Philippine Journal of Science
139 (1): 79-90, June 2010
ISSN 0031 - 7683

Acid -Tolerant Rhizobia of *Phaseolus vulgaris* L. from the Intensively Cropped Soils of La Trinidad, Benguet, Philippines

Rosemary M. Gutierrez[1,*] and **Wilfredo L. Barraquio**[2]

[1]Department of Biology, College of Science, University of the Philippines Baguio, Gov. Pack Road 2600, Baguio City, Philippines
[2]Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines

The common bean (*Phaseolus vulgaris* L.) has long been intensively cultivated on the agricultural soils of La Trinidad, Benguet, Philippines. The soil of the site has become acidic (pH 5-6) because of chemical nitrogen fertilization at early stages of plant growth. The present study reports the diversity of rhizobia isolated from Benguet -*Phaseolus* and the selection of acid-tolerant strains that may have the potential for future inoculation trials. One hundred eighty nine (189) rhizobial strains were isolated from *Phaseolus* nodules and initially screened for acid tolerance in an unbuffered Glutamate Sucrose Minimal Medium (GSM) adjusted to pH 4.5. Further gnotobiotic evaluation of these strains based on symbiotic effectiveness and growth behavior at pH 4.5 yielded two most acid-tolerant strains (RG-136 and RG-137). Polyphasic characterization identified the most acid-tolerant strains as *Rhizobium etli*.

Key Words: diversity, legumes, nodulation assays, pH tolerance, phylogeny, *Rhizobium*

INTRODUCTION

The common bean, *Phaseolus vulgaris* L. is a semi-temperate legume cultivated in the Philippines. It is a common source of protein in the Filipino diet and is planted on about 2,644 hectares of land in the country in areas with medium to high elevation (Delfin et al. 1999). La Trinidad, Benguet is one major agricultural area in the country where common beans or baguio beans, as it is locally called have long been intensively cultivated. Its soil has become acidic because of chemical nitrogen fertilization at early stages of plant growth. Earlier soil investigation studies in La Trinidad covering 1,420 hectares have indicated that there were two major soil types in La Trinidad: the La Trinidad silt loam and the La Trinidad loamy sand. The pH of the soil samples from these sites were described to be acidic (pH 3.0 to 6.0).

Legume nodules and their *Rhizobium* symbiotic partners face typical environmental stresses like acidic soils with low nutrient status (Zahran 1999). Acid soil conditions constrain nodulation and nitrogen fixation by beans over large areas of Latin America, Africa, Western Australia and in some areas of the U.S. and it may also be the case in the Philippines. Continuous search for acid – tolerant (AT) rhizobial strains are being done to improve production of legumes grown on acidic soils and to extend the range of soil upon which adapted legumes may be grown (Howieson et al. 1988). The best described AT rhizobial strain is *Rhizobium. tropici* CIAT899 (*R. tropici*. UMR1899) which was isolated from bean nodules (Graham et al. 1982) and produced isolated colonies on agar medium of pH 4.0 (Graham et al. 1994). This strain has been used successfully as inoculant in

*Corresponding author: rosemarymgutz@yahoo.com
rmgutierrez@upb.edu.ph

acid soils (Hungria et al. 2003). Furthermore, selection of AT rhizobial strains associated with other legumes have also been studied in other countries (Howieson & Ewing 1986; Del Papa et al. 1999) since AT rhizobial strains are considered to be of utmost agronomic and ecological importance that could address the need for a more environment friendly technology.

The diversity of rhizobia associated with beans has been studied with emphasis on those of Mesoamerican origin (Bernal & Graham 2001). Andrade et al. (2002) did a long term study on the diversity of *Phaseolus*-nodulating rhizobial populations in acid soils in Brazil using molecular methods. The authors hypothesized that the abundance and diversity of rhizobial species and of strain types within the species would decrease with increasing soil acidity stress. Aguilar et al. (2001) did a similar study in Argentina by characterizing their isolates using *nif*H-PCR of genes coding for 16S rRNA and *nod*C as well as REP fingerprinting. It was revealed that *Rhizobium etli* was the predominant species in the common bean nodules and a high degree of genetic diversity was observed within the species. Many other studies have indicated that there is an enormous diversity of bean nodulating bacteria in the centers of origin of *P. vulgaris* located in Middle America and South America, wherein the predominant species of rhizobia present in bean nodules was reported to be *Rhizobium etli*. In addition to *R. etli,* other species found to be present in bean nodules are some other rhizobial species like *R. leguminosarum* bv. *phaseoli*, *R. gallicum* bv. *phaseoli* and *R. giardinii* bv *phaseoli* (Martinez-Romero 2003). No studies have been done yet on the isolation of AT rhizobial strains as well as the diversity of *Phaseolus* rhizobial strains in the Philippines. This present study reports the diversity of *Phaseolus* rhizobia and the isolation, selection, characterization and identification of a superior strain from the bean fields of La Trinidad Benguet, Philippines that may have the potential to promote better nodulation and plant growth in acid soils.

MATERIALS AND METHODS

Isolation and cultural conditions

Nodules were collected from the roots of two to three month old common bean plants from farmers' field from four different geographical locations in La Trinidad, Benguet. Soil samples were also collected from these farms and based on some personal communications with the farmers, these sites had no history of inoculation with rhizobia, but were constantly applied with chemical fertilizers and pesticides. The soil samples were submitted to the Department of Science and Technology-Cordillera Administrative Region (DOST-CAR), La Trinidad, Benguet Analytical Laboratory for analysis of its physico-chemical characteristics (Table 1). After collection, healthy nodules were immediately processed in the laboratory as follows: nodules were washed five times with sterile distilled water and were immersed in 1% Chloramine T for 20 min. After several washings in sterile distilled water, nodules were macerated in sterile mortar and pestle and a loopful of each macerate was streaked onto yeast extract mannitol (YEM) agar (Vincent 1970).

Single, isolated colonies were selected, purified, and kept in YEM agar slants immersed in sterile paraffin oil and in YEM broth with 20% glycerol at -20^{o} C. Colony morphologies and pH reactions were examined for all the isolates by gram staining and by growing the isolates in YEM agar medium amended with congo red (25 μg/mL) and bromthymol blue (25 μg/mL).

The isolates were authenticated following the method of Somasegaran and Hoben (1994). The modified Jensen's N - free medium (Roughley 1970) was used as growth medium. Non-inoculated controls, with and without the mineral N were included in the authentication set- ups.

Screening for acid tolerance

Initial screening of isolates for acid tolerance was done using the glutamate-sucrose minimal agar medium or GSM (Del Papa et al. 1999). The pH of the medium was adjusted to 4.5 with HCl before autoclaving and was checked and adjusted more precisely after the addition of filter sterilized vitamins and micronutrients after autoclaving. Three day old cultures of rhizobia were streaked into the GSM plates adjusted to pH 4.5, pH 6.0 and pH 7.0, before incubation at 28^{o} C for seven days. Strains that grew single isolated colonies in the GSM medium adjusted to pH 4.5 were considered AT strains (Graham et al. 1994).

