Asian Pacific Journal of Molecular Biology and Biotechnology, 1998
Vol. 6 (1) : 13-20

Screening of acid-aluminium tolerant *Bradyrhizobium japonicum* strains : analysis of marker genes and competition *in planta*

Aris Tri Wahyudi, Antonius Suwanto*, Tedja Imas and Aris Tjahjoleksono

Department of Biology, Faculty of Mathematics and Sciences, Bogor Agricultural University, Jl. Raya Pajajaran, Bogor 16144, Indonesia.

(Received 9 August 1997/ Accepted 18 November 1997)

Abstract. The genes, *inaZ* from *Pseudomonas syringae*, *xylE* from *P. putida* and the kanamycin resistance gene (Km^R) of mini-Tn5Km1, were studied for their capabilities and reliabilities as molecular markers in three acid tolerant *Bradyrhizobium japonicum* strains. Conjugation was used to transfer each of these genes to *B. japonicum* strains. Mating was conducted on membrane filter (0.45 µm, Millipore) using modified Luria Agar. The results showed that all of the genes were able to be transferred to acid tolerant *B. japonicum* strains by conjugation. All of these bacteria were able to express the *inaZ* and Km^R genes. However, none of the exconjugants was able to express the *xylE* gene. Acid tolerant *B. japonicum* strains that carried each of these genes were able to form root nodules in either siratro or soybean plant. The Km^R gene had high stability as tested after nodulation experiments. This gene was subsequently used as a genetic marker in a competition study of acid tolerant *B. japonicum* strains for nodule occupancy in soybean plant. This study was conducted using acid tolerant soybean cultivar (B-09) grown in Leonard jars using nitrogen-free nutrient solution (pH 4.5 + Al 50 µM). Mixtures of acid tolerant *B. japonicum* strains and USDA 110 were inoculated in 1:1 ratio and nodules were harvested 30 days after planting. The results indicated that one exconjugant strain (11.7Km) dominated root nodules. Thus this strain has the potential to be developed as soybean inoculant in acid soils.

Key Words: Acid-Aluminium Tolerance, Bradyrhizobium Japonicum, Marker Genes, Competition

INTRODUCTION

Molecular marker genes are powerful biological tools with diverse applications. They may be used to substitute a structural gene of interest and hence to report on the regulation of gene expression through creation of a gene fusion. They are also used in microbial ecology to facilitate detection of marked individual strains of bacteria (Wilson *et al.*, 1995). Additionally they can be used to report on the activities of the bacteria in the environment. One of the advantages of marker genes as tools in bacterial ecology is to enable closely related strains of bacteria to be readily distinguished, and thus provide a rapid method for identifying the strain of interest. These advantages depend on the properties of the marker genes employed.

Bradyrhizobium japonicum is one of the nitrogen-fixing bacteria which can symbiose with soybean plant through root nodule formation. Most of the nitrogen source required by soybean plant could be provided by this symbiosis. Effectively *B. japonicum* can fulfill approximately 74% of the nitrogen requirement of soybean plant (Yutono, 1985). Competitive strains of *B. japonicum* could be found by selected by employing competition study amongst the isolated strains and other strains or indigenous strains for nodule occupancy in soybean plants. This analysis needs a stable molecular marker to study the ability of a particular bacterial isolate to form root nodule in soybean plants.

*Author for Corresponding, Mailing Address: Department of Biology, Faculty of Mathematics and Sciences, Bogor Agricultural University, Jl. Raya Pajajaran, Bogor 16144, Indonesia.
Tel.: 62-251-625965, Fax: 62-251-621724
E-mail: asuwanto@indo.net.id

Some of the molecular marker genes had been used to genetically mark bacteria. Three of them are *inaZ* (Georgakopoulos *et al.*, 1994), *xylE* (Prosser, 1994) and the kanamycin resistance gene (Km^R) carried by transposon mini-Tn5 (Herrero *et al.*, 1990). Usually the plasmids carrying each of these genes can be transferred by conjugation techniques. These genes have been shown to be stable and reliable molecular markers or reporter genes for studying bacterial activities in the environment.

