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Published online: 17 May 2012.

To cite this article: Khin Myat Soe, Ampan Bhromsiri, Dumnern Karladee & Takeo Yamakawa (2012) Effects of endophytic actinomycetes and Bradyrhizobium japonicum strains on growth, nodulation, nitrogen fixation and seed weight of different soybean varieties, Soil Science and Plant Nutrition, 58:3, 319-325, DOI: 10.1080/00380768.2012.682044

To link to this article: http://dx.doi.org/10.1080/00380768.2012.682044

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Effects of endophytic actinomycetes and \textit{Bradyrhizobium japonicum} strains on growth, nodulation, nitrogen fixation and seed weight of different soybean varieties

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Abstract

Effects of endophytic actinomycetes and two \textit{Bradyrhizobium japonicum} (Kirchner 1896) Jordan 1982 strains on growth, nodulation, nitrogen (N) fixation and seed weight of different soybean (\textit{Glycine max} L. Merr.) varieties from Myanmar (Hinthada), Thailand (SJ5) and Cambodia (DT84) were studied in pot experiments in Thailand. The six treatments consisted of an uninoculated control (T1), a single inoculation with endophytic actinomycetes (\textit{Streptomyces} sp. strain, P4) (T2), a single inoculation with \textit{Bradyrhizobium japonicum} strains, USDA110 (T3) or THA7 (T4) and a dual inoculation of P4 with USDA110 (T5) or THA7 (T6). Sterile soil was used for cultivation of all treatments under open field conditions, using tap water for irrigation. N$_2$ fixation activity of soybean was evaluated by the relative ureide method using xylem solute from the root bleeding sap at the early pod-fill stage. It was found that the single inoculation of P4 did not show significant effects on nodulation, N$_2$ fixation, shoot dry weight and seed weight at harvest of all soybeans compared with the uninoculated control. In the Hinthada soybean, single inoculation of USDA110 or THA7 increased seed weight while USDA110 improved N$_2$ fixation ($P<0.05$). Dual inoculation of USDA110 and P4 showed the highest shoot N accumulation and seed weight among treatments. In the SJ5 soybean, similar trends to Hinthada were shown, although the THA7 and P4 combination showed the highest shoot N accumulation and seed weight. On the other hand, in the DT84 soybean, there were no significant differences of N$_2$ fixation and seed weight between USDA110 and THA7 (both single and dual inoculation). In conclusion, dual inoculation of endophytic actinomycetes strain P4 may increase the nodulation and nitrogen fixation in some varieties; however, it is not effective for other varieties.

Key words: \textit{Bradyrhizobium japonicum}, endophytic actinomycetes, nitrogen fixation, nodulation, soybean.

INTRODUCTION

Soybean (\textit{Glycine max} L. Merr.) is a globally important oilseed crop and an important protein and oil source. It is a good meat and fish substitute because of its comparatively high protein content. According to a report by the FAO (2011), world soybean production in 2010 was 261.6 million metric tons. Soybean has been cultivated in Southeast Asia for at least 2000 years (Hymowitz 1970),
and is a traditional crop of Northern Thailand, where crops are grown in both wet and cool-dry (irrigated) seasons. In Myanmar, soybean is one of the important cash crops for domestic consumption and export (CSO 2006). It is the main crop after rice and it contributed the equivalent of 0.6% of GDP in 2005 in Cambodia, where 90% of total soybean production is exported to Vietnam and Thailand and only 10% is used for domestic consumption (MAFF 2009).

*Bradyrhizobium japonicum* (Kirchner 1896) Jordan 1982 is the nitrogen-fixing, root nodule symbiont of soybeans. Results from several studies have shown that both the bacterial and host genotypes influence the symbiotic interaction with soybean (Tripplett and Sadowsky 1992). The nitrogen (N) fixation from symbiosis between rhizobia and legume is a cheaper source of N and legume inoculant is usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crops (Zahran 1999). The fixed N from biological nitrogen fixation (BNF) in soybean production can be increased by the selection of effective *Bradyrhizobium japonicum* strains and the efficient soybean cultivar-bacterial strain pairs (Duong et al. 1984; Rennie and Dubetz 1984; Israel et al. 1986; Kucey et al. 1988).

Microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their existence are defined as endophytes (Wilson 1993; Saikkonen et al. 2004). Endophytic actinomycetes are attractive because their secondary metabolites might be promising sources of novel antibiotics and growth regulators of other organisms, as suggested by Matsukuma et al. (1994) and Okazaki et al. (1995). Tokala et al. (2002) stated that endophytic actinomycetes (*Streptomyces* sp.) influenced pea root nodulation by increasing root nodulating frequency. They also stated that the vigor of bacteroids within the nodules was also improved because endophytic actinomycetes enhanced nodular assimilation of iron and possibly other soil nutrients. Endophytic actinomycetes (*Streptomyces* sp., P4 strain) were isolated from pea (*Pisum sativum* L.) root at Kurima, Tsu-City, JAPAN on May 2001 by Thapanapongworakul (2003). Scanning electron microscopy revealed that P4 formed a spiral chain composed of more than 10 spores on aerial mycelia. Spores had a rough surface with a number of tiny spiny projections. The spore-bearing style was verticillate: true verticillates have main axes of wider diameter than branching hyphae, and have unformed spacing between verticiles. Based on these morphological characteristics, P4 was considered to belong to the genus *Streptomyces* (Thapanapongworakul 2003). When this strain was identified by full 16S rRNA sequencing, *Streptomyces* sp. P4 was mostly related to *Streptomyces griseoflavus* (Krainsky 1914) Waksman and Henrici 1948 (99.7% identical score) with the GenBank database accession number JN102356 (Tang-um and Niamsup 2012). Strain P4 was previously tested as an effective antagonistic microbe against various fungal diseases and its ability to infect other leguminous host plants such as navy bean (*Phaseolus vulgaris* L.), red kidney bean (*Phaseolus vulgaris* L.), adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), cowpea (*Vigna unguiculata* (L.) Walp.), soybean and Thai sweet pea (*Lathyrus odoratus* L.) (Thapanapongworakul 2003). In her study, significant stimulation effects of dual inoculation of P4 and *Bradyrhizobium japonicum* on N uptake of adzuki bean and Thai sweet pea as compare to bradyrhizobial inoculation were found. The experimental data reported by Thapanapongworakul (2003) are attractive for further testing with soybean in a pot experiment. The evaluation of N2 fixation by legumes is necessary for gaining an understanding of their contributions to the N econmies of agricultural and forestry systems (Herridge and Peoples 2002). According to Herridge and Peoples (2002), the seasonal percentage of plant N derived from N2 fixation can be estimated using the xylem solute (ureide) method from a single sampling of xylem sap during early pod-fill stage (R3.5), and this simplification of the protocol of the technique may encourage expanded use.

Thus the main objectives of the present study were (1) to evaluate the compatibility of endophytic actinomycetes (*Streptomyces* sp. strain, P4) with *Bradyrhizobium japonicum* strains, and (2) to determine the responses of soybean varieties to endophytic actinomycetes (*Streptomyces* sp. strain, P4) and *Bradyrhizobium japonicum* strains.

**MATERIALS AND METHODS**

**Plants**

Three soybean varieties from Myanmar (Hinthada), Thailand (SJ5) and Cambodia (DT84) were used for pot experiments. The Hinthada soybean variety was obtained from the Department of Agricultural Research, Ministry of Agriculture and Irrigation, Myanmar, while the SJ5 variety was obtained from the Department of Agronomy, Faculty of Agriculture, Chiang Mai University, Thailand, and the DT84 variety was obtained from the Camboid Agricultural Research and Development Institute, Ministry of Agriculture, Forestry and Fisheries, Cambodia.

**Endophytic actinomycetes and *Bradyrhizobium japonicum* strains**

The stock culture of *Streptomyces* sp. P4 was stored at 4°C in IMA-2 medium (Shimizu et al. 2000), and
cultured in its broth medium on a rotary shaker (100 rpm) at 30°C for 7 days. The *Bradyrhizobium japonicum* strain USDA110 was obtained from the USDA Rhizobium Culture Collection, U.S.A. THA7 was obtained from the Soil Microbiological Laboratory, Faculty of Agriculture, Chiang Mai University, Thailand. *Bradyrhizobium japonicum* strains USDA110 and THA7 were stored at 4°C on yeast extract mannitol agar slant (Vincent 1970) and were cultured in yeast extract mannitol broth on a rotary shaker (100 rpm) at 30°C for 7 days, after which a cell suspension of each *Bradyrhizobium japonicum* strain was prepared (>10⁹ colony forming unit (cfu) mL⁻¹). Fresh liquid culture of each microbe was inoculated to the seeds of each soybean variety germinated in a plastic seedling tray, using a sterile peat substrate. The rate of inoculation was 10⁶ cfu seed⁻¹ for both P4 and *Bradyrhizobium japonicum* strains.