Thirty six (36) strains initially selected from the first screening were evaluated for acid tolerance by nodulation assays as follows: *Phaseolus vulgaris* L. seeds (Bush Blue Lake variety, from the Bureau of Plant Industry (BPI), Guisad, Baguio City) were surface sterilized with 1% Chloramine T and pre-germinated in water-agar for two days. Single seeds were then planted in big tubes containing 30 mL of buffered Jensen's N-free agar medium adjusted to pH 4.5 and 7.0. Buffers used were HOMOPIPES for pH 4.5, and PIPES for pH 7.0 (Del Papa et al. 1999). Strains that were selected in the initial screening procedures using GSM plates were grown in a shaker for three days in YEM broth with cell density of approximately 1 x 10^{8} cells/mL. One ml each of the

presumptive AT strains were inoculated in the tubes with seeds. The tubes were incubated in a growth room at temperature ranging from 28 to 30° C for two weeks at 12/12 day/night cycle with a light intensity of 22 μmol $m^{-1}s^{-1}$. Non-inoculated controls, with and without the mineral N, and a control inoculated with *R. tropici* UMR1899 were included. Tubes were arranged in a randomized complete block design (RCBD) with three replicates, and the results were statistically analyzed by the Two-way ANOVA and the means were compared using the Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

Strains that performed well in the nodulation tests were screened further in liquid media. (Table 2). Further screening for acid tolerance was done in buffered liquid GSM adjusted to pH 4.5, 5.5, and 7.0. Buffers used were HOMOPIPES for pH 4.5, MES for pH 5.5 and PIPES for pH 7.0 (Del Papa et al. 1999). One hundred fifty (150) mL of buffered liquid GSM media in 250 mL erlenmeyer flasks were inoculated with AT strains previously grown in YEM broth (with a cell density of approximately 1 x 10^8 cells/mL) and were incubated in a rotary shaker at 28° C for five days. Strains that gave the highest optical density (OD) measurements for this screening procedure in liquid media were selected for growth curve monitoring studies. Growth curve studies were undertaken for the designated AT strains. All OD measurements were made at 500 nm (Del Papa et al. 1999).

Characterization and identification of rhizobial isolates

Genotypic characterization

DNA isolation

Genomic DNAs of the selected rhizobial strains were isolated using the Promega Wizard Genomic DNA Purification Kit and the protocol for isolation was followed as recommended by the manufacturer.

Restriction enzyme analysis of PCR-amplified 16S rDNA genes

Total DNA samples were amplified using the primers 27f (5'AGAGTTTGATCCTGGCTCAG 3')and 1492r (5' TACGGCTACCTTGTTACGACTT 3') (Lane 1991) which were complimentary to conserved regions of the bacterial 16S rRNA gene. Amplifications were carried out in 25 μL reaction volumes containing the following: 2.5 μL 10X PCR reaction buffer, (100mM Tris-HCl, pH 8.3, 500 mM KCL) and 1.5 μL 25 mM $MgCl_2$ solution (Applied Biosystems, Foster City USA), 4.0 μL 1.25 mM, dNTPs, 0.5 μL of 27f primer (200ng/μL), 0.5 μL of 1492r primer (200ng/ μl), 0.1 μL AmpliTaq Gold DNA polymerase and 1 or 2 μL of DNA as template. Reaction volumes were made up to 25 μL with sterile milli Q ELGA water. DNA amplifications were conducted using the PTC 100 Programmable Thermal Controller (MJ Research, Inc. Watertown, Massachusetts, USA).

The thermal cycling profile was based on the procedure described by Del Papa et al. (1999) with some modifications: it consisted of an initial denaturation step at 94° C for 3 min, followed by 28 cycles of denaturation (30 s at 94° C), annealing at 30 s at 58° C, and extension of 45 s at 78° C, followed by a final extension at 72° C for 10 min.

Amplification products were then visualized under UV light following electrophoresis on 1% agarose gel at 90v/cm for 30 min. Gels were made with 1 x TAE (0.04M Tris acetate, 0.001M EDTA, pH 8.0) and contained 0.2 μg/ml ethidium bromide. A 1 Kb Plus DNA Ladder was used as molecular marker (Life Technologies, Australia).

PCR products were digested (10 μL) with the restriction enzyme *Msp*I by following the procedure recommended by the manufacturer. Analyses of digestion products were done by electrophoresis in 2% agarose gel and was ran at 80v/cm for two h. Banding patterns were scored directly from gel photographs and the isolates were grouped through visual inspection of the banding patterns.

BOX-PCR DNA fingerprinting

Amplification for BOX-PCR was done using the following components: 2.5 μL of 10X PCR reaction buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3.2 μL of 25 mM $MgCl_2$, 4.0 μL of 1.25 mM dNTPs (Biotech International Ltd, Perth Australia), 50 pmol of BOXA1R primer (5' CTACGGCAAGGCGACGCTGACG 3') (Versalovic et al. 1994), 0.2 μL of Ampli Taq Gold DNA polymerase with GeneAmp, and 2.0 μL DNA template. Reaction volumes were made up to 25 μL with sterile milli Q ELGA water. PCR amplification was performed using the following heating profile: initial denaturation of 94° C for 10 min; 30 cycles of denaturation at 94° C for 1 min; annealing at 53° C for 1 min and extension at 65° C for 8 min. Final extension was at 65 ° C for 16 min.

Gel electrophoresis was then carried out to separate the PCR products. A 2% agarose gel was prepared in 1 x Tris Borate EDTA buffer. After electrophoresis the gel was stained in 10 mg/mL ethidium bromide for 15 min and was destained in distilled water for 10 min. The BOX-PCR amplification products were observed on a UV transilluminator. A 1 Kb Plus DNA Ladder (Life Technologies, Australia) was used as molecular marker. BOX-PCR fingerprints were analyzed using the Cluster Analysis with the Dice similarity coefficient and UPGMA

(Unweighted Pair-Group Method with Arithmetic Averages). Clustering was calculated by the Bionumerics 5.1 Software (Applied-Maths, Belgium). A 1% of pattern length was used for band matching.

Partial sequencing of 16S rDNA

For DNA sequencing, PCR products were purified using the Qiagen Purification Kit. After purification the PCR products were again subjected to electrophoresis to check for purity and molecular size. Purified PCR amplified 16S rDNA fragments were sequenced using the Big Dye Terminator Chemistry version 3. Ten µL reaction volumes were used containing the following: 4 µL of the terminator premix, 1.0 µL of primer (3.2 pmol), 2 µL of PCR purified product and 3.0 µL of milli Q ELGA water. Primers used for sequencing were the following: 27f (5' AGAGTTTGATCCTGGCTCAG 3') 1492r (5' TACGGCTACCTTGTTACGACTT 3'), 530f (5' GTGCCAGCCGCCGCGG 3') and 907r (5' CCGTCAATTCCTTTAAGTTT 3') (Lane 1991). The sequencing thermal profile consisted of an initial denaturation at 96° C for 10 s, cooled to 50° C for 15 s and extension at 60° C for 3 min. Sequencing reactions were carried out in a PTC 100 Programmable Thermal Controller.