The gene *inaZ* is one of the molecular markers which could be used as reporter for the analysis of gene expression of the bacteria in natural habitats (Loper and Lindow, 1994). Furthermore, Mariani (1995) have reported that *inaZ* could be used to screen the bacterial phylosphere as a biocontrol for *Xanthomonas campestris* pv. *glycines* in soybean plant. The method was relatively simple and reproducible. *inaZ* encodes the ice nucleation protein (Gurian-Sherman and Lindow, 1993), and its expression could be employed as a very sensitive molecular marker (Drianas *et al.*, 1995). It can be expressed in some species of gram negative bacteria, such as *Agrobacterium tumefaciens, Rhizobium meliloti,* and *Pseudomonas syringae pv phaseolicola* (Lindgren *et al.*, 1989). Another marker gene, *xylE,* is one of the genes encoding *catechol 2,3 dioxygenase.* This enzyme converts catechol to *hydroxymuconic semialdehyde.* The use of the *xylE* as a molecular marker in either gram negative (*Pseudomonas putida*) or gram positive bacteria (*Streptomyces lividans*) has been reported (MacNaughton *et al.*, 1992; Buell and Anderson, 1993).

Transposon is a DNA fragment which can transpose from one site to other sites in the genome. It has a wide application in the genetic engineering of gram negative bacteria such as in mutagenesis to determine the physical location of genes of interest. Recently, a ß-glucuronidase (*gus*) transposon for ecological and genetic studies of rhizobia and other gram negative bacteria has been reported by Wilson *et al.* (1995). In this research transposon mini-Tn*5*Km1, carrying kanamycin resistance gene (Km^R) as a molecular marker (de Lorenzo *et al.*, 1990; Herrero *et al.*, 1990) was used to mark *B. japonicum* in a competition study.

This study demonstrated the introduction of three genes, *inaZ* from *P. syringae*, *xylE* from *P. putida*, and Km^R (pUTmini-Tn*5*Km1) into three strains of acid-Al tolerant *B. japonicum*. Expression of each of these genes in *B. japonicum* before and after root nodulation of plants were also determined. The gene having high stability was used as a molecular marker for competition study in nodule occupancy in *Glycine max*. It was carried out to look for potential strains which can be recommended as inoculants in acid soils.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study and their relevant characteristics are described in Table 1.

Three acid-Al tolerant *B. japonicum* strains which have been previously reported (Endarini *et al.*, 1995) were routinely grown in Modified Luria Broth (MLB) (tryptone 5.0 g/l; NaCl 1.0 g/l; yeast extract 1.0 g/) or Yeast Extract Mannitol Agar (YMA) supplemented with Congo Red (CR) 0.0025% (Vincent, 1970) at room temperature (± 28°C). *Escherichia coli* strains were routinely grown in Luria Broth (LB) (trypton 5.0 g/l; NaCl 10.0 g/l; Yeast extract 5.0 g/l) or MLB at temperature 37°C. Antibiotic was supplemented whenever appropriate in the following concentrations: Kanamycin (Km) 50 µg/ml and 100 µg/ml (for *E. coli*) and Rifampicin (Rif) 100 µg/ml (for *B. japonicum*). Four plasmids, pJL1703 (Loper and Lindow, 1994), pRK2013 (Ditta *et al.*, 1980), pXYLE1 (Stein, 1992), and pUTmini-Tn*5*Km1 (Herrero *et al.*, 1990) were used in this study and all of them carried the kanamycin resistance (Km^R) gene.

Bacterial Mating. The recipients of acid-Al tolerant *B. japonicum* cultures were grown in MLB supplemented with Rif 100 µg/ml. All cultures were incubated aerobically at room temperature (± 28°C) agitated at 200 rpm for 48 hours (approximately 10^8 to 10^9 cells/ml). The donor, *E. coli* strains were grown in MLB supplemented with Km 100 µg/ml for DH5α (pJL1703) and HB101 (pRK2013), and 50 µg/ml for DH5α (pXYLE1) and S17-1λ *pir* (pUTmini-Tn*5*Km1). These cultures were incubated aerobically at 37°C and agitated at 200 rpm for 18 to 20 hours (approximately 10^8 to 10^9 cells/ml). Conjugation to transfer plasmids from *E. coli* carrying pJL1703 or pXYLE1, into *B. japonicum* was performed by *triparental mating* using *E. coli* HB101 (pRK2013) as a helper strain (Ditta *et al.*, 1980). pUTmini-Tn*5*Km1 was introduced into *B. japonicum* through *diparental mating* (Rostas *et al.*, 1984). All matings were carried out on membrane filters (0.45 µm, Millipore) using Modified Luria Agar (MLA) without antibiotic and incubated overnight at room temperature. The exconjugants were selected on YMA supplemented with Rif (100 µg/ml), Km (100 µg/ml) and CR (0.0025%) for pJL1703, while for pXYLE1 and pUTmini-Tn*5*Km1 we used YMA + Rif 100 µg/ml + Km 50 µg/ml + CR 0.0025%. *B. japonicum* exconjugants carrying pJL1703 were assayed for ice

