ACID TOLERANCE AND ANTIBIOTIC RESISTANCE OF SOME STRAINS OF *BRADYRHIZOBIUM* APPLIED IN THAILAND

N. Teaumroong[1*], Y. Murooka[2] and N. Boonkerd[3]

Abstract

The pH sensitivity and antibiotic resistance of 20 strains of *Bradyrhizobium* spp. and 16 strains of *B. japonicum* were characterized. The characteristics of acid tolerance among *Bradyrhizobium* can be observed after being cultured in a defined medium for 10 days of growth. One mechanism which provides the ability to tolerate low pH conditions is alkali producing, such as *Bradyrhizobium* spp. strain TAL 305 isolated from *Vigna radiata*. The antibiotic resistance profiles when applied with Carbenicillin (500 µg/ml), Chloramphenical (500 µg/ml), Streptomycin (100 µg/ml), Tetracycline (100 µg/ml) and Trimethoprim (50 µg/ml) were established. To differentiate them by using antibiotic resistance property, the results showed that this can be a rapid and efficient method for phenotypically distinguishing strains of *Bradyrhizobium*. The results obtained from this study will be combined with the data from primers-based technique.

Key words : *Bradyrhizobium*, antibiotic resistance, pH sensitivity, *Vigna radiata*

Rhizobia belonging to the genus Bradyrhizobium are slow-growing, gram-negative, heterotrophic bacteria which can form root nodules on several leguminous plants. DNA-DNA homology studies, physiological characteristics, and nodulation host-range phenotypes have been used to divide this genus into *Bradyrhizobium japonicum* and *Bradyrhizobium* spp. (Hollis et al., 1981; Huber et al., 1984; Kuykendal et. al., 1988.)

Phenotypic differences among strains of *Bradyrhizobium* spp. and *B. japonicum* have been reported for a broad range of traits (Somasegaran and B. Hoben., 1994). These diversities among strains were one of the original criteria used to separate isolates into various groupings.

Previous reports have shown that antibiotic resistance in root nodule bacteria is common (Abdel et al., 1991; van Berkum et al., 1993) although there is a considerable amount of variation within and between species. Cole and Elkan (1973) suggested that multiple antibiotic resistance is a characteristic of *B. japonicum*. Differentiation based on multiple resistance markers provides a rapid and efficient method for distinguishing between strains of *Bradyrhizobium* with distinct genetic and phenotypic backgrounds and may prove to be valuable for surveying microsymbiont populations in field assays of symbiotic competence or competitiveness (Kuykendall et al., 1988).

Soil acidity is a major factor limiting legume growth and nitrogen fixation because of its adverse effects on the growth of the host plant, its root nodule bacteria and symbiotic development (Pankhurst et al., 1982). Species of root nodule bacteria vary significantly in their tolerance to low pH in laboratory media (Glenn et al., 1994). The slow growing *Bradyrhizobium* are more tolerant to low pH than the faster-growing *Rhizobia* (Graham,

[1,3] *Ph.D., School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000.*

[2] *Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Higashi-Hiroshim 724, Japan.*

* *Corresponding Author*

1982). Even within the same species, strains differ considerably in their tolerance to acidity in culture (Graham, 1982) This variation of acid tolerance of root nodule bacteria becomes the basis for screening naturally occurring variants with greater acid tolerance for use as field inoculants.

These experiments were conducted to determine the phenotypic differences in acid tolerance and antibiotic resistance of some *Bradyrhizobium* spp. and *B. japonicum* strains applied in Thailand.

Materials and Methods

Bacterial strains and growth media

The strains of *Bradyrhizobium* spp. and *B. japonicum* used in this study are listed in Table 1. Stationary-phase broth cultures with a population of 10^9 cells/ml were used as inocula. One hundred microliters of inoculum was added to the liquid medium while 10 µl of each strain was plated on solid medium. The cultures were incubated at 28°C for 10 days .