Gel separation of the sequences was completed on an Applied Biosystems Model 377 automatic DNA sequencer (Applied Biosystems, Foster City, California). The sequencing was done at the Australian Genome Research Facility (AGRF), University of Queensland, Australia. Homology searches were carried out using the NCBI BLAST (Basic Local Alignment Search Tool). Further confirmation of the identification of selected rhizobial isolates was done using the BIOLOG MicroPlates and BIOLOG software (Hayward, Ca, USA) for microbial identification. Selected rhizobial strains were also submitted for 16S rRNA sequencing to the Laboratory of General Microbiology at the University of Bremen, in Bremen Germany. Homology searches were carried out using the NCBI BLAST (Basic Local Alignment Search Tool). Sequence alignment and phylogenetic tree construction was undertaken using the NJ-JC (Jukes-Cantor) BS1025 software, and the file was converted using the MEGA2 package.

Characterization of Selected AT strains

Carbohydrate and amino acid utilization profiling

Cultures of the two most AT strains (RG136 and RG 137) and a non- AT strain, (RG-59) and reference strains (Table 4) were grown for three days on YEM agar slants at 28° C. The isolates were then suspended in sterile 0.85% saline solution, washed two times in saline solution and resuspended in Cohen-Bazire medium (Stanier et al. 1966). OD of inoculum was adjusted to give approximately 1.0 x 10^8 cells/mL. Three hundred microliters (300 µL) of the inoculum suspension were then inoculated into three ml Cohen-Bazire medium in test tubes containing various amino acids and carbohydrates. The cultures where then incubated in a rotary shaker at 28° C. OD measurements were taken at a wavelength of 540 nm at 0 hour and after five days of incubation. Growth was scored positive if there was a significant increase in OD after five days and negative if there was no significant increase in OD measurements after five days of incubation. Amino acids and carbohydrate stock solutions were prepared as 10 % (w/v) stock solutions. Twenty three (23) different amino acids were used as follows: L-lysine, L-ornithine, L- threonine, L-serine, L-alanine, L-glutamic acid, L-glycine, L-proline, L-arginine, L-histidine, L-valine, L-phenylalanine, L-cycsteine, L-methionine, L-aspartic acid, L- isoleucine, L-leucine, L-cysteine, L-tryptophan, L-tyrosine, L-asparagine, L- glutamine and α-keto glutaric acid. Thirty three (33) different carbohydrates were used as follows: fructose, sucrose, lactose, sodium succinate, sodium butyrate, sodium fumarate, sodium acetate, sodium citrate, gluconic acid, calcium acetate, D- sorbitol, maltose, D-xylose, arabinose, dulcitol, cellobiose, melibiose, D-melizitose, D-ribose, inositol, L-rhamnose, D-raffinose, L-sorbose, meso- erythritol, adonitol, D- arabitol, mannitol, glucose, pectin, cellulose, salicin, inulin and esculin. Based on the results of the profiles, % similarities were calculated by using the formula:

$$\% \text{ similarity} = \frac{\begin{array}{c}\text{number of similar characters}\\ \text{[(+/-) scores] for a reference}\\ \text{strain \& a strain being compared}\end{array}}{\begin{array}{c}\text{total number of amino acids}\\ \text{and carbohydrates used}\end{array}} \times 100$$

Antibiotic resistance profiling

Antibiotic resistance profiling was done using glucose-glutamic acid peptone yeast extract (GGPYE) (James et al. 1994) supplemented with various antibiotics. Inocula used were selected rhizobial strains incubated for 24 hours in a rotary shaker. The ability of the strains to grow in the presence of antibiotics was tested by streaking the rhizobial isolates and reference strains in GGPYE plates containing filter sterilized antibiotics. Plates were incubated at 28° C for 3-5 days.

Strains were recorded resistant when growth occurred (+ score) and sensitive when no growth or very poor growth occurred (- score). Twenty six (26) different antibiotics were used as follows: ampicillin, chlortetracycline, erythromycin, nalidixic acid, novobiocin, polymixin-B, spectinomycin, kanamycin, rifampicin, chloramphenicol,

streptomycin, vancomycin-HCl, tetracycline, cycloheximide, oxytetracycline, penicillin-G, oxacillin, amphotericin-B, bacitrin, gentamicin, carbenicillin, clindamycin, tobramycin, lincomycin, nitrofurantoin and clarithromycin with concentrations of 50 and 100 μg/ml for each antibiotic used. Based on the results of the profiles, % similarities were calculated by using the formula:

$$\% \text{ similarity} = \frac{\text{number of similar characters [(+/-) scores] for a reference strain \& a strain being compared}}{\text{total number of antibiotics used}} \times 100$$

Physiological Characteristics

Growth on various media were investigated by streaking three day old rhizobial strains grown in YEM agar slants in two different agar plates: Luria Bertani (LB) medium, (Ausubel et al. 1990) and Tryptone Yeast medium (Somasegaran and Hoben 1994). Colony sizes were also observed and measured after three days of incubation at 28° C in YEM plates. YEM plates were prepared to test the tolerance of the isolates to high NaCl concentrations. Plates in duplicate were streaked with a loopful of three-day old cultures on YEM agar plates amended with NaCl to final concentrations (w/v) ranging from 0.1%, 0.75%, 1.0%, 2.0%, 3.0% and 4.0%. The plates were then incubated at 28° C for seven days.

RESULTS

Soil physico-chemical characteristics

Table 1 shows the physico-chemical characteristics of the soil samples collected from four different bean plantation sites in La Trinidad, Benguet, Philippines. La Trinidad is the administrative capital of Benguet province, situated about 250 kilometers north of Metro Manila and is composed of 16 barangays (brgys). However, collection of nodules and soil samples were done in four brgys only namely: Brgy. Alno in the north, Brgy. Puguis in the west, Brgy. Beckel in the east, and Brgy. Balili in the central portion of the municipality, since these were the major planting sites of common beans. Aside from beans, the land of these farms are intensively cultivated since its also being planted with other vegetables such as lettuce, sweet potatoes, garden peas, tomatoes, carrots, strawberries and some fruit trees and ornamentals. Fairly acceptable values were obtained for % organic matter and cation exchange capacity while % total nitrogen content of the soils were observed to be low. The pH of the soil ranged from 5.19 to 6.35 confirming that the soil in the different barangays of La Trinidad were slightly acidic.

Table 1 . Physico-chemical characteristics of the soil samples from the collection sites in La Trinidad, Benguet.