Table 1. Bacterial Strains and Plasmids

Strains or Plasmid	Relevant Characteristics	Source or Reference
***B. japonicum* strains:**		
11	Wild type, *nod*+, Rif^R, acid tolerance	Endarini *et al.*, 1995
33	Wild type, *nod*+, Rif^R, acid tolerance	Endarini *et al.*, 1995
43	Wild type, *nod*+, Rif^R, acid tolerance	Endarini *et al.*, 1995
11.3Km, 11.7Km	*nod*$^+$, Rif^R Km^R, acid tolerance exconjugant of pUTmini-Tn5Km1 x 11	This work
33.2Km, 33.4Km	*nod*$^+$, Rif^R Km^R, acid tolerance exconjugant of pUTmini-Tn5Km1 x 33	This work
43.3Km, 43.4Km	*nod*$^+$, Rif^R Km^R, acid tolerance, exconjugant of pUTmini-Tn5Km1 x 43	This work
USDA 110	*nod*$^+$, Rif^R, acid tolerance	
***E. coli* strains:**		
DH5α	F^-, *lacZ*M15*recA*1*hsd*R17	Sambrook *et al.*, 1989
HB101	Res^-, Mod^-*recA*13 Sm^R	Sambrook *et al.*, 1989
S17(λ *pir*)	Pro^- Res^- Mod^+ *recA* integrated plasmid RP4-Tc::Mu-Km::Tn7	Herrero *et al.*, 1990
Plasmid		
pJL1703	*inaZ* with own promoter on pVSP61, Km^R	Loper and Lindow, 1994
pRK2013	ColE1 replicon, *Tra*$^+$ of RK2, Km^R	Ditta *et al.*, 1980
pXYLE1	Cathecol 2,3 dioxygenase, Km^R	Stein, 1992
pUTmini-Tn5Km1	Mini-Tn5Km1 in plasmid pUT: Km^R, Amp^R, *tnp*$^+$, *ori*R6K, RP4 mob	Herrero *et al.*, 1990

nucleation in reaction tubes containing 10 ml phosphate buffer (0.01 M, pH 7.0) at temperature -8°C, using circulating alcohol bath as described previously (Lindow, 1990). Cathecol 2,3 dioxygenase assay of pXYLE1 exconjugants were conducted using catechol 1% sprayed on colonies that have previously streaked on filter paper (0.45 μm, Whatman). Exconjugants from pUTmini-Tn5Km1 treatment were screened by growing the colonies on YMA supplemented with Rif (100 μg/ml), Km (50 μg/ml) and CR (0.0025%).

Root Nodulation Test. All of the exconjugants were tested for root nodulation of either Siratro or soybean plants. The exconjugants containing pXYLE1 and pUTmini-Tn5Km1 were separately tested for their capability to form root nodules in Siratro (Macroptilium atropurpureum) which were planted in 30 x 200 mm reaction tubes (Wahyudi, 1993), using media as described by Speidel and Wollum (1980) solidified with 0.085% of bacteriological agar. On the other hand, the exconjugants containing pJL1703 were tested for root nodule formation either in siratro or soybean (Glycine max) B-09 (this soybean cultivar is acid-Al tolerance, and obtained from irradiation treatment, BATAN-Jakarta). The soybeans were planted in Leonard jar using N-free nutrient solution pH 4.5 described by Alva *et al.* (1988). The nodules of each plant formed by the exconjugants of pJL1703 were suspended in one mililitre of 0.01 M phosphate buffer to be used as samples in ice nucleation assay described previously. Suspensions of the nodules formed by B. japonicum exconjugants of pXYLE1 and pUTmini-Tn5Km1 were streaked on YMA supplemented with Rif, Km, and CR. Colonies which appeared on the media (from suspension of the nodules formed by B. japonicum exconjugants of pXYLE1) were sprayed with catechol 1% as described earlier. Molecular markers which showed high stability of expression were subsequently used as molecular markers for the competition study of *B. japonicum* in nodule occupancy.