### Plant cultivation

Three pot experiments were conducted outdoors at the Faculty of Agriculture, University of Chiang Mai, Thailand, during November 2008 to March 2009. The top soil (0—15 cm) of Kamphaeng Saen soil was collected from the Mae Hae Research and Training Center, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand (18.48 N, 98.59 E) and used. This soil is a member of the fine-silty mixed, semi-active isohyperthermic Typic Haplustalfs. It is silt loam with pH 7.6. The soil was sterilized by autoclaving at 120°C for 1 hour before using for soybean cultivation in order to eliminate the native population of root nodule bacteria in the soil. The autoclaved soil had 55 mg kg⁻¹ of ammonium-nitrogen (NH₄-N), 15 mg kg⁻¹ of nitrate-nitrogen (NO₃-N), 40 mg kg⁻¹ of available phosphorus (P), 87 mg kg⁻¹ of exchangeable potassium (K), 1447 mg kg⁻¹ of exchangeable calcium (Ca) and 101 mg kg⁻¹ of exchangeable magnesium (Mg). The soybean varieties Hintada, SJ5 and DT84 were grown in each pot experiment, using randomized complete block design (RCB) with three replications and six treatments. The treatments were: an uninoculated control treatment (Control) (T1), a single inoculation with endophytic actinomycetes (*Streptomyces* sp. strain, P4) (P4) (T2), two single inoculation treatments of *Bradyrhizobium japonicum* strains, (USDA110) (T3) or (THA7) (T4), and two dual inoculated treatments of P4 with USDA110 or THA7 (P4+USDA110) (T5) or (P4+THA7) (T6). The soybean plants were grown in plastic pots for 3 sets of growing stages, such as the six unfolded trifoliate leaves stage (V6 stage), the R3.5 stage and the maturing stage. Each plastic pot was filled with 3 kg of soil per pot (20.3 cm inches in diameter) for V6 stage and 12 kg of soil per pot (25.4—30.5 cm inches in diameter) for R3.5 stage and maturing stage, respectively. Tap water was ordinarily used for irrigation throughout soybean cultivation.

### Xylem sap collection and plant sampling

For xylem sap collection at the R3.5 stage, root bleeding sap samples of the plants from each pot were collected. To collect the root bleeding sap, the shoot just under the cotyledonic node of each plant was cut with a very sharp blade or cutter according to the method of Peoples *et al.* (1989). Sap samples were stabilized immediately after collection by mixing with an equal volume of ethanol in the collection tube, and then put into a freezer. After finishing all sap collection, all collected sap samples were kept at −15°C for long-term storage (Peoples *et al.* 1989).

For plant sampling at V6 and R3.5 stages, plants were harvested and separated into shoots, roots and nodules. Nodules and roots were carefully taken to remove the soil by sieving through a 1-mm sieve. Nodules, roots and shoots of the plants from each pot were oven-dried at 70°C for at least 72 h for dry weight determination. At harvesting time, seed weight was recorded from each plant.

### Ureide index measurement

The root bleeding sap samples were analyzed for amino-N (Herridge 1984), NO₃-N, NO₂-N (Cataldo *et al.* 1975) and ureide-N (Young and Conway 1942). Relative ureide index (RUI) of root bleeding sap at the R3.5 stage was calculated according to the following formula (Peoples *et al.* 1989):

\[
\text{Relative ureide index} (\%) = \left(4 \times \text{ureide} \right) \times 100 / \left(4 \times \text{ureide} + \text{amino acid} + \text{nitrate} \right)
\]

The total N accumulation of the shoot at R3.5 stage was calculated according to the formula as follows:

\[
\text{N accumulation of plant (g per plant)} = \% N \times \text{shoot dry wt.} \ (g) \times 100
\]

The %N was calculated by the colorimeter method.

### Statistical analysis

The data on dry weight of nodule and shoot, seed weight per plant, % RUI, shoot N accumulation and correlation coefficient (r) value were statistically analyzed and the means were compared by Tukey’s multiple comparison test at *P* < 0.05.
RESULTS

In the Hinthada soybean variety, the tested microbial inoculations had significant effects on nodule dry weight at V6 and R3.5 stages, RUI, shoot N accumulation and seed weight at harvest (Table 1). A single inoculation of P4 did not improve nodulation, shoot dry weight, seed weight and RUI compared to the uninoculated control treatment ($P<0.05$). The single inoculation of USDA110 and THA7 did not differ significantly from the single inoculation of P4 and the uninoculated control treatment for the effects on almost all studied parameters, but these two single inoculations of bradyrhizobia increased the seed weight of Hinthada soybean compared to the uninoculated control ($P<0.05$). Dual inoculation of P4 and each bradyrhizobial strain did not show any beneficial effects over single inoculation of bradyrhizobial treatment on all studied parameters ($P<0.05$), but the effect on nodule dry weight at V6 and R3.5 stages and shoot N accumulation of dual inoculation of P4 and USDA110 was considered the best treatment because it was the only treatment which could significantly increase those two studied parameters compared to the uninoculated control. A significant difference in the effect on $N_2$ fixation between the USDA110 and THA7 strains was not found, but USDA110 seemed to be more effective than THA7, for the reason that the single inoculation of USDA110 resulted in a significant increase of RUI as compared to the uninoculated control.

