ISOLATION AND CHARACTERIZATION OF RHIZOBIA FROM ROOT NODULES OF SOYBEAN (GLYCINE MAX L. MERR.) GROWN IN FERRALSOLS OF DAKNONG AND DAKLAK PROVINCE, VIETNAM

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ABSTRACT
Total of 44 isolates were isolated from nodules of soybean grown on ferralsols of CuJut district (24 isolates) and Buonho town (20 isolates). The microsymbionts causing nodules on the roots of soybean (Glycine max L. Merr.) and 34/44 rhizobial isolates were analysed with 16S-23S intergenic spacer and PCR-RFLP for repeated sequence RSα a 1195-bp DNA fragment, indole acetic acid production and nitrogen fixing activity. Almost isolates produced IAA but they had the different nitrogen fixation capacity. All the strains were fast-growing soybean rhizobia (Sinorhizobium [Ensifer) fredii} except two isolates (CJ02 and CJ09) were slow-growing soybean rhizobia (they were identified with sequence RSα as molecular marker for Bradyrhizobium japonicum). The study also showed the low presence of soybean nodulating slow growers in ferralsols of western highland of Vietnam.

KEYWORDS: Bradyrhizobium, ferralsols, Sinorhizobium, soybean, western highland of Vietnam.

INTRODUCTION
Soybean [Glycine max (L.) Merr.], is an important protein and oil source and is one of the most important grain legumes in the world. Soybean, like other legumes, fixes atmospheric nitrogen in association with gram-negative soil bacteria of the genera Bradyrhizobium and
Sinorhizobium (Jordan, 1982; Scholla and Elkan, 1984). In 1982, the fast-growing rhizobial strains were also isolated from soybean nodules and from soil of People’s Republic of China, within the center of origin and diversity of this legume (Keyser et al., 1982). Later, fast-growing strains were isolated from other primary nd secondary centers of soybean origin (e.g., Xu and Ge, 1984; Dowdle and Bohlool, 1985; Young et al. 1988; Rodriguez-Navarro et al., 1996). The fast growers were classified as the new species Rhizobium fredii (Scholla and Elkan, 1984), later reclassified as Sinorhizobium fredii and Sinorhizobium xinjiangensis (Chen et al., 1988), and recently proposed to change to genus Ensifer (Young, 2003). Although it was originally thought that Sinorhizobium (Ensifer) fredii was specific for Asian soybean lines (Keyser et al., 1982; Devine, 1985).

Soybean was introduced in Vietnam for a long time, and it has become a crop which to produce many kinds of food for human everyday. According to Shurleff and Aoyaki (2010), Soybean products (soy sauce) are first ordered for use in Hanoi, Vietnam by Dutch East India Company officers in 1652; soybean cultivation is first reported in the Van-dai Loai-ngu (Encyclopedia of Vietnam), by Le Quy Don (discovered by Tran Van Lai 1993, p. 143) and soybean cultivation reported again (Jao de Loureiro 1790, in Latin). He calls it Dolichos Soja, Dau nanh, Hoam te1u (yellow bean) and he also mentions soy sauce and tofu (Tau hu). Vietnam also has small local production, but the country’s booming feed industry imported 600,000 tonnes of soybean meal in 2002. Vietnam is the only country in the region experiencing double-digit growth in grain demand. Soybean milk requirement of Vietnamese is more and more with 613 million litres (2014)[3rd country in the world drink soy-milk] and Vietnam imported over 1.2 million tonnes soybean seed. Soybean cultivation also introduced on red yellow latosol (ferralsols) in the begining of 1965-1970 with new cultivars from USDA at EakMat experimental station at Ban Me Thuot (Know, 1969). An area of soybean cultivation of two provinces (DakNong and DakLak) of western highland of Vietnam reduced from 20.000 ha (2000) to 8.000 ha (2014) with 15.000 tonnes soybean production (Trac, 2015)(http://daunanhdinhduonglanh.vn/).