The defined medium (Table 2) used for screening the tolerance of the strains to acidity was based on Keyser and Munns (1973). For solid media, the indicator dyes bromcresol purple and bromcresol green were added to the control (pH 7.0) and acid (pH less than 7.0) plates, respectively. Acid media were acidified with HCl before autoclaving. The modified arabinose-gluconate medium was used for the antibiotic resistance experiment (Somasegran and Hoben, 1994). Three ml of the media were used for cultivation.

Tolerance to acidity

The strains were tested for response to low pH in liquid and solid media. Four pH levels were used; namely, 4.5, 5.0, 5.5 and 7.0. The strains which were able to grow in pH 4.5 were further screened for growth at pH 4.0 using the procedure mentioned above.

Antibiotic resistance

Plates containing an antibiotic, as well as a nonselective control plate, were inoculated with 10 µl of inoculum. Resistance to a particular concentration of antibiotic was defined as the ability of a strain to form colonies at that concentration. Intrinsic resistance is clearly distinguished from mutant selection since in the latter case only a few colonies are formed per 10^7 to 10^8 cells plated (Kuykendall et al., 1988). The antibiotics and the concentration used in this study are listed in Table 3. All preparations were filter sterilized by using 0.45 µm membrane filters. Antibiotics were added to molten agar after sterilization and cooling to 50°C

Results and Discussion

Acid tolerance and antibiotic resistance properties of *Bradyrhizobium* spp. and *B. japonicum* are some of criteria to determine phenotypic differences among them. Twenty *Bradyrhizobium* spp. strains and sixteen strains of *B. japonicum* were isolated from root nodules of *Vigna radiata*, *Arachis hypogaea* and *Glycine max*, respectively. For the

Table 1. *Bradyrhizobium* spp. and *B. japonicum* strains used in this study.

Host plant of origin	Strain
Vigna radiata	*Bradyrhizobium* spp. TAL 209, USAD 3267, TAL 306, TAL 305, TA L 301, THA 302, TAL 442, THA 304 , TAL 425 and TAL 441
Arachis hypogaea	*Bradyrhizobium* spp. E-7-1, E-17-1, 280 A, NE-36-19, N-22-18, M-47-12, M-43-10, NE-41-15, M-50-2 and 22-2A
Glycine max	*B. japonicum* TAL 102 (USAD 110), TAL 432, THA 2, TAL 379, TAL 944, TAL 212, TAL 211, USAD 8-T, TAL 220, USAD 94, USAD 35, USAD 117, TAL 377, THA7, THA 5 and TAL 216

Table 2. Defined medium for screening strains/ isolates for acid tolerance.

Component	Concentration
$MgSO_4 . 7H_2O$	300 µM
$CaCl_2 . 2H_2O$	300 µM
Fe EDTA	10 µM
KCl	1.5 mM
KH_2PO_4	5 µM
$MnCl_2 . 4H_2O$	1 µM
$ZnSO_4 . 7H_2O$	0.4 µM
$CuCl_2 . 2H_2O$	0.1 µM
$Na_2MoO_4 . 2H_2O$	0.02 µM
Arabinose	5 g/l
Galactose	5 g/l
Sodium glutamate	1.8 g/l

acid tolerance test, every strain of *Bradyrhizobium* spp. which was isolated from *Vigna radiata* exhibited the ability to grow at pH 4.5, while eight strains

Table 3. Type and concentration of antibiotic used in the study.

Antibiotic	Concentration (µg/ml)	Solvent
Carbenicillin (Car)	500	- Distilled water
Chloramphenicol (Chl)	500	- Distilled water
Erythromycin (Ery)	250	- 50% ethanol
Nalidixic acid (Nal)	50	- 0.35 N NaOH
Streptomycin (Str)	100	- Distilled water
Tetracycline (Tet)	100	- 50% ethanol
Trimethoprim (Tmp)	50	- 70% ethanol

Table 4. pH sensitivity profile of *Bradyrhizobium* spp. in liquid and solid media.