Collection Site	%OM[a]	CEC[b] m.e/100g soil	Total N[c] %	Soil pH[d]	No. of authenticated isolates[e]
Brgy. Alno	3.32	27.17	0.17	6.35	66
Brgy. Balili	3.74	39.55	0.22	5.33	25
Brgy. Beckel	2.74	33.72	0.15	5.92	28
Brgy. Puguis	4.38	37.88	0.24	5.19	70

[a] % Organic matter was analyzed by the Walkley-Black Method
[b] Cation exchange capacity was analyzed by the Ammonium Acetate method
[c] % Total Nitrogen was analyzed by the Kjeldahl method
[d] Soil pH was checked in a 1:1 soil - water mixture
[e] Total no of authenticated isolates = 189

Isolation and screening for acid tolerance

A total of 189 strains (Table 1) were isolated and authenticated as *Rhizobium* strains from four geographically distinct locations from La Trinidad, Benguet, a major agricultural area in the northern part of the Philippines. All rhizobial strains isolated were gram negative and all of them were acid producers, as indicated by the observation that there was a color change from blue to yellow in YEM agar plates containing bromthymol blue. All 189 isolates were also fast growers wherein single isolated colonies could be observed in YEM agar plates after three to five days of incubation at 28° C.

Initial screening for acid tolerance using the defined medium, (GSM) yielded 36 AT strains (19%) that produced single isolated colonies on unbuffered GSM adjusted to pH 4.5 after seven days of incubation at 28° C. These 36 AT strains were tested for their nodulation abilities at pH 4.5 and 7.0 and results are shown on Table 2. Only nine strains out of the initial 36 presumptive AT strains (25%) showed promising nodulating abilities at pH 4.5 and 7.0. The other strains (75%) exhibited poor nodulating abilities or did not nodulate in the nodulation assays and therefore were not included in the statistical analyses. Results of data analyses revealed that in terms of nodule number, there were significant differences among treatment means at .05 alpha level of significance due to the strains used in this particular experiment. However, there were no significant differences among treatment means due to the pH (pH 4.5 and pH 7.0) and due to the interaction of the strains and the pH used. However, by comparing the treatment means using the Duncan's Multiple Range Test (DMRT), it is interesting to note that at pH 4.5, there was one strain that showed significant difference among the other strains used in the experiment in terms of nodulating ability and this was strain RG-136. However, in terms of shoot dry weights, no significant differences were observed among treatment means due

Table 2 . Average number of nodules and shoot dry weights (mg) of aseptically raised *Phaseolus vulgaris* inoculated with AT strains in buffered Jensen's N-free media.

Strains	pH 4.5		pH 7.0	
	No. of nodules/* plant	Shoot dry* w/plant	No. of nodules/* plant	Shoot dry* w/plant
RG-59	20.33b	117.70	39.67b	152.70
RG-13	35.00b	103.00	14.33b	106.30
RG-133	29.33b	92.33	5.33b	81.00
RG-136	92.33a	122.00	28.00b	134.30
RG-137	34.33b	44.67	10.67b	51.00
RG-60	21.67b	122.70	20.00b	153.70
RG-57	5.33b	85.33	16.00b	114.67
RG-54	11.67b	119.00	18.66b	89.00
RG-75	2.0b	121.30	19.67b	129.30
UMR1899	20.0b	124.30	20.33b	138.70
Control	0b	120.30	0b	124.00
N^+ Control	0b	138.70	0b	116.00
LSD	34.48	ns	34.48	ns

*Values are means of 3 replicates and when followed by the same letter in the same column, did not show statistical difference (Duncan, $p < 0.05$);
ns- not significant at 5% level of significance

to the strains and due to the pH used in the experiments.

Further screening of the nine promising AT strains using buffered liquid GSM adjusted to pH 4.5, 5.5 and 7.0 indicated that there were two strains, RG-136 and RG-137, that gave high OD at pH 4.5 after 5 days of incubation in a rotary shaker next to the well studied AT reference strain *Rhizobium tropici* UMR1899 (data not shown).

Furthermore, results of growth curve monitoring experiments (Table 3) showed that strain RG-136 had the shortest generation time, g, of 4.8 hrs and highest specific growth rate, μ, 0.145 per hour at pH 4.5 as compared to RG-137 and *Rhizobium tropici* UMR1899 strains. Although the cultures were buffered to maintain the initial pH, we observed an increase in the pH of the extracellular medium by 0.5 units after four hrs of growth. Then after eight hrs, a further increase in the pH of the medium by 0.3 units was observed and was maintained until the end of the growth period for five days. This increase in pH was observed in all the pH ranges (4.5, 5.5, 7.0) used in these growth curve monitoring experiments.

Genomic characters

A single DNA fragment of about 1500 base pairs representing the 16S rRNA gene was amplified for 31 out or the 36 AT strains which corresponded to the expected size of the 16S rRNA gene among eubacteria.

Table 3 . Specific growth rate (μ), and generation time (g), of acid-tolerant strains at different pH levels.

Strain	μ, hr^{-1}			g, hr		
	pH 4.5	pH 5.5	pH 7.0	pH 4.5	pH 5.5	pH 7.0
RG-136	0.145	0.099	0.067	4.8	6.9	10.3
RG-137	0.121	0.097	0.075	5.7	7.2	9.2
UMR1899	0.112	0.072	0.112	6.2	9.7	6.2

Restriction enzyme analyses of the 16S rRNA gene amplification products using *Msp*I revealed only three distinct RFLP patterns for the 36 AT strains. The restriction enzyme patterns of representative strains are shown in Fig 1. Nineteen strains out of the 36 AT strains (52.7%) exhibited restriction pattern A; the three reference strains used (UQ373 *(R. trifoli)*; UQ2230 (*R. leguminosarum*

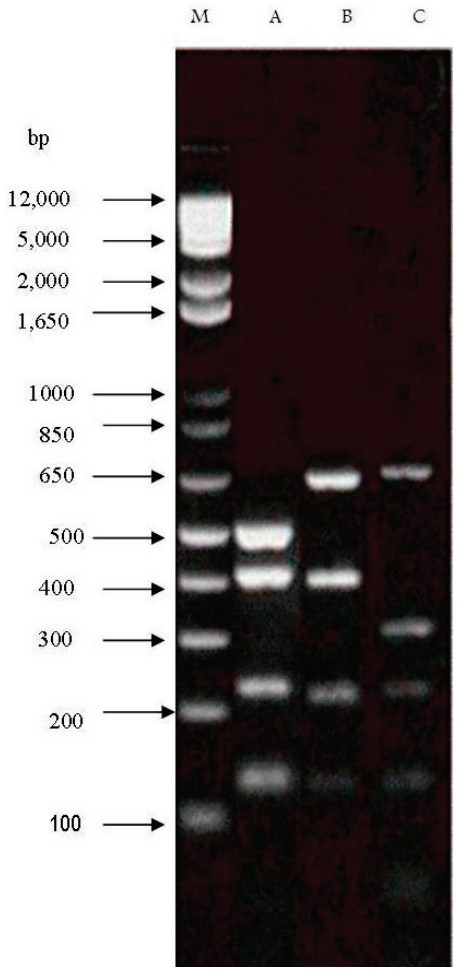

Figure 1. Restriction enzyme pattern analysis of 16S rDNA PCR products using *Msp* I revealed only three patterns for the 36 AT strains. RFLP patterns of the representative strains are shown (M, molecular marker; A, restriction pattern A from strain RG-136; B, restriction pattern B from strain RG-85 ; C, restriction pattern C from strain RG-33)