In the SJ5 Thai soybean variety the tested microbial inoculated treatments had significant effects on only two parameters, RUI and seed weight (Table 2). Inoculation of P4 and each bradyrhizobial strain did not have any effects on all studied parameters compared to the uninoculated control. Dual inoculation of each bradyrhizobial strain and P4 did not differ significantly from single inoculation trials. However, dual inoculated treatments seemed to be more effective because they could significantly increase RUI and seed weight over those of the uninoculated control treatment.

In DT84, the Cambodian soybean variety, the microbial inoculated treatments had significant effects on shoot dry weight at V6 and RUI (Table 3). Single inoculation of P4 did not have significant effects on both shoot dry weight and seed yields of DT84 soybean compared to the uninoculated control. There were no significant differences of RUI and seed weight of this soybean variety between USDA110 and THA7 in single and dual inoculations.

### Table 1
**Effects of microbial inoculations on nodule and shoot dry weight, relative ureide index (RUI), shoot nitrogen (N) accumulation at R3.5 and seed weight at harvest of Hinthada (Myanmar) soybean**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodule dry weight (g plant$^{-1}$)</th>
<th>Shoot dry weight (g plant$^{-1}$)</th>
<th>RUI (%)</th>
<th>N accumulation at R3.5 (mg N plant$^{-1}$)</th>
<th>Seed weight at harvest (g plant$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V6</td>
<td>R3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.17 b</td>
<td>0.26 b</td>
<td></td>
<td>53.97 c</td>
<td>152 b</td>
</tr>
<tr>
<td>P4</td>
<td>0.19 ab</td>
<td>0.29 ab</td>
<td>5.39 a</td>
<td>7.88 a</td>
<td>58.75 bc</td>
</tr>
<tr>
<td>USDA110</td>
<td>0.27 ab</td>
<td>0.42 ab</td>
<td>5.33 a</td>
<td>9.41 a</td>
<td>80.28 bc</td>
</tr>
<tr>
<td>THA7</td>
<td>0.24 ab</td>
<td>0.37 ab</td>
<td>4.49 a</td>
<td>9.28 a</td>
<td>71.73 abc</td>
</tr>
<tr>
<td>P4+USDA110</td>
<td>0.31 a</td>
<td>0.47 a</td>
<td>6.31 a</td>
<td>10.75 a</td>
<td>90.43 a</td>
</tr>
<tr>
<td>P4+THA7</td>
<td>0.30 ab</td>
<td>0.45 ab</td>
<td>5.34 a</td>
<td>9.95 a</td>
<td>88.87 a</td>
</tr>
</tbody>
</table>

Means in each column followed by different letters differed significantly at $P<0.05$ (Tukey’s test), V6 means V6 growth stage and R3.5 means R3.5 growth stage.

### Table 2
**Effects of microbial inoculations on nodule and shoot dry weight, relative ureide index (RUI), shoot nitrogen (N) accumulation at R3.5 and seed weight at harvest of SJ5 (Thailand) soybean**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodule dry weight (g plant$^{-1}$)</th>
<th>Shoot dry weight (g plant$^{-1}$)</th>
<th>RUI (%)</th>
<th>N accumulation at R3.5 (mg N plant$^{-1}$)</th>
<th>Seed weight at harvest (g plant$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V6</td>
<td>R3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.22 a</td>
<td>0.33 a</td>
<td></td>
<td>48.69 b</td>
<td>107 a</td>
</tr>
<tr>
<td>P4</td>
<td>0.24 a</td>
<td>0.34 a</td>
<td>5.01 a</td>
<td>7.26 a</td>
<td>55.12 ab</td>
</tr>
<tr>
<td>USDA110</td>
<td>0.35 a</td>
<td>0.37 a</td>
<td>5.24 a</td>
<td>11.38 a</td>
<td>71.82 ab</td>
</tr>
<tr>
<td>THA7</td>
<td>0.36 a</td>
<td>0.53 a</td>
<td>6.46 a</td>
<td>12.21 a</td>
<td>73.10 ab</td>
</tr>
<tr>
<td>P4+USDA110</td>
<td>0.35 a</td>
<td>0.62 a</td>
<td>5.79 a</td>
<td>12.73 a</td>
<td>79.53 a</td>
</tr>
<tr>
<td>P4+THA7</td>
<td>0.42 a</td>
<td>0.63 a</td>
<td>7.10 a</td>
<td>13.27 a</td>
<td>80.82 a</td>
</tr>
</tbody>
</table>

