, only *B. japonicum* A1017kas^r + *P. fluorescens* 2137gus co-inoculation treatment increased the ARA of root nodules by 2.1 times higher than with single inoculation of *B. japonicum* A1017kas^r at 20 DAI, while the ARA in *B. japonicum* A1017kas^r + *P. fluorescens* WCS365gus treatment was decreased to 1/16.6 compared with that of *B. japonicum* A1017kas^r alone (Figure 9). Addition of *P. fluorescens* 2137gus sterilized supernatant significantly increased the growth of *B. japonicum* A1017kas^r in YM growth medium 48, 72, 96, and 120 hours after incubation (Figure 10), suggesting that *P. fluorescens* might have

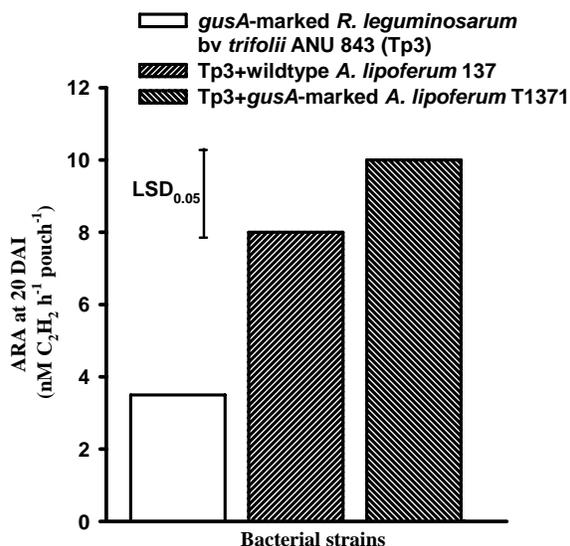


Figure 6. Effect of co-inoculation of *R. leguminosarum* bv. *trifolii* and *A. lipoferum* on the ARA of white clover 20 days after inoculation. Values are means of 5 replications.

co-inoculated with *P. fluorescens* 2137gus and 5.1-8.7% when co-inoculated with *P. fluorescens* WS365gus (Table 1).

The number of nodules in *P. fluorescens* 2137gus and *B. japonicum* A1017kas^r co-inoculation treatment was 3.7 times higher than in single inoculation of *B. japonicum* A1017kas^r at 10 DAI, and 1.5 times higher at 20 DAI, but *A. agilis* 125gus+*B. japonicum*

produced substances that stimulated the growth of *B. japonicum* A1017kas^r.

Plant growth-promoting bacteria are of great interest because of their high colonization ability and beneficial properties such as phytohormones, their antibiotic production and fixation of molecular nitrogen (Kloepper et al 1980, Lugtenberg et al 1991, O'Sullivan & O'Gara 1992, Okon & Labandera-

Gonzalez,1994). For an effective establishment of plant-bacteria interaction, colonization of plant roots by the introduced bacteria is an important step. Under natural conditions, there are many factors affecting plant root colonization by inoculated bacteria. Scher et al (1985) showed that rhizobacterial motility and chemotactic movement toward soybean roots play important roles in the colonization of roots. Generally,

population density of introduced *gusA*-marked bacteria on the soybean roots varied from 7.5 to 8.6 log cfu/gram root (Table 1). Moreover, the rhizobacterial strains we used in this study had varying ability to colonize soybean roots. Whether inoculated alone or co-inoculated with *B. japonicum*, *P. fluorescens* 2137 possessed the highest root colonization ability among the rhizobacteria studied. Although *P. fluorescens*

Table 1. Colonization rate of *gusA*-marked rhizobacteria on soybean roots when inoculated alone or co-inoculated with *B. japonicum* A1017kas^r

Inoculation Experiment	Bacterial Population Density			
	<i>Rhizobacteria</i>		<i>Bradyrhizobia</i>	
	10 DAI	20 DAI	10 DAI	20 DAI
Single inoculation experiment				
<i>Azomonas agilis</i> 125gus	8.45 a	8.36 b		
<i>Pseudomonas fluorescens</i> 2137gus	8.36 ab	8.62 a		
<i>Pseudomonas fluorescens</i> WCS365gus		8.34 b		
Co-inoculation experiment				
<i>Bradyrhizobium japonicum</i> A1017kas ^r alone			7.58 c	7.76 e
<i>B. japonicum</i> A1017 + <i>A. agilis</i> 125gus		7.88 b		8.57 b
<i>B. japonicum</i> A1017 + <i>P. fluorescens</i> 2137gus	7.90	8.34 a	7.68 c	9.23 a
<i>B. japonicum</i> A1017 + <i>P. fluorescens</i> WCS365gus	7.97	7.98 b	9.11 a	8.16 d
			8.24 b	