bv *phaseoli*); UQ2720 (*R. leguminosarum*) also had restriction pattern A; 11 strains (31%) had restriction profile B, while only one strain, RG-33, exhibited restriction profile C. The two most AT strains, RG-136 and RG-137, belong to the biggest group of 19 strains that had restriction profile A. However, there were five strains that did not amplify for 16S PCR analysis namely: RG-21, RG-30, RG-60, RG-76 and RG-87. Amplification of these strains using different DNA extraction methods and lysis procedures as well as different primers were not successful.

The cluster analysis based on BOXA1R-PCR DNA fingerprints is shown in the dendogram in Fig 2. This UPGMA clustering analysis of the BOXA1R-PCR DNA patterns resulted in five main clusters. It is interesting to note that isolates coming from the same sampling sites clustered together. For instance, Cluster I contained two strains (RG- 30 and RG 33) which were both isolated from Brgy. Puguis. Cluster II contained four strains (RG-61, RG-75, RG-96 and RG-76) which were all isolated from Brgy. Alno. The biggest Cluster, Cluster V contained seven strains (RG-85, RG- 90, RG-94, RG-115, RG-116 RG-105, RG-78) and all of these seven strains were also from Brgy. Alno. Cluster IV contained five strains, and all of these strains except for one (RG-56) were isolated from Brgy. Beckel, while RG-56 was isolated from Brgy Balili. Four of the six strains (RG-58, RG-59, RG-54, RG-51) in Cluster III were isolated from Brgy. Balili while RG-2 was isolated from Brgy Beckel and RG-133 was from Brgy. Puguis. There were 12 other strains that gave unique BOXA1R- PCR fingerprints and therefore did not belong to these five clusters. The three reference strains used in the experiment also had unique BOXA1R-PCR fingerprints and did not group with any of the five main clusters. Strain RG-124, with a unique pattern showed about 60% similarity with reference strain *R. leguminosarum,* while strain RG-87 also with a unique pattern showed 60% similarity with the reference strain *R. phaseoli.* The two most AT strains RG-136 and RG-137 have unique BOXA1R-PCR DNA fingerprints.

Identification of selected rhizobial strains and Characterization of AT rhizobia

RG-136 was selected as an AT strain with good nodulating ability at pH 4.5 and grew well in buffered liquid media also at pH 4.5, so the strain was selected for partial rRNA sequencing. BLAST analyses revealed that this AT strain RG-136 (also coded as Buy2-10) had 98% similarity with *Rhizobium etli*. Result of this identification was confirmed using the BIOLOG identification system and the BIOLOG software identified this strain as *Rhizobium etli.*

Results of screening for acid tolerance revealed that there were two strains that grew well in buffered liquid media and performed well in the nodulation assays. These two strains namely RG-136 and RG-137 were designated as the most AT strains. These results prompted us to further characterize these two strains in comparison with other reference strains. Results of phenotypic characterization experiments are shown in Table 4. The phenotypic characterization of the two most AT strains showed high % similarities with *Rhizobium etli*. In terms of the antibiotic profile, the two most AT strain gave high %

Table 4 . Strains used in the study and their relevant and discriminating phenotypic characters.

Strains	Source	Growth on		Growth on YEM w/ NaCl conc >1%	Resistance to Amp	Resistance to Chloram	Resistance to NA	Growth on Dulcitol	Utilization of Glycine	Gummy colonies	Colony size (mm)*
		LB	TY								
RG-136	This study	–	+	–	–	–	–	+	–	+	2.5 – 5.0
RG-137	This study	–	+	–	–	–	–	+	+	+	2.0 – 4.0
RG-59	This study	+	+	+	+	+	+	–	–	+	1.5 – 4.0
UMR1899	UMRRL	+	+	+	+	+	+	–	+	+	2.0 – 6.0
UMR1862	UMRRL	–	+	–	–	–	–	+	–	+	2.0 – 4.0
UMR6917	UMRRL	–	+	–	–	–	–	–	–	+	0.5 – 1.5
UMR6918	UMRRL	–	+	–	+	+	+	+	–	–	3.0 – 5.0
UQ373	UQMPL	–	+	–	ND	ND	ND	ND	ND	–	3.0 – 5.0
UQ2230	UQMPL	–	+	–	ND	ND	ND	ND	ND	+	0.5 – 1.0
UQ2730	UQMPL	–	+	–	ND	ND	ND	ND	ND	+	2.5 – 5.0

ND- not determined; Amp- ampicillin; Chloram-chloramphenicol; NA-nalidixic acid
UMRRL-University of Minnesota *Rhizobium* Research Laboratory
UQMPL-University of Queensland Microbiology and Parasitology Laboratory
UMR1899 (*R. tropici*); UMR1632 (*R. etli*); UMR 6917 (*R. giardini*); UMR 6918 (*R. gallicum*)
UQ373(*R. trifoli)*; UQ2230 (*R. leguminosarum* bv *phaseoli*); UQ2720 (*R. leguminosarum*)
* measured after 3 days of incubation in YEM plates