The aim of this study were (i) isolation and selection good brady(rhizobia) isolates, (ii) analysis of characterization and identification of soybean brady(rhizobia)(slow-growing or fast-growing soybean rhizobia) from nodules of soybean grown on ferralsols of western highland of Vietnam by PCR 16S-23S IGS and PCR of RSα methods.
MATERIALS AND METHODS

**Sampling of nodules and isolation of brady(rhizobia) strains**

In the present study, root nodules were collected from soybean plants (at the flowering stage) grown in 25 sites in two provinces (DakNong and DakLak) mainly at CuJut district (DakNong) and BuonHo town (DakLak). DakNong and DakLak provinces are situated in the western highland of Vietnam, CuJut district is a district of DakNong which locates the north of DakNong (107°44’44” E and 12°40’56” N) and Buon Ho is a town of Daklak province, it locates the north of province (108°16’13” E and 12°51’16” N)(Figure 1).

**Isolation of brady(rhizobium)**

The rhizobia were isolated from nodules and purified with the standard protocol using yeast extract-mannitol agar (YMA) supplied with 0.02% congo red (Vincent, 1970). The nodules were surface-sterilized in 95% ethanol for 1 min, and then in 1% sodium hypochlorite for 50 min. After the nodules were washed in sterile water, each nodule was pricked by a sterile needle, streaked onto a YMA agar plate containing Congo red and incubated in the dark at 30°C until the appearance of single colonies, which were selected after 24 h of showing different morpholotypes and subcultured until the purity of cultures was confirmed (Marsudi et al. 1999). Pure cultures were stored in 20% glycerol at – 80°C. Cell shape and size were
observed under light microscope by taking a drop of bacterial culture suspension in saline. Gram’s reaction was performed according to Vincent (1970).

**Nodulation test**

The nodulation capacity of the strains on soybean was confirmed by inoculation tests as described previously (Vincent, 1970) with sterile black rice-hull ash (substitutive vermiculite). Leonard jar assemblies consist of plastic bottle [drinking water bottle 500mL], bottom of bottle was cut and up-down in another bottle which was cut at neck of bottle; above bottle was filled by sterile sand and mouth bottle was put with sterile cotton (connecting from above bottle to jar or under bottle) to irrigate mineral solution N-free (Winarno and Lie, 1979)(Figure 2).

![Figure 2 Leonard jar assemblies with sterile black rice-hull ash and sand](image)

Briefly, the *Glycine max* (cv. CuJut) seeds were surface sterilized with ethanol 70% in 5 min, washed with sterile water 5 times and then germinated on 0.8% agar-water plates in the dark at 30°C for 24 to 36 h. Two germinated seeds were placed in the drinking bottle 500-ml filled with sterile black rice-hull ash (Leonard jar model) and moistened with nutrient solution free-N (Winarno and Lie, 1979). Two days later, the seedlings were inoculated with each of the isolates (approx. 10⁶ CFU per bottle). The negative controls were inoculated with sterile water. All the plants were grown in a greenhouse with temperature and light control. Nodules were checked and harvested after 28 days. Effectiveness of the nodules was identified by observing the pink color within nodules and dark green color of leaves, especially nodules presented near tap-roots as good isolates together with their size and dry weight.
**Nitrogen fixation capacity**

The good isolates were chosen from “nodulation test” experiment, they were evaluated their effectiveness in Leonard jar assembly model (Krisnan and Puepphe, 1991). Leonard jar assemblies consisted of plastic bottle [drinking water bottle 500mL], bottom of bottle was cut and up-down in another bootle which was cut at neck of bottle; above bottle was filled by sterile sand and mouth bottle was put with sterile cotton (connecting from above bottle to jar or under bottle) to irrigate mineral solution N-free (Winarno and Lie, 1979). These systems were heat sterilized (70°C) in 48 hours before use. In order to select the isolates having high effectiveness, The experiment was designed with positive control (with N fertilizer) and negative control with four replications. Nodules were checked and harvested after 40 days. Effectiveness of the nodules was identified by observing the pink color within nodules and dark green color of leaves, especially nodules presented near tap-roots as good isolates together with their size and dry weight. Shoot and Root dry weight of each treatment were recorded at the harvesting stage.

**IAA production**

The qualitative detection of indole-3-acetic acid (IAA) production was carried out based on the colorimetric method (Gordon and Weber 1951). Precultures were grown in Burk’s N free (100 ml) without tryptophan in 250mL-flask at 30°C on a roller at 100 rpm and samples were taken from at 2, 4, 6, and 8 DAI, cell free supernatants were mixed 2:1 with Salkowki reagent (0.01 M FeCl₃ in 35% perchloric acid) and incubated in the dark for 20 min at RT. IAA-containing solutions were indicated by reddish color with an absorption peak at 530 nm on Genesys 10 uv Thermo Scientific spectrophotometer.

