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# RHIZOBACTERIA ACTS AS BIOENHANCER FOR GROWTH OF SOYBEAN (*GLYCINE MAX* (L.) MERR.) UNDER GROWTH CHAMBER CONDITION

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## Abstract

Inoculations of plant growth promoting rhizobacteria (PGPR) have been shown to produce beneficial effects through growth stimulation in legumes. An attempt has been made to use rhizobacteria in soybean. Two experiments were conducted to observe the effects of PGPR inoculation on root stimulation and colonization of soybean seedlings. The PGPR strains (UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14) were evaluated for their $N_2$ fixing capacities in association with soybean roots by acetylene reduction assay (ARA) and IAA production. The results showed that roots of soybean inoculated with indigenous PGPRs isolates of UPMB10 and UPMB12 fixed $N_2$ and produced higher ARA and IAA values, consequently, improved the bioenhancing activity by increasing root growth, nodulation and nitrogen percentage.

## Introduction

Nitrogen (N) is the key nutrient required for plant growth (Muthukumarasamy *et al.* 2002). Most of the Malaysian soils are deficient in available N because of high temperature, rainfall and leaching nutrient. Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots, increase nutrient uptake and enhance plant growth by a wide variety of mechanisms (Pareek *et al.* 2002).

Five indigenous and nitrogen fixer isolates of PGPR designated as *Bacillus* sp. strain UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14 were successfully isolated and characterized from oil palm roots in Selangor soils. Isolates of PGPR induced production of plant phytohormones (IAA), phosphate solubilization and biological $N_2$-fixation (nitrogenase enzyme) to enhance plant growth. Some of the isolates previously tested for dissolving deficient of N in Malaysian soil with rice and banana (Mia *et al.* 2012, Tan *et al*. 2014), but it is for the first time they were tested with soybean plant in this area. Soybean (*Glycine max* (L.) Merr.) is the most important staple food; and chemical fertilizers are the most important input required for soybean cultivation (Sciarappa 2005). In order to make its cultivation sustainable and less dependent on chemical fertilizers in Malaysian soil, it is important to use PGPR that could contribute to the improvement of soybean growth. For that reason, an experiment was conducted with indigenous isolates of PGPRs viz. UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14 as biofertilizer to identify the best performance on root growth promotion, colonization pattern of PGPRs, nodulation and growth of soybean seedlings.

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**Material and Methods**

Three experiments were conducted in the laboratory and growth chamber of Soil Microbiology Laboratory, Department of Land Management and glasshouse of Universiti Putra Malaysia (UPM). Laboratory experiment was conducted to evaluate the viability and activity of all five indigenous PGPR isolates (UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14), isolated from oil palm roots. The quantitative measurement of $N_2$-fixation rate of the selected bacterial strains was done using Acetylene Reduction Assay (ARA) method according to Hardy *et al.* (1968) and Somasegaran and Hoben (1985).

Fully grown bacterial cultures were inoculated in 100 ml Tryptic Soy Broth (TSB) and shaken on an orbital shaker for 24 hrs. One ml of bacterial culture was transferred into new 100 ml TSB with the addition of 5 ml L-Tryptophan as the precursor of indole-3-acetic acid. 1.5 ml of bacterial culture at was transferred into sterile Eppendorf tube and centrifuged at 7000 rpm for 7 minutes. The supernatant (1 ml) was mixed with 2 ml of Salkowsky reagent (2% of 0.5 M FeCl3 in 35% perchloric acid) according to the method of Gordon and Weber (1951).

The isolated culture was grown on Pikoskaya medium to determine the ability to solubilize phosphate (Pikovskaya 1948). The ability to solubilize phosphate was observed by the formation of a halo zone around the colony (10 µl inoculums, $1\times10^8$ CFU ml) after 24 hrs of incubation. (Sitepu *et al.* 2007). Chrome Azurol S (CAS) agar was prepared following Schwyn and Neilands (1987) to detect the siderophore production by bacteria. Carboxy Methtl Cellulose (CMC) agar plates were prepared by screening for cellulose enzyme production according to the method by Kasana *et al.* 2008. Pectinase agar plates were prepared to screen for pectinase enzyme production, according to the method by Yogesh *et al.* (2009). Growth chamber experiment carried out to testing viability and activity of PGPR isolates on soil media and their effects on root distribution and growth of soybean seedling.