In each column and among strains within inoculation experiment, means followed by the same letters are not significantly different at 5% level of significance using DMRT. DAI = days after inoculation

population density of introduced PGPR in the soil varied from 1.6 to 7.4 log cfu/gram root (de Weger et al 1987, Andrade et al 1998). When soybean plants were grown under greenhouse condition, the population density of PGPR was higher compared than that in the field by 1.0 to 1.5 log cfu/gram root. According to de Weger et al (1989), the population of *Pseudomonas* spp. that were firmly bound to 3-cm segments of potato roots under sterile conditions ranged from 5.2 to 6.4 log cfu.

In our experiment using growth pouches, the

strain 2137 exhibited comparably high motility with strain WCS365), the latter had lower colonization ability than the former. Besides, there are other bacterial properties which might have differed between these strains, and are important factors influencing an effective root colonization of rhizobacteria. Examples of these are the presence of flagella and O-antigen of lipopolysaccharide and as the ability to synthesize amino acids (de Weger et al 1987, de Weger et al 1989, Simons et al 1997).

The lower population of rhizobacteria in co-

inoculation experiment than in single strain inoculation was expected because of the competition for attachment sites on the root surface with bradyrhizobia. According to Vande Broek et al (1998), during the first days of the association, bacteria are not uniformly spread on the entire root surface but are specifically concentrated in the root hair zones and at the bases of lateral roots of wheat. Our study on soybean root staining in X-Gluc solution showed that

but not *A. lipoferum*. Sindhu et al (1999) have observed that the culture supernatant of the *Pseudomonas* isolates contained some fluorescent compounds which influenced root flavonoid content of green gram (*Vigna radiata*) and concluded that the production of flavonoid-like compounds by plant roots because of co-inoculation with rhizobacteria, probably augments the nodule formation by bradyrhizobia.

In this study, the higher population density of

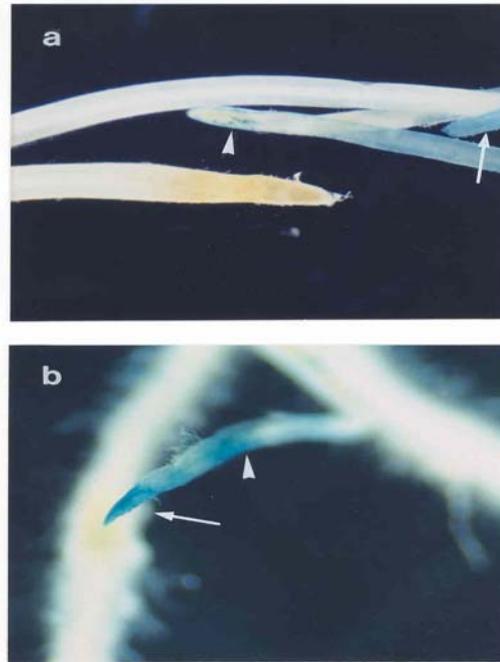


Figure 7. Colonization of soybean roots by *gusA*-marked *P. fluorescens* 2137gus at 10 DAI (a) and 20 DAI (b). Blue staining indicates GUS activity of the bacteria.

gusA-marked rhizobacteria abundantly colonized root tips or root surface near the tip (Figure 7).

It has been reported that co-inoculation of soybean with *B. japonicum* and rhizobacteria increased nodulation, ARA, plant dry matter, and grain yield (Polonenko et al 1987, Dashti et al 1998). It has been suggested that rhizobacteria can stimulate additional infection sites that are later occupied by rhizobia (Plazinski & Rolfe 1985). Bashan (1999) has reported that co-inoculation of *A. lipoferum* and *B. japonicum* in the soil strongly simulated the growth of *B. japonicum*

bradyrhizobia and higher colonization ability of *P. fluorescens* 2137gus increased the number, size, and ARA of nodule formed on soybean roots compared with that of *B. japonicum* inoculation alone. This suggests that the colonization of *P. fluorescens* 2137 and its possible production of growth-promoting compound(s) for *B. japonicum* A1017 enhanced the nitrogen fixation of soybean.