**PCR 16S-23S IGS rDNA sequence (Massol-Deya et al., 1995)**

The isolates were grown on TY medium (Beringer, 1974) on agar slants in 2 days, a loopful from each tube (each isolate) was aseptically suspended in a Eppendorf vial 1.5ml containing one sterile nano-pure water by vortexing, this suspension was washed with 1 ml NaCl 1M two times and 1 ml nano-pure water one time, adding 30-50 µl lysis buffere (NaOH 50 mM + 2.5% SDS)(Rodriguez-Navarro et al., 1996) and inoculated at 70°C in 2 hours, centifuge 12000 rpm in 2 minutes, adding 50 µl sterile nano-pure water into suspension and it was used as a template for PCR.

Reactions were performed in a Perkin-Elmer 9700 thermocycler, USA; A 50 µl reaction volume contained 25 pmol primer pHr and 25 pmol primer p23SRO1, 5 µl 10X PCR buffer
(0.5 M KCl, 0.1 Tris-HCl, pH=8.3), 1.5 µl 2.5 mM MgCl$_2$, 1.25 µl W1-detergent, 2.5 µl 5 mM dNTP, 32 µl sterile nano-pure water, 2.5 U of Taq DNA polymerase and 2.5 µl cell suspension as template. The following temperature profile was used for PCR amplification: 94°C 7 minutes for an initial denaturation, 35 cycles of denaturation at 94°C in 20 sec, annealing at 52°C for 20 sec and extension for 40 sec at 68°C and a final extension at 68°C for 7 minutes. Amplified 16S-23S IGS rDNA was examined by electrophoresis in 1.5% agarose NA gel with 5 µl aliquots of PCR produces, gels were stained with ethidium bromide and photographed under UV illumination. Electrophoresis was carried at 80 volt for 60 minutes with standard (6 by 8 cm).

**Amplication of RSα (Annapura et al., 2007)**

Total genomic DNA was isolated as described above. Amplication of RSα was carried out by PCR using a Perkin-Elmer 9700 thermocycler. The expected size of the amplification product was 1195 bp. Primers RSAL 1 (23 mers, 5’ AGC GGG CGC GGA TAG TTC TGT TG 3’) and RSAR 1 (23 mers, 5’ GGC TCG GCT CTG TCG TTG TAT GC 3’) were synthesized by Phu Sa Co (Vietnam). Amplification was carried out in a 50-µl reaction mixture containing: 150 ng genomic DNA as template, 5 µl of PCR buffer (with 15 mM MgCl$_2$), 2.5 µl of dNTP mix (2 mM), 50 ng of each primer, and 0.4 µl of Taq polymerase (3U l$^{-1}$). The cycling parameters used were initial denaturation at 95°C for 5 min, 20 cycles of denaturation for 40 s at 94°C, annealing at 60°C for 90 s, and extension at 74°C for 3 min, and 10 cycles of denaturation for 40 s at 94°C, annealing at 60°C for 90 s, and extension at 74°C for 6 min, and a final extension at 74°C for 15 min. Electrophoresis was carried at 80 volt for 60 minutes and gels were visualized under UV and photographed with Gel documentation system.

**Data analyses**

Data from nodule number/plant, dry weight of nodule, dry weight of shoot and IAA concentrations in media were analysed in completely randomized design with three replicates and Duncan test at P=0.01 or P=0.05 were used to differentiate between statistically different means using SPSS version 16.

**RESULTS AND DISCUSSION**

**Isolation of brady(rhizobium) and nodulation test**

Total of 44 isolates were isolated from nodules of soybean grown on ferralsols of CuJut district (24 isolates) and Buonho town (20 isolates). The isolates grew on YMA with Congo
red and their colonies were stained with red color clearly, and this result recognized rhizobia in comparison to other bacteria (Figure 3). Furthermore, two isolates (CJ02 and CJ09) grew very slow onto YMA and single colonies only appeared after 4 or 5 days incubation; especially their colonies had red color very dark (Figure 3a).