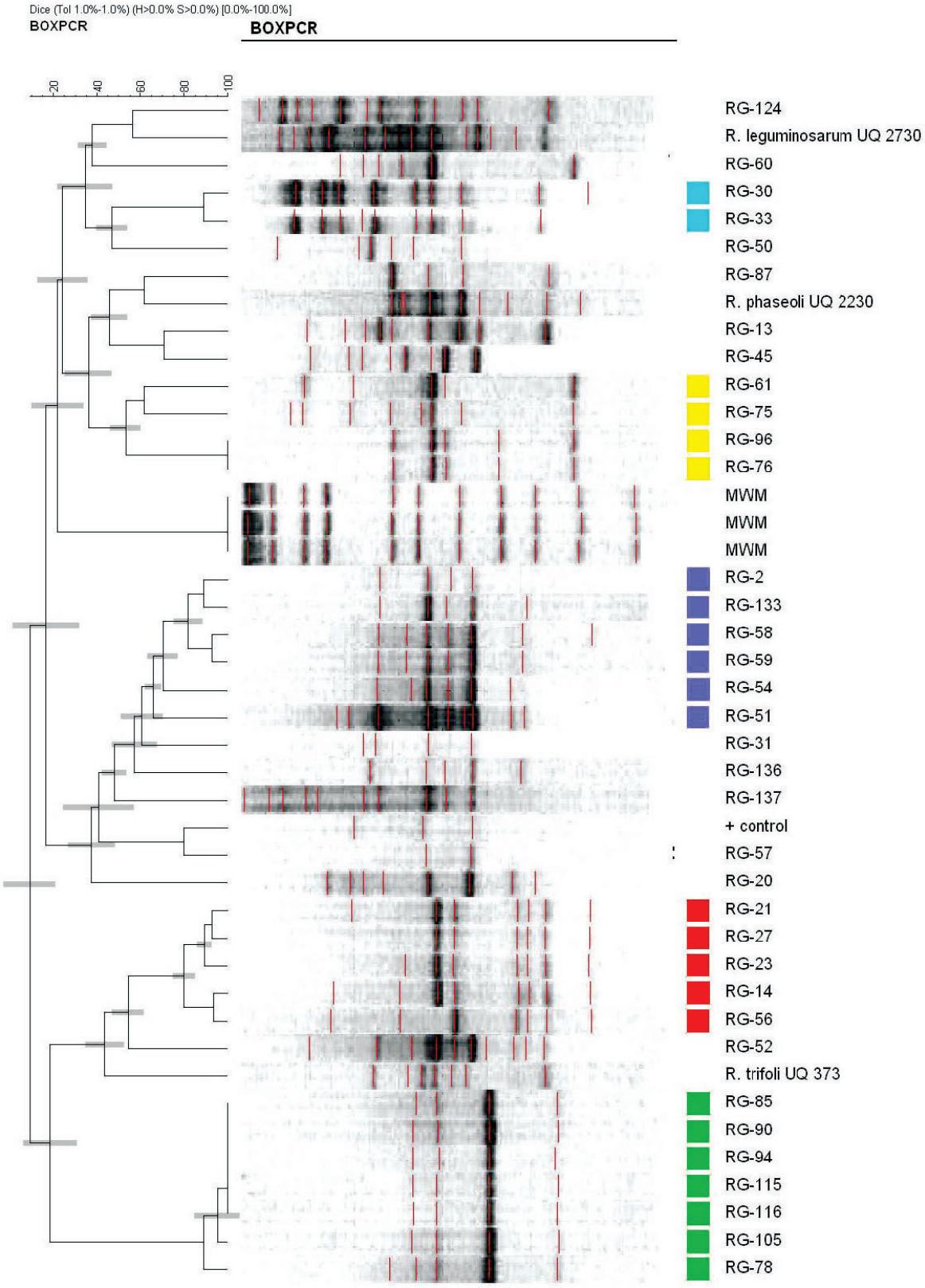

Figure 2. Dendogram of the BOXA1R-PCR products clustered with dice similarity coefficient and the UPGMA method of 36 AT strains from La Trinidad, Benguet and of three bean rhizobial species.

similarities with *R. etli* (97.6 % for RG-136 and 92.9% for RG-137). In terms of carbohydrate and amino acid profiles, RG-136 gave 71.5 % similarity with *R. tropici*, while RG-137 gave 72.7 % similarity with *R. etli*. When their minimal but discriminating phenotypic characters were investigated, RG-136 gave 100% similarity with *R. etli* while RG-137 gave 87.5% similarity with *R. etli*. Results of our identification of the most AT strain RG-136 were consistent with our phenotypic characterization of these two most AT strains, since the two most AT strains showed similar characteristics with *Rhizobium etli.*

Phylogenetic analyses

Representative *Rhizobium* isolates were submitted for 16S rRNA sequencing at the Laboratory of General Microbiology at the University of Bremen, in Bremen Germany. A phylogenetic tree was created and is shown in Fig 3. Based on this dendogram our most AT strains RG-136 (also coded as Buy2-10) and RG-137 (also coded as BalC-24) were clustered with *Rhizobium etli*. Another isolate RG-59 (also coded as BalC-30) was clustered with *Agrobacterium*. Isolate RG-59 was included in the phylogenetic analyses because it was one of the nine strains that nodulated well in the nodulation assays but nodulated more at pH 7.0 compared with pH 4.5 (Table 3), so we presumed that it maybe an acid-sensitive or non-AT strain, hence it was also included in our phenotypic characterization experiments for comparison with the most AT strains (Table 4).

DISCUSSION

In this study we describe the screening for acid tolerance in a population of rhizobia isolated from the nodules of *P. vulgaris* plants grown in slightly acidic soils (pH 5.0 to 6.0) of La Trinidad, Benguet, Philippines. Thirty six (36) presumptive AT isolates were initially gathered but only 9 or 25% of these initial AT strains were able to exhibit nodulating abilities at pH 4.5 and 7.0 in the laboratory condition. The rest of the strains either exhibited poor nodulating ability (less than 10 nodules/plant) or did not nodulate at all. It is possible that these initial AT strains were not genetically stable and might have lost their nodulating abilities. This was also observed by Mostasso et al. (2002) in their selection of rhizobial strains for inoculation of bean fields in Brazil. The physiological and genetic basis for acid tolerance in rhizobia has not been fully understood (Graham et al. 1994, Glenn et al, 1999). Most of the work done for acid tolerance on rhizobia has been with *Rhizobium tropici* CIAT899 (Vinuesa et al. 2003) and with *Sinorhizobium meliloti* WSM419 (*Rhizobium (sic) meliloli* WSM419) (Glenn et al. 1999). Both strains have successfully been used as inoculants in acid soils. A physiological trait shared by AT rhizobia is their ability to maintain a slightly alkaline intracellular pH (pHi) when cultivated on media buffered at moderate acidity, while in acid-sensitive strains the pHi decreases with the pH of the medium (Ricillo et al. 2000).

It is interesting to note that the two most AT strains screened in liquid media, RG-136 and RG-137, were isolated from Brgy Puguis (Table 1), that had the lowest soil pH of 5.19 among the isolation sites in La Trinidad. Dilworth et al.(2001) pointed out that the most logical place to obtain putatively AT root nodule bacterial strains will be from acid soils on which suitable legumes carry nodules. An example of this is the AT strain *Rhizobium meliloti* isolated from the acidic soils of Sardinia, Western Australia (Howieson & Ewing 1986).

Results of restriction enzyme analysis and BOXA1R-PCR fingerprinting analysis may suggest that the AT isolates from the beans of La Trinidad were genetically diverse. However, since this is the first study to describe the genetic diversity of the rhizobial strains from the acidic soils of La Trinidad, more studies are needed to assess the genetic and phenotypic diversity of *Phaseolus* rhizobia from other sites in La Trinidad. The result of the clustering of rhizobia based on BOXA1R-PCR fingerprinting patterns depended upon the region or geographic location from which the isolates were obtained. This observation was consistent with the findings of other authors (Bernal & Graham, 2001, Bernal et al. 2004, Tlusty et al. 2005).