Means in each column followed by different letters differed significantly at $P<0.05$ (Tukey’s test), V6 means V6 growth stage and R3.5 means R3.5 growth stage.
inoculation. However, single inoculation of USDA110 resulted in significant increase of shoot dry weight and N$_2$ fixation of DT84 soybean. Single inoculation of THA7 increased N$_2$ fixation ($P < 0.05$) compared to the uninoculated control while less stimulative effects were observed in dual inoculated treatments.

Relation between RUI and seed weight

In all tested soybean varieties, significant correlation ($P < 0.05$) between RUI and seed weight at harvest was observed with correlation coefficients ($r$) of 0.68, 0.77 and 0.63 for Hinthada, SJ5 and DT84 soybean varieties respectively (Fig. 1).

**DISCUSSION**

According to Wani et al. (1995), within grain legume species, genotypic variability affected nodule number or nitrogenase activity. In this study single inoculation of the examined bradyrhizobial strain resulted in significant improvement of N$_2$ fixation of Hinthada and DT84
soybean varieties while in SJ5 soybean only a positive trend was observed which supported the report of Wani et al. (1995).

From the study of Tokala et al. (2002), pea root colonization of endophytic actinomycetes (Streptomyces lydicas WYEC 108) resulted in increasing root nodulation frequency, possibly at the level of infection by Rhizobium sp. In this study, dual inoculation of P4+USDA110 significantly improved nodule dry weight of Hinthada soybean, which was in accordance with the findings of Tokala et al. (2002). The existence of the examined P4 in the tissue of soybean plant was not investigated in this study, but the ability of this P4 to colonize the soybean host plant was previously tested by Thapanapongworakul (2003). Tokala et al. (2002) reported that when endophytic actinomycetes (Streptomyces lydicas WYEC 108) colonized pea roots the average size of the nodules that formed were increased. The vigor of bacteroids within the nodules was also improved because endophytic actinomycetes enhanced nodule assimilation of iron and possibly other soil nutrients. In this study, single inoculation of the selected P4 and USDA110 or THA7 bradyrhizobial strains significantly improved N2 fixation of Hinthada and SJ5 soybean varieties compared with the uninoculated control, while single inoculation of each bradyrhizobial strain showed only a positive trend. None of the mechanisms as stated by Tokala et al. (2002) was tested in our study, but such mechanisms could occur in the case that synergistic effects of dual inoculation of P4 and each of the tested bradyrhizobial strain were observed.

Yield is more directly related to mineral N assimilation in the first stage of the reproductive growth period and to high N2 fixation rates at the R6 stage, according to Fabre and Planchon (2000). Their finding confirmed the importance of symbiotic N2 fixation in yield and seed protein content of soybean. In our study, significant correlation between N2 fixation at the R3.5 stage and seed weight was also observed in each soybean variety which supported Fabre and Planchon (2000) and agreed with the findings of Meghvansi et al. (2010).

Milic et al. (2002) reported that there was variability in performance of different bradyrhizobial strains in dry matter mass and N content in the nodules of the soybean varieties. Such variations may be attributed to the variations in the genomic constitutions of the host or bacteria, or both, which control symbiosis, or there might be more than one affinity group within the legume rhizobia leading to such variation. According to Okereke et al. (2001) the inoculation response of Bradyrhizobium in different soybean cultivars was cultivar-specific and site-specific. In the present study, variation among the three tested soybean varieties for their responses to bradyrhizobial strain was also observed.

Conclusively, in this experiment, synergistic effects of P4 coinoculation with Bradyrhizobium strains were found in Myanmar and Thailand soybean varieties. These positive interactions were observed under the proper environmental conditions, and when the proper varieties and proper nodulated bacteria were used with strain P4. Our experimental results confirmed the finding of Akarapisan et al. (2008) and Soe (2009) that P4 is one of the effective endophytic actinomycetes which can be used in combination with selective root nodule bacterial strains for production of leguminous crops of economic importance as biological means. In future, the infection behavior and parts of promoting effects by P4 will be investigated using green fluorescent protein under a fluorescence microscope. We will also study the acetylene reduction activity (ARA) of the plant which is caused by P4.

ACKNOWLEDGMENTS

We thank the Thailand International Development Cooperation Agency (TICA), Royal Thailand Government, for granting a scholarship in this research.

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