Besides that, these isolates were determined capacity of nodulation on soybean plants (Leonard jar assemblies) and they were also selected through nodule number/plant and dry weight (DW) of one nodule (Table 1).

Figure:3 The colonies of several isolates on YMA with congo red, the rhizobial isolates were identified through red colonies

CJ02 (CuJut) and BH18 isolates (Buonho) nodulated the highest nodule numbers on soybean plants (cv. CuJut) but the highest DW of nodule/plant of CJ10 (CuJut) and BH07 isolate (Buonho) and especially the BH07 isolate also had bigger nodules than others while CJ14 isolate had the biggest nodules in comparison to 23 isolates (CuJut)(Table 1). At Cujut, many isolates nodulated many nodules/plant and high DW of nodules as isolate: CJ02, CJ06, CJ08, CJ10 but the highest DW of one nodule was CJ14 isolate (the lowest nodule number/plant) however many isolates had high DW of one nodule as CJ03, CJ13 and CJ28, this results showed that these rhizobial isolates nodulated many nodules but small nodule size. At Buonho, 11/20 rhizobial isolates nodulated many nodules/plant but there were 4 isolates had high DW of nodules, especially BH07 isolate had the highest DW of one nodule but DW of one nodule of this isolate was nearly half (47.8%) of DW of nodule of CJ14 isolate.
Furthermore, two isolates (CJ02 and CJ09), originated from nodules of soybean grown on ferralsols of CuJut district, nodulated nodules early and near tap-root of soybean plants this showed that they had high effectiveness of biological nitrogen fixation because these rhizobia isolates supported the early nodulation and host soybean plant symbiosis while rhizobia originated from nodules of soybean grown on ferralsols of Buonho town which nodulated nodules slowly, nodules did not present near tap-root of soybean plant, they nodulated nodules on rootlets (far from tap-root)(Figure 4).

Table:1 Nodule number/plant, dry weight (DW) of nodule/plant and DW of one nodule of rhizobia isolates were isolated from nodules of soybean cultivated on ferralsols of CuJut district (DakNong province) and Buonho town (DakLak province) in Leonard jar assemblies with sterile black rice-hull ash

<table>
<thead>
<tr>
<th>Rhizobia isolate</th>
<th>Cu-Jut district</th>
<th>Buonho town</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number nodule /plant</td>
<td>DW of nodule /plant (mg)</td>
</tr>
<tr>
<td>Control</td>
<td>3.33 f</td>
<td>4.91 f</td>
</tr>
<tr>
<td>CJ01</td>
<td>20.01 cd</td>
<td>42.27 c</td>
</tr>
<tr>
<td>CJ02</td>
<td>39.67 a</td>
<td>64.27 b</td>
</tr>
<tr>
<td>CJ03</td>
<td>20.33 c</td>
<td>62.97 b</td>
</tr>
<tr>
<td>CJ04</td>
<td>22.67 c</td>
<td>38.71 cd</td>
</tr>
<tr>
<td>CJ05</td>
<td>18.67 d</td>
<td>29.57 d</td>
</tr>
<tr>
<td>CJ06</td>
<td>34.01 ab</td>
<td>46.37 c</td>
</tr>
<tr>
<td>CJ07</td>
<td>22.33 c</td>
<td>48.11 c</td>
</tr>
<tr>
<td>CJ08</td>
<td>33.01 ab</td>
<td>51.17 bc</td>
</tr>
<tr>
<td>CJ09</td>
<td>25.33 c</td>
<td>64.01 b</td>
</tr>
<tr>
<td>CJ10</td>
<td>34.67 ab</td>
<td>82.57 a</td>
</tr>
<tr>
<td>CJ11</td>
<td>12.67 e</td>
<td>21.87 d</td>
</tr>
<tr>
<td>CJ12</td>
<td>10.67 e</td>
<td>14.73 e</td>
</tr>
<tr>
<td>CJ13</td>
<td>11.33 e</td>
<td>31.47 cd</td>
</tr>
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<td>CJ14</td>
<td>7.01 ef</td>
<td>21.17 d</td>
</tr>
<tr>
<td>CJ15</td>
<td>13.01 e</td>
<td>20.51 e</td>
</tr>
<tr>
<td>CJ16</td>
<td>23.67 c</td>
<td>34.41 cd</td>
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<td>CJ18</td>
<td>27.67 bc</td>
<td>37.97 cd</td>
</tr>
<tr>
<td>CJ19</td>
<td>22.67 c</td>
<td>41.83 c</td>
</tr>
<tr>
<td>CJ20</td>
<td>21.01 cd</td>
<td>35.43 cd</td>
</tr>
<tr>
<td>CJ26</td>
<td>25.67 c</td>
<td>44.87 c</td>
</tr>
<tr>
<td>CJ27</td>
<td>11.01 e</td>
<td>23.27 de</td>
</tr>
<tr>
<td>CJ28</td>
<td>10.01 e</td>
<td>27.17 de</td>
</tr>
<tr>
<td>CJ29</td>
<td>19.67 cd</td>
<td>52.91 bc</td>
</tr>
<tr>
<td>C.V (%)</td>
<td>16.47</td>
<td>15.93</td>
</tr>
</tbody>
</table>