Acknowledgment

The authors would like to thank Dr L Vassiyuk and Dr N Makarova (All Russia Research Institute for Agricultural Microbiology, Russia) for providing us *A. lipoferum* 137, *P. fluorescens* 2137, and *Azomonas agilis*; Dr I Kennedy (University of Sydney, Australia) for *R. leguminosarum* bv. *trifolii* ANU 843; Dr R Ridge (International Christian University, Japan) for *R.l. bv. trifolii* ANU 843 [Tp3]; Prof I Maruyama (Tokyo University, Japan) for *B. japonicum* A1017 and *B. japonicum* A1017kas^r; and Dr G Bloemberg (Leiden University, The Netherlands) for *P. fluorescens* WCS365.

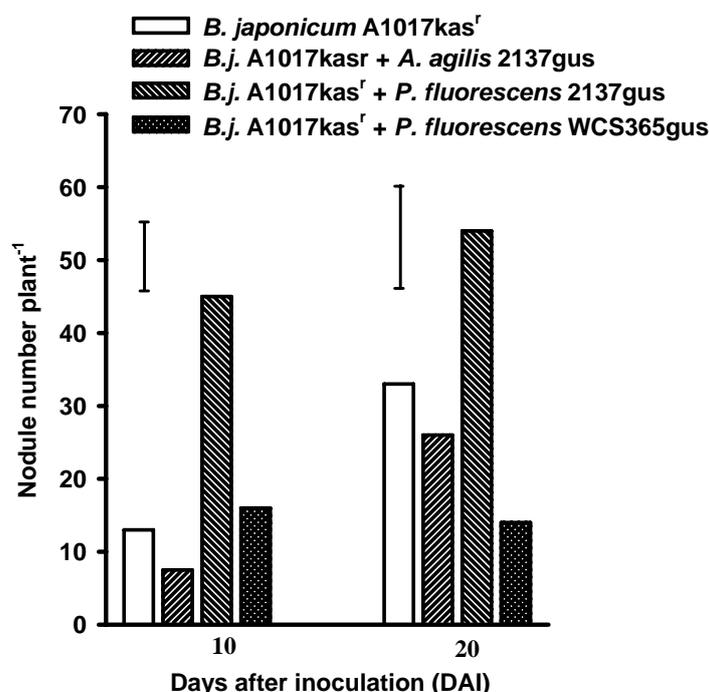


Figure 8. Effect of co-inoculation of *B. japonicum* A1017kas^r and rhizobacteria on the nodulation of soybean 10 and 20 days after inoculation. Values are means of three replications. Bars represent LSD_{0.05}.

LITERATURE CITED

- Akao S & H Kouchi. 1989. Light microscopic observation of root hair curling of soybean induced by *Rhizobium* infection. *Japanese Journal of Soil Science and Plant Nutrition* 60: 53-55
- Andrade G, FA de Leij & JM Lynch. 1998. Plant mediated interactions between *Pseudomonas fluorescens*, *Rhizobium leguminosarum* and arbuscular mycorrhizae on pea. *Letters In Applied Microbiology* 26: 311-316
- Bashan Y. 1999. Interactions of *Azospirillum* spp. in soils: A review. *Biology and Fertility of Soils* 29: 246-256
- Dashti N, F Zhang, R Hynes & DL Smith. 1998. Plant growth promoting rhizobacteria accelerate nodulation and increase nitrogen fixation activity by field grown soybean (*Glycine max* (L.) Merr.) under short season conditions. *Plant and Soil* 200: 205-213
- De Weger LA, CIM van der Vlugt, AHM Wijfjes, PAHM Bakker, B Schippers & B Lugtenberg. 1987. Flagella of a plant growth-stimulating *Pseudomonas fluorescens* are required for colonization of potato roots. *Journal of Bacteriology* 169: 2769-2773
- De Weger LA, MCM van Loosdrecht, HE Klaasen & B Lugtenberg. 1989. Mutational changes

in physicochemical cell surface properties of plant-growth-stimulating *Pseudomonas* spp. do not influence the attachment properties of the cells. *Journal of Bacteriology* 171: 2756-2761