The five PGPR strains used in this experiment were cultured in Tryptic Soy Broth (TSB) in 250 ml flasks shaken at 250 rpm at room temperature (21 - 23$^o$C). At loge phase (2 days for PGPR), each of the PGPR strains was adjusted with distilled water to an $O.D._{420}$ value giving a cell density of $10^8$ cell/ml. Before inoculation PGPR strains were incubated for at least half an hour at room temperature without shaking. The inocula was then cooled and applied by pipette to the soybean seeds.

Soybean seeds were obtained from the Laboratory of seed technology, Crop Science Department, Faculty of Agriculture, UPM. Pots were filled with 1 kg soil samples of clay loam (pH 5.5 - 6) that was air dried and sieved. Moreover, aqueous nutrient solution containing complete formulation of nutrient (Hoagland and Aronon 1950) was applied at 2 ml twice per day to each pot with a pipette for feeding plants during 5 weeks. All implements used in this experiment were sterilized with alcohol before placed in autoclave.

For root measurements (root length, root surface area, average root diameter, root weight and root colonization) a device Win RHIZO Pro 2007 (Regent Instrument Inc Company) was used and root colonization was calculated by total plate count (TPC) technique (Somasegaran and Hoben 1985). Root dry weight all samples weighted by digital of balance after oven drying at 70ºC for at least 48 hrs.

The roots and rooting medium (rhizosphere) samples were collected 35 DAI for enumerating of bacteria in rhizosphere. The number of PGPR cells in the rooting medium was determined by transferring 1 g of the rooting medium into a 100 ml Erlenmeyer flask, containing 99 ml of sterile distilled water.

All nodules separated from the roots of each treatment and counted after 6 seedlings in each replication were uprooted at the end of seedling stage (35 DAI). Then nodule dry weight (NDW)

was taken. Nitrogen percentage in nodules and shoots were determined by device named TruMac CNS/NS Determinator. Product of LECO, USA.

The collected data were analyzed statistically using the Statistical Analysis System (SAS Institute Inc. 2007). The data were computed by Complete Randomized Design (CRD). Following the ANOVA, differences among treatment means were determined using DMRT comparison method (whenever applicable) at 5% level of significance.

## Results and Discussion

Performance of five isolates of plant growth promoting rhizobacteria were evaluated in laboratory and growth chamber experiments for $N_2$-fixing activity, IAA production, phosphate solubilization, siderophore production, hydrolyzing enzyme production, root traits, nodulation and nitrogen percentage. The Acetylene Reduction Assay (ARA) is an indirect method to quantify biological nitrogen fixation (BNF) rate measured in the experiment. The results indicated that the ethylene produced by native isolates UPMB10 and UPMB12 were higher than UPMB14, UPMB13 and UPMB11 by descending manner. Katupitiya *et al.* (1995) reported that plant inoculated with *Azospirillum* (Sp7) which produced only 2.3 nmol $C_2H_4$ per plant/hr. This is also true when compared with diazotroph isolates from rice by Naher *et al.* (2009) which ranges from $6.1 \times 10^{-8}$ to $1.2 \times 10^{-3}$ nmol $C_2H_4$ per cfu/hr. In addition, Tan *et al*. (2014) reported that PGPR and rhizobial isolates from rice which ranges from 0.683 to 23.681 nmol $C_2H_4$ per ml/hr.