Competition Study for Nodule Occupancy. Six exconjugants of *B. japonicum* strains obtained from conjugation using transposon mini-Tn5Km1, designated (11.3Km, 11.7Km, 33.2Km, 33.4Km, 43.3Km, and 43.4Km) were used for competition study. *B. japonicum* USDA 110 was used as competitor and acid tolerant soybeans B-09 was used as host plants. Competition study was carried out using modified Leonard jars located in a greenhouse. Each bottle was planted with two plants supplemented with nitrogen-free nutrient solution (pH 4.5) and 50 μM Al (Alva *et al.*, 1988). The nitrogen-free nutrient solution was maintained at pH 4.5 until 30 days after planting. Mixtures of acid tolerant *B. japonicum* exconjugants and USDA 110 were inoculated in 1:1 ratio (approximately 10^7 cells/ml) into soybean plants. The nodules were harvested 30 days after planting. Suspension of each nodule was streaked onto YMA + Rif 100 μg/ml + CR 0.0025% and YMA + Rif 100 μg/ml + Km 50 μg/ml + CR 0.0025% to determine whether the nodules which were formed solely by Km^R exconjugants strains or mixtures of Km^R exconjugants and USDA 110. Suspensions which showed the growth of *B. japonicum* on the latter media were serially diluted and plated onto YMA + Rif 100 μg/ml + CR 0.0025% to determine the dominant bacterial strains occupying the nodules.

RESULTS AND DISCUSSION

Gene Transfer and Root Nodulating Assay. The genes, inaZ (pJl1703), xylE (pXYLE1), and Km^R gene carried by transposon mini-Tn5, were successfully transferred into three strains of acid-Al tolerant *B. japonicum* using conjugation. The genes *inaZ* and *xylE* were transferred into *B. japonicum* by *triparental mating* using pRK2013 as a helper plasmid. Neither pJL1703 nor pXYLE1 has the gene *tra* which is required for conjugative transfer. Therefore, they can be introduced into *B. japonicum* using *tra* gene products which are provided *in trans* from another plasmid such as pRK2013. This plasmid, pRK2013, has a narrow host range and it cannot be stably maintained in most non *E. coli* hosts (Puhler, 1993). The Km^R gene carried by transposon mini-Tn5 (pUTmini-Tn5Km1) was transferred into *B. japonicum* by *diparental mating*. This plasmid has the origin of replication (*ori*) from plasmid R6K. It therefore can replicate only in a host providing a *pir* protein, such as S17-1 λ pir (Herrero *et al.*, 1990; Puhler, 1993). Transfer into the recipient was achieved utilizing RP4 *mob* which can be driven by the products of *tra* genes provided *in trans* in plasmid RP4, such as in *E. coli* S17-1 λ pir (Herrero *et al.*, 1990). Frequency of conjugation of each marker genes shows in Table 2.

Twenty five *B. japonicum inaZ* exconjugants: ten exconjugants of strain 11, six exconjugants of strain 33, and nine exconjugants of strain 43, were able to grow on YMA supplemented with Rif, Km and CR. Ice nucleation assay of the exconjugants were able to nucleate ice at temperature -8°C except for three exconjugants of strain 43 which were not able to show detectable ice nucleation activity (INA). Ice nucleation activity of 22 exconjugants were caused by the presence of *inaZ* carried in plasmid pJL1703. Expression of this gene in *B. japonicum* yielded ice nucleation active (INA) protein which plays an important role to orient the water molecules to be frozen (Gurian-Sherman and Lindow, 1993). The origin of *inaZ* used in this study came from *P. syringae* and for its proper function, the INA protein has to be inserted in the outer membrane of the bacterial cell.

All of the exconjugants were able to form root nodules either in siratro or soybean plants. The number of nodules formed were between 1 to 5 per plant of siratro, and 20 to 47 per two plants of soybean, respectively, and the majority of the nodules were located in primary roots (data not shown). Data from the ice nucleation assay of the nodules of siratro and soybean is shown in Table 3. The results indicated that plasmid pJL1703 (*inaZ*) was not stable after root nodule formation. The loss of the plasmid might have occured after bacteroid formation. These observations suggested that *inaZ* carried by plasmid pJL1703 could not be effectively used as a marker in *B. japonicum* to monitor strains involved in root nodulation.