Dreyfus BL, C Elmerich & YR Dommergues. 1983. Free-living *Rhizobium* strain able to grow on N₂ as the sole nitrogen source. *Applied and Environmental Microbiology* 45: 711-713

Francisco PB, S Akao & M Kokubun. 1992. Irradiance and nitrate effects on growth and symbiotic parameters of the supernodulating and nitrate-tolerant soybean mutant En6500 and its parent cultivar Enrei. *Journal of Plant Physiology* 140: 453-459

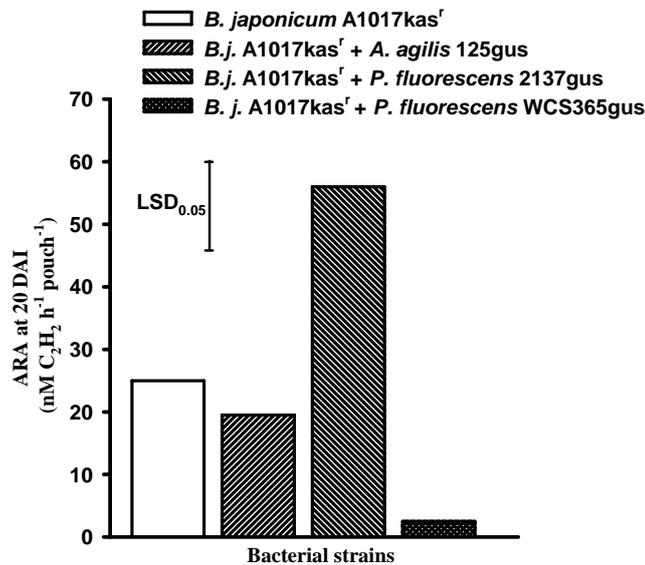


Figure 9. Effect of co-inoculation of *B. japonicum* A1017kas^r and rhizobacteria on the acetylene reduction activity of soybean nodules 20 days after inoculation. Data are means of three replications.

Holguin G & Y Bashan. 1996. Nitrogen fixation by *Azospirillum brasilense* Cd is promoted when co-cultured with mangrove rhizosphere bacterium (*Staphylococcus* sp.). *Soil Biology and Biochemistry* 28: 1651-1660

Jefferson RA, TA Kavanagh & MW Bevan. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* 6: 3901-3907

Kang UG, JH Choi, JS Lee & YT Jung. 1991. Studies on the development acid tolerant and superior nitrogen fixation symbiont for pasture hilly land. III. Inoculation effect of *R. meliloti* "YA03" to alfalfa on hilly acid soil. *Journal of Korean Society of Soil Science and Fertilizer* 24: 219-224

Kloepper JW, MN Schroth & TD Miller. 1980. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. *Phytopathology* 70: 1078-1082

Lugtenberg BJJ, LA de Weger & JW Bennett. 1991. Microbial stimulation of plant growth and protection from disease. *Current Opinion in Biotechnology* 2: 457-464

O'Sullivan DJ & F O'Gara. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiology Review* 56: 662-676

Okon Y & C Labandera-Gonzales. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation experiments. *Soil Biology and Biochemistry* 26: 1591-1601

Oliveira A, M Ferreira & ME Pampulha. 1997. Nitrogen fixation, nodulation and yield of clover plants co-inoculated with root-colonizing bacteria. *Symbiosis* 23: 35-42

Patriquin DG, J Döbereiner & DK Jain. 1983. Sites and processes of association between diazotrophs and grasses. *Canadian Journal of Microbiology* 29: 900-915

- Plazinski J & BG Rolfe BG. 1985. Influence of *Azospirillum* strains on the nodulation of clovers by *Rhizobium* strains. *Applied and Environmental Microbiology* 49: 84-89
- Polonenko DR, FM Scher, JW Kloepper, CA Singleton, M Laliberte & I Zaleska. 1987. Effects of root colonizing bacteria on nodulation of soybean roots by *Bradyrhizobium japonicum*. *Canadian Journal of Microbiology* 33: 498-503
- Scher FM, JW Kloepper JW & CA Singleton. 1985. Chemotaxis of fluorescent *Pseudomonas* spp. to soybean seed exudates in vitro and in soil. *Canadian Journal of Microbiology* 31: 570-574