Strain/Identification	pH sensitivity profile[a]							
	Liquid media				Solid media			
	4.5	5.0	5.5	7.0	4.5	5.0	5.5	7.0
TAL 209	+/-	+/-	+	+	-	+	+	+
TAL 305	+	+	+	+	+	+	+	+
THA 301	+	+	+	+	+/-	+	+	+
USDA 3267	+/-	+	+	+	+/-	+	+	+
TAL 441	+/-	+	+	+	+/-	+	+	+
THA 302	+/-	+/-	+	+	+/-	+	+	+
TAL 442	+/-	+/-	+/-	+	+/-	+	+	+
TAL 425	+	+	+	+	+/-	+/-	+/-	+/-
THA 304	+/-	+/-	+/-	+	+/-	+	+	+
TAL 306	+/-	+	+	+	+/-	+	+	+
E-17-1	-	+/-	+/-	+	-	+/-	+	+
280A	-	+/-	+	+	-	+	+	+
E-7-1	-	+/-	+/-	+	-	+	+	+
NE-36-19	-	+/-	+	+	-	+	+	+
N-22-18	-	+/-	+	+	-	+	+	+
M-47-12	-	+/-	+/-	+	-	+	+	+
M-43-10	+/-	+	+	+	-	+/	-+	+
NE-41-15	-	+/-	+/-	+	-	+/-	+	+
M-50-2	-	-	+/-	+	-	+	+	+
22-2A	+	+	+	+	-	+	+	+

[a]*Growth rating : +, growth; +/-, very poor growth; -. no growth*

from ten isolated from *Arachis hypogaea* were unable to tolerate pH as low as 4.5. Ten *B. japonicum* strains from sixteen can grow at pH level 4.5. The results are shown in Tables 4 and 5. To determine the condition causing to them survive at low pH, the final pH was observed after 10 days of growth. We can divide them from determination of final pH as acid producer, alkali producer and pH independent group. The results which were indicated by changing pH value after 10 days of growth are depicted in Tables 6 and 7.

The antibiotic resistance profiles of *Bradyrhizobium* spp. and *B. japonicum* strains are shown in Tables 8 and 9. The resistant, susceptible and susceptible with mutated colony characteristics of each strain against seven antibiotics used in this experiment can differentiate them. In the example, ten strains of *Bradyrhizobium* spp. isolated from *Vigna radiata*, THA 301 and THA 302 strain showed the same profile of antibiotics resistance, whereas the rest performed differently. *Bradyrhizobium* spp. from *Arachis hypogaea*, E-7-1 and M-43-10 showed the

Table 6. pH of the broth culture of Bradyrhizobium japonicum after 10 days of growth.

Strain/Identification	pH			
	4.5	5.0	5.5	7.0
TAL 102	4.6	6.0	7.1	7.2
TAL 432	4.6	5.6	5.5	7.0
THA 2	4.4	4.5	4.5	4.8
TAL 379	4.6	5.7	6.7	7.0
TAL 944	4.6	5.8	7.0	7.2
TAL 212	4.8	6.6	7.1	7.3
TAL 211	4.8	6.3	7.1	7.2
TAL 220	4.5	5.2	6.3	6.6
USDA 94	4.2	5.0	5.4	6.2
USDA 35	4.5	5.2	5.8	7.0
USDA 117	4.4	4.7	4.9	4.9
TAL 117	4.5	5.7	6.8	7.0
TAL 377	4.5	4.8	4.9	4.9
THA 7	4.5	4.8	4.9	4.9
THA 5	4.3	4.7	4.9	5.0
TAL 216	4.7	6.8	7.0	7.3

Table 5. pH sensitivity profile of *B. japonicum* in liquid and solid media.