Thirteen exconjugants of *B. japonicum* found from conjugation of pXYLE1 (*xylE*) (five exconjugants of strain 11, three exconjugants of strain 33, and five exconjugants of strain 43), were able to grow on YMA supplemented with Rif, Km, and CR. None of the exconjugants was able to express *xylE*. However, the Kan^R gene in the same plasmid (pXYLE1) could be expressed in *B. japonicum* either before or after root nodulation experiments. The failure of *B. japonicum* to express *xylE* gene might be caused by the inability of the host bacterium to recognize *xylE* promoter, which came from *lacZ* promoter of *E. coli*. Plasmid pXYLE1 is constructed from promoterless *xylE* gene of TOL plasmid of *P. putida*, fused with plasmid pKAN18 (Stein, 1992). Therefore, this result suggested that *xylE* carried by plasmid pXYLE1 could not be used as a molecular marker in *B. japonicum*.

The Km^R gene carried by transposon mini-Tn5 on the plasmid pUTmini-Tn5Km1 was also succesfully introduced into *B. japonicum*. Twenty five exconjugants were able to grow on YMA supplemented with Rif, Km, and CR possibly due to Km^R

Table 2. Frequencies of Conjugation of *inaZ* (pJI1703), *xylE1*(pXYLE1), and Km^R (pUTmini-Tn5Km1) from *E.coli* into *B. japonicum*

Bacterial mating[a]	Frequency[b]
B.j. 11 x *E.c.* DH_5 (pJL1703) x *E.c.* HB101 (pRK2013)	1.9×10^{-7}
B.j. 33 x *E.c.* DH_5 (pJL1703) x *E.c.* HB101 (pRK2013)	6.8×10^{-8}
B.j. 43 x *E.c.* DH_5 (pJL1703) x *E.c.* HB101 (pRK2013)	5.6×10^{-7}
B.j. 11 x *E.c.* DH_5 (pXYLE1) x *E.c.* HB101 (pRK2013)	2.8×10^{-8}
B.j. 33 x *E.c.* DH_5 (pXYLE1) x *E.c.* HB101 (pRK2013)	1.9×10^{-8}
B.j. 43 x *E.c.* DH_5 (pXYLE1) x *E.c.* HB101 (pRK2013)	3.9×10^{-8}
B.j. 11 x *E.c.* S17-1 x pir (pUTmini-Tn5Km1)	3.6×10^{-9}
B.j. 33 x *E.c.* S17-1 x pir (pUTmini-Tn5Km1)	2.8×10^{-9}
B.j. 43 x *E.c.* S17-1 x pir (pUTmini-Tn5Km1)	5.7×10^{-9}

[a] *B.j.* : *Bradyrhizobium japonicum*, *E.c.* : *Escherichia coli*
[b] Frequency of conjugation is calculated per recipient.
The values are means of two replicates.

Table 3. Ice nucleation assay of root nodules in Siratro and Soybean Plant

Inoculum of exconjugant Number	Root nodule Ice nucleation assay[a]	Growth of root nodule on YMA+Rif+CR[b]	YMA+Rif+Km+CR[b]	Colony Ice nucleation Assay[a]
Siratro				
6 (ice$^+$)	+	+	-	-
3 (ice$^+$)	+	+	+	+
13 (ice$^+$)	-	+	-	-
3 (ice$^-$)	-	+	+	-
Soybean				
5(ice$^+$)	-	+	+	-
3 (ice$^+$)	+	+	+	+
14 (ice$^+$)	-	+	-	-
3 (ice$^-$)	-	+	+	-

[a] + : formed ice crystal; - : did not form ice crystal
[b] + : growth ; - : no growth

gene insertion into chromosomal DNA of *B. japonicum*. After all of the exconjugants which were infected in siratro, 24 exconjugants were able to form root nodules, but one exconjugant could not. The inability of miniTn5-Km1 exconjugant to form nodule might be caused by insertion of mini-Tn5Km1 transposon into a *nod* gene (gene for nodulation) or a nodulation regulatory gene. The insertion of this transposon will inactivate *nod* gene(s) (Brock *et al.*, 1994). Suspension of the root nodules formed by 24 exconjugants after streaking on YMA supplemented with Rif, Km, and CR showed that all of them grew very well. This result indicated that the insertion of transposon mini-Tn5Km1 in the bacterial chromosome of *B. japonicum* was very stable and could be due to separation of transposase gene (*tnp*$^+$) from the transposed element (Herrero *et al.*, 1990).