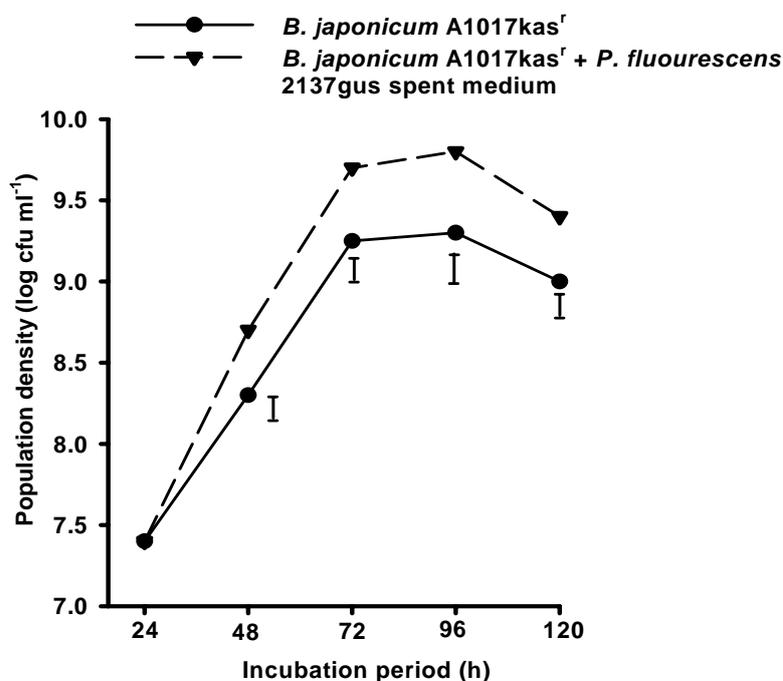


Figure 10. Effect of spent medium of *P. fluorescens* 2137gus on the growth of *B. japonicum* A1017kas^r in yeast mannitol broth. Data are means of 3 replications. Bars represent LSD_{0.05}.

- Schippers B, AW Bakker & PAHM Bakker. 1987. Interaction of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology* 23: 339-358
- Simons M, AJ Van der Bij, J Brand, LA de Weger, CA Wijffelman & BJJ Lugtenberg. 1997. Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Molecular Plant Microbe Interactions* 9: 600-607
- Sindhu SS, SK Gupta & KR Dadarwal. 1999. Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of growth of green gram (*Vigna radiata*). *Biology and Fertility of Soils* 29: 62-68
- Tomashow LS & DM Weller. 1995. Current concepts in the use of introduced bacteria for biological disease control: Mechanisms and antifungal metabolites. In Plant-Microbe Interaction, G. Stacey & NT Keen (ed), Chapman & Hall, New York, pp 187-235
- Umali-Garcia M, DH Hubbel, MH Gaskins MH & FB Dazzo. 1980. Association of *Azospirillum* with grass roots. *Applied and Environmental Microbiology* 39: 219-226
- Vande Broek A, M Lambrecht & J Vanderleyden. 1998. Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. *Microbiology* 144: 2599-2606
- Vassiyuk LF, AV Borovkov, AE Khal'chitskii, SV Ionkova SV & ZV Chmeleva. 1990. Bacteria of the genus *Azospirillum* and their influence on the productivity of non-leguminous plants. *Microbiology (English Translation of Mikrobiologiya)* January: 522-528

- Wilson KJ, A Sessitch, JC Corbo, KE Giller, ADL Akkermans & RA Jefferson. 1995. β -glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria. *Microbiology* 141: 1691-1705
- Wilson KJ, SG Hughes & RA Jefferson. 1992. The *Escherichia coli gus* operon: Induction and expression of the *gus* operon in *E. coli* and the occurrence and use of GUS in other bacteria. **In GUS Protocol: Using The GUS Gene As A Reporter Of Gene Expression**, SR Gallagher (ed), Academic Press, San Diego, California, pp 7-22
- Yahalom E, Y Okon Y & A Dovrat. 1987. *Azospirillum* effects on susceptibility to *Rhizobium* nodulation and on nitrogen fixation of several forage legumes. *Canadian Journal of Microbiology* 33: 510-514