Strain/Identification	pH sensitivity profile[a]							
	Liquid media				Solid media			
	4.5	5.0	5.5	7.0	4.5	5.0	5.5	7.0
TAL 102	-	+	+	+	-	+/-	+/-	+
TAL 432	-	-	-	+	-	+	+	+
THA 2	+/-	+	+	+	+/-	+/-	+	+
TAL 379	+/-	+	+	+	-	+/-	+	+
TAL 944	+/-	+	+	+	-	+	+	+
TAL 212	+/-	+	+	+	+/-	+	+	+
TAL 211	+	+	+	-	+/-	+	+	+
USDA 8-T	-	+/-	+/-	-	-	+/-	+	+
TAL 212	+/-	+	+	-	+/-	+	+	+
TAL 220	+/-	+	+	-	+/-	+	+	+
USDA 94	+/-	+	+	-	+/-	+	+	+
USDA 35	-	+/-	+/-	+	-	+/-	+	+
USDA 117	-	+/-	+/-	+	+/-	+/-	+	+
TAL 377	-	+/-	+/-	+	+/-	+/	+	+
THA 7	+/-	+/-	+/-	+	+/	+/-	+	+
TAL 5	+/-	+/-	+/-	+	+/-	+	+	+
TAL 216	+	+	+	+	+/-	+	+	+

[a]*Growth rating : +, growth; +/- , very poor growth; - , no growth.*

Table 7. pH of the broth culture of *Bradyrhibium* spp. after 10 days of growth.

Strain/Identification	pH			
	4.5	5.0	5.5	7.0
TAL 209	4.2	4.6	4.6	4.9
TAL 305	6.5	7.1	7.4	7.5
TAL 301	4.7	5.3	5.3	5.8
USDA 3267	4.6	5.0	5.5	6.8
TAL 441	4.6	4.9	4.9	4.9
THA 302	4.6	5.1	5.0	6.9
TAL 442	4.7	5.1	5.0	5.0
TAL 425	4.6	5.2	5.3	6.9
THA 304	4.6	5.1	5.4	5.4
TAL 306	4.6	4.9	5.1	5.5
E-17-1	4.6	5.1	5.2	5.2
280A	4.7	5.0	5.4	5.4
E-7-1	4.5	4.9	5.1	5.3
NE-36-19	4.5	5.1	5.3	5.3
N-22-18	4.6	5.3	6.0	6.6
M-47-12	4.6	5.3	6.0	6.6
M-43-10	4.5	5.0	6.7	5.5
NE-41-15	4.5	5.1	5.7	6.9
M-50-2	4.5	5.0	4.9	4.7
22-2A	4.8	6.6	7.1	7.3

same profile of antibiotic resistance. In addition, another pair with the same antibiotic resistance profile is N-22-18 and M-47-12, while the rest are different. For sixteen strains of *B. japonicum*, we found that strain TAL 379 and TAL 211 shared the same characteristics of antibiotic resistance whereas the rest performed differently.

The results obtained from pH sensitivity profiles and characteristics of antibiotic resistance might be useful for distinguishing between strains and development of acid tolerance strain by breeding. For more precise differentiation among strains, the PCR technique, together with the appropiate primers will be conducted and analyzed with phenotypic characteristics obtained from this study.

References

Abdel Basit, H., Angle, J.S., Salem, S., Gewaily, E.M., Kotob, S. I. and van Berkum, P. (1991). Phenotypic diversity among strains of *Bradyrhizobium jopoicum*. Appl. Environ. Microbiol. 57: 570-1572.

Cole, M.A. and Elkan, G.H. (1973). Transmissible resistance to penicillin G, neomycin and Chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother 4: 248-253.

Glenn. A.R. and Dilworth, M.J. (1994). Periplasmic proteins of *Rhizobium:* variation with growth conditions and the use in strain identification. FEMS Microbiol. Let. 123: 1-10.

Graham, P.H. (1992). Stress tolerance of *Rhizobium* and *Bradyrhizobium* and nodulation under adverse soil conditions. Can. J. Microbiol. 38: 485-492.

Hollis, A.B., Kloos, W.E. Elkan, and G.H. (1981). DNA-DNA hybridization studies of *Rhizobium japonicum*, and related Rhizobiaceae. J. Gen. Micro-Microbiol. 123: 215-222 (1981).