Competition Study for Nodule Occupancy. Six strains of representative colonies among the acid-Al tolerant *B.japonicum* exconjugants that carried Km^R gene from transposition of mini-Tn5Km1 (11.3Km, 11.7Km, 33.2Km, 33.4Km, 43.3Km, and 43.4Km), were used for competition study for nodule occupancy in a green-

Table 4. Root nodulating of soybean plant inoculated by mixtures of *Bradyrhizobium japonicum* strains

Inoculum Strains	Nodule number[b]	Suspension of nodule growing on[c] YMA+Rif+CR	YMA+Rif+Km+CR	Number of nodule formed by A	B	A+B
Mixture of strains (A+B)[a]						
11.3Km + USDA 110	38±7.00	30	6	5	24	1(1A)[d]
11.7Km + USDA 110	38±4.93	30	17	14	12	4(3A,1B)
33.2Km + USDA 110	31±1.00	30	4	4	26	0
33.4Km + USDA 110	31±1.15	30	6	5	24	1 (1A)
43.3Km + USDA 110	38±6.08	30	8	5	22	3(2A,1B)
43.4Km + USDA 110	37±1.53	30	5	5	25	0
Single strains						
11.3Km	54±6.24	30	30	30	0	0
11.7Km	39±2.31	30	30	30	0	0
33.2Km	43±6.60	30	30	30	0	0
33.4Km	36±6.56	30	30	30	0	0
43.3Km	50±2.52	30	30	30	0	0
43.4Km	46±4.04	30	30	30	0	0
USDA 110	34±4.04	30	0	0	30	0

[a] A: Strains of exconjugant; B: Strain USDA 110; [b] The values are mean ± SD of three replicates
[c] Analyzed 30 nodules; [d] Strain dominating nodules formed by mixtures of strains

house. A known as acid tolerant *B. japonicum* strain USDA 110 was used as a competitor (Taylor *et al.*, 1991). The nitrogen-free nutrient solution used in the Leonard jars contained Al (50 μM; pH 4.5). The soybean plants grew well when inoculated with *B. japonicum* and all of them were able to form root nodules in acid condition. Each mixture of acid tolerant *B. japonicum* strains and USDA 110, inoculated in a 1:1 ratio, produced 31 to 38 root nodules per two plants. As much as 30 root nodules (10 root nodules of each replicate formed by mixtures of *B. japonicum*) were resuspended and spread onto YMA supplemented with Rif and CR, and YMA supplemented with Rif, Km and CR. All colonies showed resistance to Rif, but only 25.6% were resistant to Km and Rif. Root nodule suspensions which yielded bacterial growth on YMA supplemented with Rif, Km, and and CR were diluted and plated onto YMA supplemented with Rif and CR to determine the dominant strain in a nodule which was presumably formed by two strains. The differences in colony performance on the media were based on type and size of colony, and the growth rate. After 10 days of incubation, colonies of strain USDA 110 showed were large and mucoid, while the Kan^R exconjugants were large and watery. USDA 110 colonies were comparatively smaller than the Km^R exconjugants. USDA 110 also grew slower than the exconjugants (Fig. 1).

One of the six Km^R exconjugants from strains 11.7Km outcompeted strain USDA 110 and dominated root nodulation whereas the others were suppressed by USDA 110. Most of the nodules were formed by one strain (95%). However this work also showed that some nodules were formed by two different strains, i.e., USDA 110 and Km^R exconjugants (Table 4, Fig. 2). Occupation of the nodules by Km^R exconjugant 11.7Km indicated that this strain was relatively more competitive than USDA 110 while the others were not. Lack of competitiveness in the five Km^R exconjugants tested might be caused by differential location of transposition into the *B. japonicum* genome which might be needed for fitness or survival of the bacteria in environment.

B. japonicum USDA 110 has been reported to be the best competitor in the competition study for nodule occupancy (Kosslack and Bohlool, 1985; George *et al.*, 1987; Moawad *et al.*, 1988), and this is also supported by this study. In addition, strain USDA 110 was known as the most tolerant strain to acid-aluminium media than the other USDA strains (Taylor *et al.*, 1991). Although *B. japonicum* USDA 110 dominated in root nodulation of soybean plants, this study demonstrated that *B. japonicum* strain 11.7 Km was an effective and competitive strain in acid condition. Therefore this strain can be recommended as an inoculant strain for soybean plants in acid soils in future studies.