The result that *Rhizobium etli* nodulates *Phaseolus vulgaris* grown in La Trinidad is veritable since other authors previously reported that *R. etli* has symbiotic relationship with *Phaseolus vulgaris.* The predominance of *R. etli* - like 16SrRNA genes in rhizobial populations associated with the common bean was also reported by other authors like Diouf et al. (2000) in West Africa, and Beyene et al. (2004) in Ethiopia. Moreover, Aguilar et al. (2004) investigated the distribution of *nodC* alleles in a worldwide collection of *R. etli* strains. They found that the common nodulation gene *nodC* was a useful molecular marker to characterize *R. etli* populations of diverse geographical origin. Their study demonstrated that *nodC* typing is a valuable indicator of co-evolution between rhizobia and wild beans in the Americas. The authors also pointed out that *R.etli* is found in regions where the common beans have been introduced as early as the 16th century, because the dissemination of microorganisms harbored by the plants and seeds during the process of export to new regions may have occurred as well. This could probably be the reason why *R. etli* exists in La Trinidad, Benguet, in the Philippines.

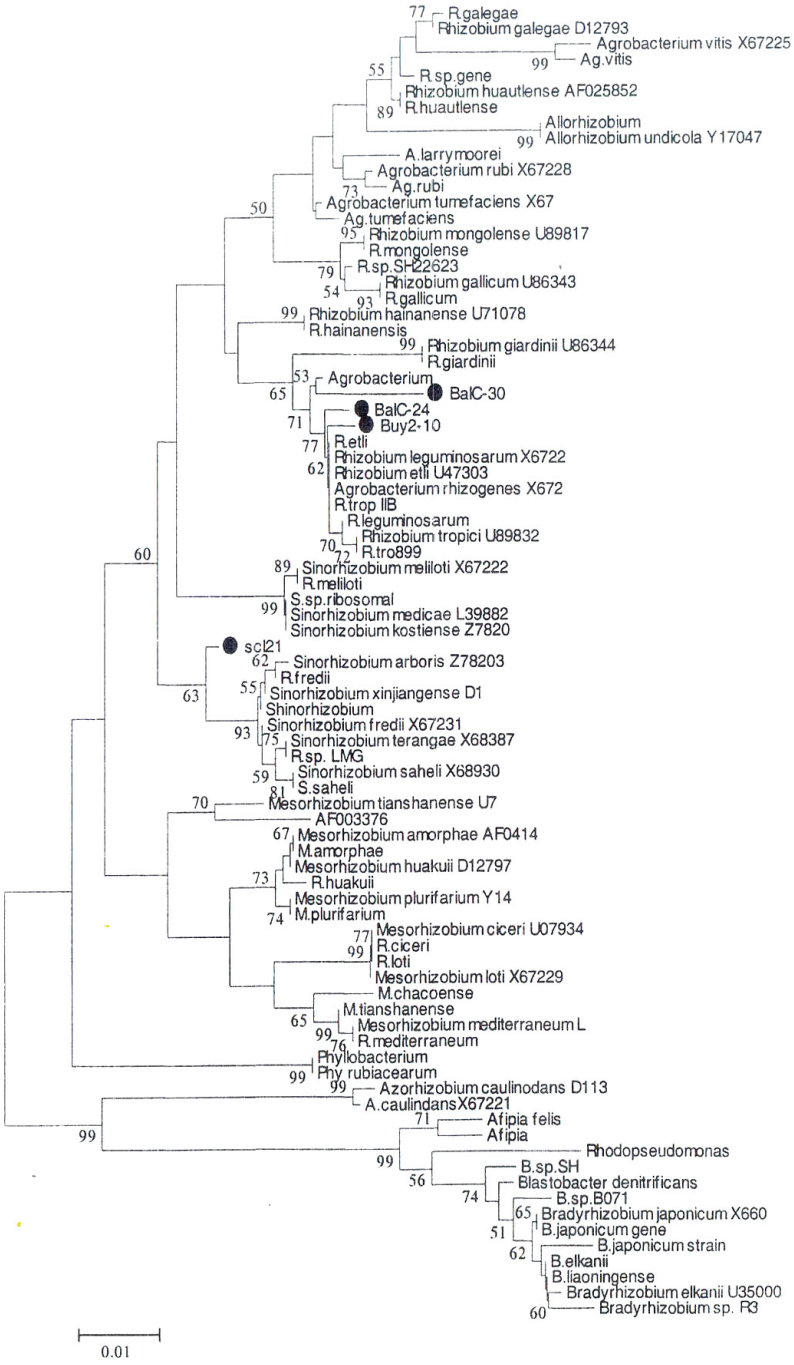

Figure 3. The phylogenetic position of the acid tolerant (AT) and non-AT strains based on their 16S rRNA sequence analysis. AT strains RG-136 (Buy2-10) and RG-137 (Bal C-24) were clustered with *Rhizobium etli* while the non-AT strain RG-59 (Bal C-30) was clustered with *Agrobacterium*.

The dendogram shown in Fig 3 indicates that our isolates RG136 (also coded as Buy2-10) and RG-137 (also coded as BalC-24) were clustered with *R. etli*. This result further confirms the relatedness of our AT isolate to *R. etli*. Another isolate, RG-59 (also coded as Bal-C30) was clustered with *Agrobacterium.* Chen et al. (2000) reported that native isolates resembling *Agrobacterium* spp. can be isolated from field grown nodules and establish an efficient symbiosis with soybean plants. Through the result of the phylogenetic analysis, we were also able

to demonstrate in this study that strains resembling *Agrobacterium* can be isolated from field grown bean nodules of La Trinidad, Benguet.

It is evident in this present study, based on the consistencies between phenotypic and genotypic as well as phylogenetic analyses and identification, that the AT rhizobial strain found in common bean rhizobia in La Trinidad Benguet is *R. etli. R. etli* has been reported to the predominant *Phaseolus vulgaris*- nodulating species in Mexico, Columbia and Argentina and it was also found in regions where common bean has been introduced such as Spain, France, Austria, Senegal, Egypt, Gambia and Tunisia. To our knowledge, this is the first study that reports the presence of *Rhizobium elti* strains isolated from the bean fields grown in the acidic soils of La Trinidad, Benguet here in the Philippines. In conclusion, since *R. etli* strain RG-136 isolated in this study was able to grow at pH 4.5 and showed better nodulating ability at pH 4.5 in the laboratory condition, it will be interesting to investigate further whether the strain could be developed as successful inoculant in acid soils.

ACKNOWLEDGEMENT

Rosemary Gutierrez would like to acknowledge the UNESCO-MIRCEN (Microbial Resource Center) Fellowship in Biotechnology and the Center for Bacterial Diversity and Identification at the University of Queensland, Brisbane, Australia headed by Dr. Lindsay Sly and Dr. Mark Fegan, wherein part of this research work was undertaken. This work was also part of a Ph.D. dissertation funded by the Philippine Council for Advanced Science and Technology Research and Development (PCASTRD), the Commission on Higher Education (CHED) and the Doctoral Studies Fund (DSF) of the University of the Philippines (UP). Heartfelt gratitude to Dr. Rosario Monsalud for her help in the nucleotide sequence analysis done at the Microbiology Laboratory, headed by Dr. Barbara Hurek at the University of Bremen, Bremen, Germany. We are grateful to Dr. Peter Graham of the Rhizobium Research Lab (RRL) of the University of Minnesota for providing some of the reference strains used in the study. Special thanks to Ms. Amelie Correces and Ms. Isabel Campus for their assistance in the laboratory experiments. Financial support extended by the University of the Philippines Baguio Ph.D. Incentive Grant is also acknowledged.