Huber, T.A., Agarwal, A.K. and Keister, D.L. (1984). Extracellular polysaccharide composition, explanta nitrogenase activety and DNA homology in *Rhizobium japonicum*. J. Bacteriol. 158: 1168-1171.

Josey D.P., Beynon, J.L., Johnston, A.W.B. and Beringer, J.E.J. Appl. Bacteriol. 46: 343-350.

Keyser, H.H. and Munns, D.N. (1984). The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. Soil Sci. Soc. Am. J. 48: 1273-1276.

Kuykendall, L.D., Roy, M.A., O'Neill, J.J. and Devine, T.E. (1988). Fatty acids, antibiotic resistance and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bacteriol. 38: 358-361.

Pankhurst, C.E., Scott, D.B. and Ronson, C.W. (1982). Correlation between rifampicin-resistance of slow-growing *Rhizobium* strains and their ability to express nitrogenase activity in culture. FEMS Microbiol Let. 15: 137-139.

Somasegaran, P and Hoben, H.J. (1994) Handbook for *Rhizobia*: Methods in Legume-Rhizo bium Technology.

van Berkum, P., Kotob, S.I., Abdel Basit, H., Salem, S., Gewaily, E.M. and Angle, J.S. (1993). Genotypic diversity among strains of *Brdyrhizobium japonicum* belonging to serogroup 110. Appl. Environ. Microbiol. 59: 3130-3133.

Table 8. Antibiotic resistance of *Bradyrhizobium* spp.

Strain/Identification	Antibiotic resistance[a]						
	Car	Chl	Ery	Nal	Str	Tmp	Tet
TAL 209	r	s	s_m	r	s_m	r	r
TAL 305	r	s_m	s_m	r	s_m	r	s
THA 301	r	s	s_m	r	s_m	r	s
USDA 3267	s	s	s	r	s	r	s
TAL 441	r	s	s	r	s	r	s
THA 302	r	s	s_m	r	s_m	r	s
TAL 442	r	s	r	r	s_m	r	s
THA 304	s_m	s	s_m	r	s_m	r	r
TAL 306	r	s	s_m	s	s_m	r	r
E-17-1	r	r	r	r	s_m	r	r
280A	r	s	s_m	s	s_m	r	r
E-7-1	r	r	s_m	r	s_m	r	r
NE-36-19	r	s	s_m	r	s_m	r	r
N-22-18	r	s	s_m	r	s_m	r	r
M-47-12	r	s	s_m	r	s_m	r	s
M-43-10	r	r	s_m	r	s_m	r	r
NE-41-15	r	s_m	s_m	r	s_m	r	r
M-50-2	r	s	s_m	s	s	r	s
22-2A	r	s	s_m	s	s_m	r	s

[a] *r - resistant; s - susceptible; s_m- susceptible with mutated colony*

Table 9. Antibiotic resistance of *B. japonicum.*

Strain/Identification	Antibiotic resistance[a]						
	Car	Chl	Ery	Nal	Str	Tmp	Tet
TAL 102(USDA 110)	r	s	r	s	s_m	r	s_m
TAL 432	r	s	s_m	r	s_m	r	s
THA 2	s	s	s_m	r	s_m	r	r
TAL 379	r	s	s_m	s_m	s_m	r	s
TAL 944	r	r	s	r	s	r	r
TAL 212	r	s	s_m	s_m	s_m	r	s
TAL 211	r	s	s_m	s	s_m	r	s
USDA 8-T	s	s	s_m	s_m	r	r	s
TAL 220	r	r	r	s	r	r	r
USDA 94	s	r	s	s	s_m	r	r
USDA 35	s	s	s_m	s_m	s_m	r	s
USDA 117	s	s	s_m	r	s	r	r
THA 377	r	s	s_m	s	r	r	s
THA 7	s	s	s_m	s_m	r	r	r
THA 5	s_m	s	s_m	r	s_m	r	r
TAL 216	r	r	s_m	s_m	s_m	r	r