Means within a column followed by the same letter/s are not significantly different at p<0.01
From each site, 10 isolates were chosen to evaluate nitrogen fixation capacity through nodulation, DW of nodules/plant and DW of one nodule.

![Figure 4](image)

**Figure: 4 Early nodulation of two isolates (CJ02 and CJ09) and nodules presented near tap-root and on main root (left), nodules presented on rootlets (far from tap-root) of soybean roots by BH06 isolate (right)**

**Nitrogen fixation capacity**

**Table: 2 Effects of 24 rhizobial isolates on nodule number/plant, DW of nodule/plant and DW of one nodule (mg) on soybean plant (cv. Cujut) cultivated on Leonard jar with sand in 35 days**

<table>
<thead>
<tr>
<th>Rhizobia isolate</th>
<th>Cujut site</th>
<th>Buonho site</th>
<th>Cujut site</th>
<th>Buonho site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>DW of</td>
<td>Number</td>
<td>DW of</td>
</tr>
<tr>
<td></td>
<td>nodule</td>
<td>nodule</td>
<td>nodule</td>
<td>nodule</td>
</tr>
<tr>
<td></td>
<td>/plant</td>
<td>/plant (mg)</td>
<td>/plant (mg)</td>
<td>/plant (mg)</td>
</tr>
<tr>
<td>Control *</td>
<td>3.33 e</td>
<td>4.49 f</td>
<td>1.467 c</td>
<td>2.17 gh</td>
</tr>
<tr>
<td>NH₄NO₃ *</td>
<td>9.33 d</td>
<td>21.57 e</td>
<td>2.311 ab</td>
<td>NH₄NO₃ *</td>
</tr>
<tr>
<td>CJ01</td>
<td>21.67 b</td>
<td>47.11 bc</td>
<td>2.183 ab</td>
<td>BH03</td>
</tr>
<tr>
<td>CJ02</td>
<td>28.67 a</td>
<td>64.43 a</td>
<td>2.254 ab</td>
<td>BH05</td>
</tr>
<tr>
<td>CJ03</td>
<td>31.01 a</td>
<td>52.57 b</td>
<td>1.706 c</td>
<td>BH07</td>
</tr>
<tr>
<td>CJ04</td>
<td>18.67 bc</td>
<td>36.53 c</td>
<td>1.973 bc</td>
<td>BH10</td>
</tr>
<tr>
<td>CJ06</td>
<td>18.33 bc</td>
<td>47.31 bc</td>
<td>2.586 a</td>
<td>BH13</td>
</tr>
<tr>
<td>CJ07</td>
<td>15.33 c</td>
<td>28.77 d</td>
<td>1.928 b</td>
<td>BH14</td>
</tr>
<tr>
<td>CJ08</td>
<td>18.33 bc</td>
<td>31.50 cd</td>
<td>1.752 bc</td>
<td>BH15a</td>
</tr>
<tr>
<td>CJ09</td>
<td>21.01 b</td>
<td>42.20 bc</td>
<td>2.037 ab</td>
<td>BH17a</td>
</tr>
<tr>
<td>CJ10</td>
<td>28.33 a</td>
<td>50.63 b</td>
<td>1.789 bc</td>
<td>BH17b</td>
</tr>
<tr>
<td>CJ19</td>
<td>18.00 bc</td>
<td>37.77 c</td>
<td>2.114 ab</td>
<td>BH18</td>
</tr>
<tr>
<td>CJ26</td>
<td>19.67 bc</td>
<td>41.23 bc</td>
<td>2.135 ab</td>
<td>BH19</td>
</tr>
<tr>
<td>CJ29</td>
<td>19.33 bc</td>
<td>41.27 bc</td>
<td>2.143 ab</td>
<td>BH20</td>
</tr>
<tr>
<td>C.V (%)</td>
<td>13.15</td>
<td>6.68</td>
<td>12.92</td>
<td>C.V (%)</td>
</tr>
</tbody>
</table>
Means within a column followed by the same letter/s are not significantly different at p<0.01