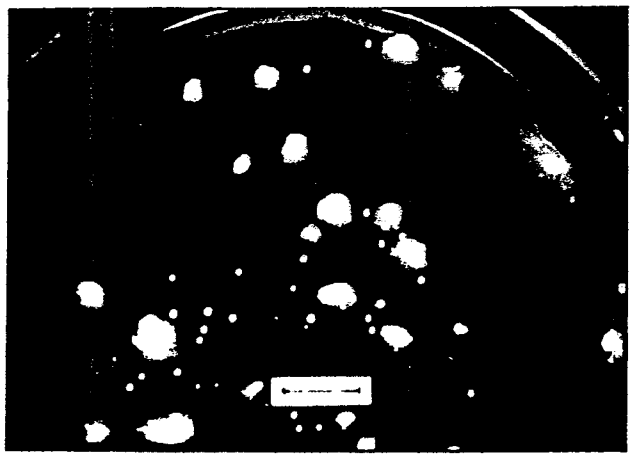

Figure 1. Performance of colonies of *B. japonicum* exconjugant strain 11.7Km (A) and *B. japonicum* USDA 110 (B) on YMA + Rif 100 ug/ml + CR 0.0025% incubated for 12 days at room temperature (±28°C).

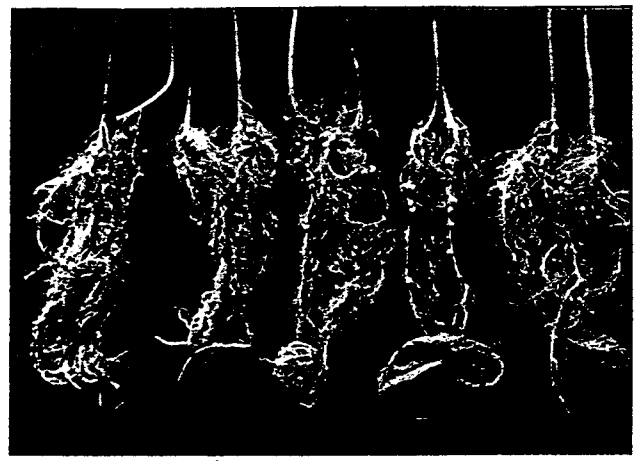

Figure 2. Performance of root nodules of soybean plants inoculated with *B. japonicum* strain USDA 110 (1), 11.7Km (2), 11.7Km, + USDA 110 (3), 43.4Km (4), and 43.4Km + USDA 110 (5) harvested 30 days after planting.

ACKNOWLEDGEMENT

This work was supported by Riset Unggulan Terpadu I (RUT-I) to TI.

REFERENCES

Alva A.K., Edwards, D.G., Caroll, B.J., Asher, C.J. and Gresshoff, P.M. 1988. Nodulation and early growth of soybean mutants with increased nodulation capacity under acid soil infertility factors. *Agronomy Journal* 80: 836-841.

Buell, C. R. and Anderson, A.J. 1993. Expression of the *AggA* locus of *Pseudomonas putida* in vitro and in planta as detected by the reporter gene, *xylE*. *Molecular Plant-Microbe Interactions* 6: 331-340.

Brock, T.D., Madigan, M.T., Martinko, J.M. and Parker, J. 1994. Biology of Microorganisms. USA: Prentice Hall International, Inc.

de Lorenzo, V., Herrero, M., Jakubzik, U. and Timmis, K.N. 1990. Tn5 transposon derivatives for insertion mutagenesis promoter probing and chromosomal insertion of cloned DNA in gram-negative eubacteria. *Journal of Bacteriology* 172: 6568-6572.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. 1980. Broad host range DNA cloning system for gram negative bacteria: Construction of a gene bank of *Rhizobium melliloti*. *Proceedings of the National Academy of Sciences, USA* 77: 7347-7351.

Drainas, A., Vartholomatos. G. and Panopoulos, N.J. 1995. The ice nucleation gene from *Pseudomonas syringae* as a sensitive gene reporter for promotor analysis in *Zymomonas mobilis*. *Applied and Environmental Microbiology* 61: 273-277.

Endarini, T., Wahyudi, A.T. and Imas, T. 1995. Seleksi galur *Bradyrhizobium japonicum* indigenos toleran media asam-Aluminium. *Hayati* 2: 74-79.

Georgakopoulos, D.G., Hendson, M., Panopaulos and N.J., Schroth, M.N. 1994. Cloning of a phenazine biosynthetic locus of *Pseudomonas aureofaciens* PGS12 and analysis of its expression. *Applied and Environmental Microbiology* 60: 2931-2938.