REFERENCES

AGUILAR OM, LOPEZ VM, RICILLO PM. 2001. The diversity of rhizobia nodulating beans in Northwest Argentina as a source of more efficient inoculant strains. J Biotechnol 91:181-188.

AGUILAR OM RIVA O, PELTZER E. 2004. Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports co-evolution in centers of host diversification. Proc Natl Acad Sci 101, (37): 13548--13553.

ANDRADE DS, MURPHY PJ, GILLER KE. 2002. The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. Appl Environ Microbiol 68: (8), 4025-4034.

AUSUBEL F., BRENT R, KINGSTON RE, MOORE DD, SEIDMAN J.D, SMITH JA, KRUHL K. 1990. Current protocols in molecular biology. Vol 1. John USA: Wiley and Sons, Inc. 672 p.

BERNAL GR, GRAHAM PH. 2001. Diversity in the rhizobia associated with *Phaseolus vulgaris* L. in Ecuador, and comparisons with Mexican bean rhizobia. Canadian Journal of Microbiology 47: 526-534.

BERNAL GR, TLUSTY B, ESTEVEZDE JC, VAN BERKUM P, GRAHAM PH. 2004 Characteristics of rhizobia nodulating beans in the central region of Minnesota. Can J Microbiol 50(12): 1023-1031.

BEYENNE D, KASSA S, AMOY F, ASSEFA F, GEBREMEDHIN T, VAN BERKUM P. 2004 Ethiopian soils harbor natural populations of rhizobia that form symbioses with the common bean (*Phaseolus vulgaris* L.) Arch Microbiol 181:129-136.

CHEN LS, FIIGEREDO A, PEDROSA FO, HUNGRIA M. 2000. Genetic characterization of soybean rhizobia from Paraguay. Appl Environ Microbiol 66, (11): 5099-5103.

DEL PAPA MF, BALAGUE LJ, SOWINSKI SC, WEGENER C, SEGUNDO E, ABARCA FM, TORO N, NIEHAUS K, PUHLER A, AGUILAR MO, DRETS GM, LAGARES A. 1999. Isolation and characterization of *Alfalfa*-nodulating rhizobia present in acid soils of Central Argentina and Uruguay. Appl Environ Microbiol 65, (4): 1420-1427.

DELFIN EF, PATERNO ES, TANDANG LL. 1999. Field estimation on nitrogen fixation of bush and pole snapbeans (*Phaseolus vulgaris* L.) using the ureide technique. Philipp J Crop Sci 24: 91-95.

DILWORTH MJ, HOWIESON JG, REEVE WG, TIWARI RP, GLENN AR. 2001. Acid tolerance in legume root

nodule bacteria and selecting for it. Aust J Exp Agric 41: 435-446.

DIOUF A, DE LAJUDIE P, NEYRA M., KERSTERS K, GILIS M, MARTINEZ-ROMERO E, GUEYE M. 2000. Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). Int J Syst Environ Microbiol 50:159-170.

GLENN AR, REEVE WG, TIWARI RP, DILWORTH MJ. 1999. Acid tolerance in root nodule bacteria. In: Bacterial responses to pH. Booth RI, ed. Chichester, England: John Wiley and Sons Ltd. p. 112-130.

GRAHAM, PH; VITERI, SE, MACKIE F; VARGAS AT, PALACIOS A.1982. Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res 5:121-128.

GRAHAM PH, DRAEGER KJ, FERREY ML, CONROY MJ, HAMMER BE, MARTINEZ E, AARONS SR, QUINTO C. 1994. Acid pH tolerance in strains of *Rhizobium, Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR 1899. Can J Microbiol 40: 198-207.

HOWIESON JG, EWING MA.1986. Acid tolerance in the *Rhizobium meliloti- Medicago* symbiosis. Aust J Agric Res 37: 55-64.

HOWIESON JG, EWING MA, D'ANTUONO MF. 1988. Selection for acid tolerance in *Rhizobium meliloti*. Plant Soil 105**,** 179-188.

HUNGRIA M, CAMO RJ, MENDES IC. 2003. Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. Biol Fertility Soils 39:88-93.

JAMES EK, REIS VM, OLIVARES FL, BALDANI JI, DABEREINER J. 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus* J Exp Bot 45: 757-766.

LANE DJ. 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt E, Goodfellow M, eds. New York, N.Y: John Wiley & Sons, Inc. p. 115-148.

MARTINEZ-ROMERO E. 2003. Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspective. Plant Soil 252: 11-23.

MOSTASSO L, MOSTASSO FL, DIAS BG, VARGAS MAT, HUNGRIA M. 2002. Selection of bean (*Phaseolus vulgaris* L) rhizobial strains for the Brazilian cerrados. Field Crops Res 73, 121-132.

RICILLO PM, MUGLIA CI, DE BRUIJN FJ, BOOTH IR, AGUILAR OM 2000. Gluthatione is involved in environmental stress responses in *Rhizobium tropici* including acid tolerance. Appl Environ Microbiol 182, (6): 748-1753.

ROUGHLEY RJ. 1970. The preparation and use of legume seed inoculants. Plant Soil. 32, 675-701.

SOMASEGARAN P, HOBEN HJ. 1994. Handbook for Rhizobia, Methods in Legume *Rhizobium* Technology New York Inc.: Springer-Verlag. 450 p.

STANIER RYN, PALLERONI NJ, DOUDOROFF M. 1966. The aerobic pseudomonads. a taxonomic study. J Gen Microbiol 43: 159-271.

TLUSTY B, VAN BERKUM P & GRAHAM PH. 2005. Characteristics of the rhizobia associated with Dalea spp. in the Ordway, Kellog-Weaver Dunes and Hayden prairies. Can J Microbiol 51 (1): 15-23.

VERSALOVIC J, SCHEIDER M, DE BRUIJN FJ, LUPSKI JR. 1994. Genomic fingerprinting of bacteria using repetitive sequence based polymerase chain reaction. Methods Cell Mol Biol 5, 25-40.

VINCENT JM. 1970. A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook no. 15. Oxford: Blackwell Scientific Publications. 164 p.

VINUESA P, SILKOW FN, BRAS CP, SPAINK HP, MARTINEZ-ROMERO E, & WERNER D. 2003. Genetic Analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol Plant Microbe Interact 16, (2): 159-168.

ZAHRAN HH. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63: (4), 968-989.