The result from Table 2 showed that there were three isolates (CJ02, CJ03, CJ10) had the highest nodule number/plant, DW of nodules/plant and DW of one nodule from CuJut site, and at Buonho site, BH15a isolate had the highest nodule number/plant and DW of nodules/plant and two isolates (BH05 and BH14) had the highest DW of nodules/plant and they did not differ with BH15a isolate significantly.

Effect of biological nitrogen fixation was evaluated through DW of shoot, the result from Figure 5 showed that CJ09 isolate supported the highest DW of soybean shoot and CJ01 and CJ10 isolates also had the high DW of shoot and they also did not differ from CJ09 isolate and soybean plant applying N inorganic fertilizer (1 mM NH$_4$NO$_3$) in rhizobial isolates which were isolated from soybean nodules cultivating in CuJut site. Furthermore, many isolates (CJ02, CJ04, CJ06, CJ26 and CJ29) also supported DW of shoot equivalent as DW of shoot from treatment applying NH$_4$NO$_3$ and all rhizobial isolates had DW of shoot higher than control treatment. This results showed that all rhizobial isolates had different effectiveness on soybean plant and the rhizobial isolates had the highest effectiveness as CJ01, CJ02, CJ04, CJ06, CJ09, CJ10, CJ26 and CJ29 and their effectiveness did not differ from with N inorganic fertilizer treatment without inoculation.

![Figure 5. Effect of rhizobial isolates (from soybean nodule of CuJut site) and N inorganic fertilizer on DW of soybean shoot cultivated on Leonard jar assemblies](image)

C.V = 7.7%
Note: control: no rhizobial isolate inoculation, no fertilizer, $\text{NH}_4\text{NO}_3$: no rhizobial isolate inoculation and applied 1 mM $\text{NH}_4\text{NO}_3$

From Figure 6 showed that DW of shoot of N inorganic fertilizer (1 mM $\text{NH}_4\text{NO}_3$) was the highest but it did not differ from DW of shoot of BH03, BH14, BH15b, BH18 significantly however there were shoot DW of rhizobial isolates lower than control treatment as BH07 and BH10 isolates. This result showed that rhizobial isolates from soybean nodules in Buonho site had effectiveness lower than rhizobial isolates from Cujut site.

Means within a column followed by the same letter/s are not significantly different at $p<0.01$

**Figure 6** Effect of rhizobial isolates (from soybean nodule of Buonho site) and N inorganic fertilizer on DW of soybean shoot cultivated on Leonard jar assemblies

Note: control: no rhizobial isolate inoculation, no fertilizer, $\text{NH}_4\text{NO}_3$: no rhizobial isolate inoculation and applied 1 mM $\text{NH}_4\text{NO}_3$