George, T., Bohlool, B.B. and Singleton, P.W. 1987. *Bradyrhizobium japonicum* environment interaction: Nodulation and interstrain competition in soils along an elevational transect *Applied and Environmental Microbiology* 53: 1113-1117.

Gurian-Sherman, D. and Lindow, S.E. 1993. Bacterial ice nucleation: Significance and molecular basis. *FASEB Journal* 7: 1338-1343.

Herrero, M., Lorenzo, V.D. and Timmis, K.N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *Journal of Bacteriology* 172: 6557-6567.

Kosslak, R.M. and Bohlool, B.B. 1985. Influence of environmental factors on interstrain competition in *Rhizobium japonicum*. *Applied and Environmental Microbiology* 49: 1128-1133.

Lindgren, B.P., Frederick, R., Govindarajan, A.G., Panapaulos, N.J., Staskawicz B.J. and Lindow, S.E. 1989. An ice nucleation reporter gene system: Identification of inducible pathogenicity genes in *Pseudomonas syringae* p.v. *phaseolicola*. *EMBO Journal* 8: 1291-1301.

Lindow, S.E. 1990. Bacterial ice nucleation activity. *In* Methods in phytobacteriology, ed. Z. Klement, K. Rudolph and D.C. Sand, pp.428-434. Budapest: Academiai Kiodo.

Loper, J.E. and Lindow, S.E. 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. *Applied and Environmental Microbiology* 60: 1934-1941.

MacNaughton, S.J., Rose D.A. and Donnel, A.G. 1992. Persistence of a *xylE* marker gene in *Pseudomonas putida* introduced into soils of differing texture. *Journal of General Microbiology* 138: 667-673.

Mariani. 1995. Isolasi dan seleksi bakteri filosfer yang berpotensi untuk biokontrol *Xanthomonas campestris pv. Glycines* 8Ra pada tanaman kedelai dengan esei nukleasi. Skripsi Faculty of Science and Mathematics, Institut Pertanian Bogor, Bogor, Indonesia.

Moawad, H., Badr Eldin, S.M.S. and Khalafallah, A. 1988. Field performance of three *Bradyrhizobium japonicum* strains with two soybean (*Glycine max* L.) cultivars. *Biology and Fertility of Soil* 6: 174-177.

Prosser, J.I. 1994. Molecular marker system for selection of genetically engineered micro-organism in the environment. *Microbiology* 140: 5-17.

Puhler, A. 1993. Genetic Engineering of Microrganisms. Federal Republic of Germany: VCH Verlagsgesellschaft mbh .

Rostas, K., Sista, D.S., Stanley, J. and Verma, D.P.S. 1984. Transposon mutagenesis of *Rhizobium japonicum*. *Moecular General Genetics* 197: 230-235.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, N.Y : Cold Spring Harbor Laboratory Press.

Speidel, K.L. and Wollum, A.G. 1990. Evaluating of leguminous inoculant quality: A manual. Department of Soil Science. North Carolina State University.

Stein, D.C. 1992. Plasmid with easily exisable *xylE* cassettes. *Gene* 117: 157-158.

Taylor, R.W., Williams, M.L. and Sistani, K.R. 1991. N_2 fixation by Soybean-*Bradyrhizobium japonicum* combinations under acidity, low P and high Al stresses. *Plant and Soil* 131: 293-300.

Vincent, J.M. 1970. A manual for the practical study of the root-nodule bacteria. International Biological Programme. Oxford: Blackwell Scientific Publication.

Wahyudi, A.T. 1993. Isolasi dan autentikasi bakteri bintil akar tumbuh lambat asal tanah Nusa Tenggara Timur. Jurusan Biologi, Faculty of Science and Mathematics, Institut Pertanian Bogor, Indonesia.

Faculty of Science and Mathematics, Institut Pertanian Bogor, Indonesia:.

Wilson, K.J., Sessitsch, A., Carbo, J.C., Giller, K.E., Akkermans, A.D.L. and Jefferson R.A. 1995. ß-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria. *Microbiology* 141: 1691-1705.

Yutono. 1985. Inokulasi *Rhizobium* pada Kedelai. *In* Kedelai, ed. S. Somaatmadja *et al.* Puslitbangtan, Bogor: Balai Penelitian dan Pengembangan Pertanian.