The relationship between nodule number/plant with DW of nodule/plant closely very significantly ($p<0.01$) and DW of nodule/plant with DW of shoot also was closely ($p<0.01$) while nodule number/plant with DW of shoot correlated significantly ($<0.05$) with rhizobial isolates isolated from nodules of CuJut site (Table 3). While nodule number/plant did not relate with Shoot DW but it had a relationship closely with nodule DW ($p<0.01$) and the relationship between nodule DW with shoot DW was closely very significantly ($p<0.01$) from rhizobial isolates isolated from soybean nodules at Buonho site (Table 3). These results showed that nodule DW is an important factor which affect to shoot DW of soybean in comparison to nodule number/plant.
### Table 3. The relationship between Nodule number/plant and Nodule DW with Shoot DW.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cujut site</th>
<th>Buonho site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight</td>
<td>( r = 0.396^* )</td>
<td>( r = 0.037 ) ns</td>
</tr>
<tr>
<td>Nodule dry weight</td>
<td>( Y = 11.517X + 687.92 )</td>
<td>( Y = 2.598X + 723.65 )</td>
</tr>
<tr>
<td>Shoot dry weight</td>
<td>( r = 0.786^{**} )</td>
<td>( r = 0.467^{**} )</td>
</tr>
<tr>
<td>Nodule dry weight</td>
<td>( Y = 1.4359X + 12.529 )</td>
<td>( Y = 6.137 + 623.84 )</td>
</tr>
<tr>
<td>Shoot dry weight</td>
<td>( r = 0.466^{**} )</td>
<td>( r = 0.542^{**} )</td>
</tr>
<tr>
<td>Nodule dry weight</td>
<td>( Y = 7.424 + 613.33 )</td>
<td>( Y = 0.917 X + 11.157 )</td>
</tr>
</tbody>
</table>

Almost rhizobial isolates showed IAA production *in-vitro* (with tryptophan) except three isolates (CJ05, CJ11 and CJ28) synthesized IAA very low.

**PCR 16S-23S IGS**

16S-23S rDNA IGS region of 16/20 rhizobial representative isolates (Buonho site) was amplified with two suitable primers and the results showed that there was a big variation in 16S-23S IGS (Figure 7) with band size of approx from 1250 to 1600 bp and 18 rhizobial isolates (Cujut site) (Figure 8) with band size from 1150 to 1500 bp.

![Figure 7 Gel electrophoresis showing variability of PCR amplified 16S-23S rDNA IGS region from 16/20 soybean rhizobial isolates (Buonho site)](image)

*Note: M: Ladder 100 bp plus*

Figure:8 Gel electrophoresis showing variability of PCR amplified 16S-23S rDNA IGS region from 8/24 soybean rhizobial isolates (Cujut site)

Note: M = ladder  
1 = CJ28 (1500 bp), 2 = CJ14 (1500 bp), 3 = CJ15 (1450 bp), 4 = CJ26 (1250 bp), 5 = CJ09 (1250 bp), 6 = CJ01 (1250 bp), 7 = CJ03 (1250 bp), 8 = CJ04 (1250 bp)

With results from gel electrophoresis of PCR amplified 16S-23S rDNA IGS showed that the isolates had different size (table 4)

| Table:4 The isolates had different size on gel electrophoresis 16S-23S |
|------------------|------------------|------------------|------------------|------------------|
| Buonho site      | Cujut site       |
| No   | Bacterial name | Size (bp) | No   | Bacterial name | Size (bp) |
| 01   | BH3            | 1300      | 01   | CJ01           | 1250      |
| 02   | BH6            | 1250      | 02   | CJ03           | 1250      |
| 03   | BH7            | 1400      | 03   | CJ04           | 1250      |
| 04   | BH9            | 1300      | 04   | CJ05           | 1150      |
| 05   | BH10           | 1400      | 05   | CJ06           | 1200      |
| 06   | BH11           | 1600      | 06   | CJ07           | 1200      |
| 07   | BH13           | 1400      | 07   | CJ08           | 1200      |
| 08   | BH14           | 1400      | 08   | CJ09           | 1250      |
| 09   | BH15a          | 1300      | 09   | CJ10           | 1200      |
| 10   | BH15b          | 1350      | 10   | CJ13           | 1500      |
| 11   | BH16           | 1400      | 11   | CJ14           | 1500      |
| 12   | BH17a          | 1400      | 12   | CJ15           | 1450      |
| 13   | BH17b          | 1500      | 13   | CJ18           | 1200      |
| 14   | BH18           | 1400      | 14   | CJ20           | 1250      |
| 15   | BH19           | 1350      | 15   | CJ26           | 1250      |
| 16   | BH20           | 1450      | 16   | CJ27           | 1250      |
| 17   |                |           | 17   | CJ28           | 1500      |
| 18   |                |           | 18   | CJ29           | 1250      |

Amplication of RSa

There were two isolates made a band at 1195 bp in 20 rhizobial isolates (Figure 9) when all of them were tested with primers RSAL 1 and RSAR 1, this results demonstrated that two isolates are Bradyrhizobium japonicum.
Hungaria et al. (2008) studied on 30 fast-growing rhizobial strains isolated from nodules of Asian and modern soybean genotypes by rep-PCR and RAPD methods, the result showed that none of the strains was related to Sinorhizobium (Ensifer) fredii, whereas most were related to Rhizobium tropici (although they were unable to nodulate Phaseolus vulgaris) and to Rhizobium genomic species Q. One strain was related to Rhizobium sp. OR 191, while two others were closely related to Agrobacterium (Rhizobium) spp.; furthermore, symbiotic effectiveness with soybean was maintained in those strains; especially 5 strains were related to Bradyrhizobium japonicum and Bradyrhizobium elkanii. However when studied on rhizobia associated with soybean grown in the subtropical and tropical regions of Chiana, Man et al. (2008) reported that 252 rhizobial strains isolated from 5 eco-regions of China with four genomic groups including of Bradyrhizobium japonicum complex (including B. liaoningense, B. japonicum and a B. japonicum related genomic species) and Bradyrhizobium elkanii as major groups. Bradyrhizobium yuanmingense and Sinorhizobium fredii as the minor groups, were identified by ribosomal/housekeeping genes analyses. Analysis of diversity of 215 rhizobia strains isolated from root nodules of Glycine max grown in Hebei province, China; All the strains were classified into nine genospecies in the genera of Bradyrhizobium and Sinorhizobium (Ensifer) except one (Mesorhizobium). While Adhikari et al. (2012) recognized the soybean rhizobial isolates isolated from root nodule in Nepal were belonged to Bradyrhizobium (slow-growing rhizobia).

Several reports have appeared that demonstrate the presence of repeated sequences (RSα) in the genome of Rhizobium species (Kuykendal et al., 1988; Kuykendall et al., 1992; Wheatcroft and Laberge, 1991). Kaluza et al. (1985) discovered two different RSα in the Bradyrhizobium genome that process the structural characteristic on prokaryotic insertion
sequence elements. Hartmann et al. (1996) demonstrated the usefulness of RSα for *Bradyrhizobium* strain identification. Annapurma et al. (2007) demonstrated that RSα can be a useful molecular marker for slow-growing soybean rhizobia.

When Keyser et al. (1982) isolated a new group of fast-growing rhizobia, from nodules of domesticated soybeans and from the wild type *Glycine soja*, in the east-central provinces of China; These rhizobia, called *Sinorhizobium fredii* (Scholla and Elkan, 1984). In Vietnam, Saeki et al. (2005) isolated 120 isolates from soybean nodules which collected from the fields of Hanoi Agricultural University (HAU), Hanoi and Can Tho University (CTU), Can Tho, Vietnam and based on 16S rDNA and 16S-23S rDNA ITS region techniques to analyse phylogenetic tree of these isolates, the results showed that most of the isolates on *Bradyrhizobium japonicum* were extra-slow-growing and their ITS were similar to that of *Bradyrhizobium japonicum* USDA 135. Using PCR-ARDRA IGS 16S-23S, we isolated 223 isolates from nodules of cultivated soybean and wild legumes cultivars in the Mekong Delta, Vietnam and the results showed that most of them were fast-growing soybean rhizobia (*Sinorhizobium fredii*) and several rhizobial strains had high nodule number/plant (>100 nodules/plant) and high DW of shoot in comparison to control and reference strain (USDA 110)(Diep et al., 2004). Their results showed that most of the isolates were *Sinorhizobium fredii* except two isolates (CJ02 and CJ04) were *Bradyrhizobium japonicum* as Annapurna et al. (2007) previous described.

**CONCLUSION**

Total of 44 root nodule isolates of soybean rhizobia were isolated from two field sites (Cujut, DakNong province and Buonho town, DakLak province). They were characterized using PCR-16S-23S rDNA IGS and PCR for repeated sequence RSα a 1195 bp DNA fragment. The results showed that two isolates (CJ02 and CJ09) were *Bradyrhizobium japonicum* and others were *Sinorhizobium fredii*. Three strains *Sinorhizobium fredii* CJ01 and CJ10 and *Bradyrhizobium japonicum* CJ09 revealed promising candidates to produce a inoculant for soybean cultivation on ferralsols.

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