**Table 1. $N_2$-fixation, IAA, pectinase, cellulase and siderophore production and, phosphate solubilization of PGPR isolates.**

| Isolates | ARA (nmol/ $C_2H_4$ ml/ hr) | IAA production (µg/ml) | Pectinase production | Cellulase production | Siderophore production | Phosphate solubilization index |
|---|---|---|---|---|---|---|
| Control | - | - | - | - | - | - |
| UPMB10 | 19.90 ± 1.21a | 21.08 ± 0.12a | +++ | +++ | ++ | 2.14 ± 0.03a |
| UPMB11 | 15.36 ± 1.62d | 13.00 ± 0.92e | - | - | + | 1.5 ± 0.17e |
| UPMB12 | 19.42 ± 1.88a | 18.57 ± 1.99b | - | ++ | + | 2.02 ± 0.09b |
| UPMB13 | 17.36 ± 0.75c | 15.07 ± 0.82d | - | - | + | 1.70 ± 0.20c |
| UPMB14 | 18.66 ± 0.22b | 16.66 ± 0.78c | - | - | + | 1.63 ± 0.15d |

Values with the same letter(s) within a column are not significantly different at $p \leq 0.05$; + = minimum growth, ++ = Goodmoderate growth, +++ = maximum growth, - = No growth.

Consequently, soybean seedling inoculated with UPMB10 and UPMB12 had higher root length, root surface area, root volume and root dry weight, nodule number and dry weight, N% in nodule and shoot, plant height, leaf area and plant biomass compared to others because mentioned isolates had larger effect on $N_2$-fixing activity and therefore, increasing N absorption from soil. The IAA production by bacterial isolates in this study was almost similar with selected isolates of *Azotobacter*, *Fluorescent pseduomonas* and *Bacillus* from rhizospheric soils of different crops, namely mustard, barseem, wheat, sugarcane, brinjal, onion, cauliflower, cabbage and root nodules of chickpea (Battacharjee *et al.* 2012) and from rice soil diazotropic isolates (Naher *et al.* 2009) which ranged from 7.03 - 22.02, 3.56 - 24.32 and 32 - 69 µg/ml, respectively, when using the same concentration of tryptophan as the precursor (1 ml/20 ml broth). Results demonstrated that all native isolates UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14 had ability to siderophore production while, isolate UPMB10 recorded strongly and positively production of siderophore compared to others (Table 1). In parallel, plant growth of soybean inoculated with

UPMB10 enhanced more than other isolates because UPMB10 by producing highest siderophore caused increasing the availability of iron near the roots for plant uptake (Alexander and Zuberer 1991).

**Table 2. Root characteristics and population of bacteria in soil of soybean inoculated with different PGPR (UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14).**

| Treatment | Root length (cm) | Root diameter (mm) | Root volume ($cm^3$) | Root surface area ($cm^2$) | Root dry weight (g) | Root colonization (CFU/g root) | Population of bacteria (CFU/g soil) |
|---|---|---|---|---|---|---|---|
| Control | 35.36±0.91$^{d}$ | 0.95±0.46$^{d}$ | 0.47±0.37$^{c}$ | 15.55±1.18$^{f}$ | 0.31±0.031$^{e}$ | 7.80±1.69$^{d}$ | 6.57±1.84$^{f}$ |
| UPMB10 | 87.41±1.23$^{a}$ | 3.78±0.47$^{a}$ | 4.34±1.26$^{a}$ | 78.37±0.046$^{a}$ | 1.80±0.015$^{a}$ | 8.84±1.49$^{a}$ | 7.54±0.84$^{a}$ |
| UPMB11 | 50.82±0.55$^{c}$ | 1.52±1.005$^{c}$ | 1.45±0.049$^{b}$ | 28.25±0.61$^{e}$ | 0.49±1.01$^{d}$ | 8.36±0.91$^{c}$ | 6.89±0.25$^{e}$ |
| UPMB12 | 72.00±0.29$^{b}$ | 2.66±1.93$^{a}$ | 3.71±0.84$^{a}$ | 59.51±1.76$^{b}$ | 1.44±1.06$^{b}$ | 8.74±0.91$^{a}$ | 7.37±0.54$^{b}$ |
| UPMB13 | 53.52±1.85$^{c}$ | 1.96±0.35$^{b}$ | 1.76±0.028$^{b}$ | 34.93±0.14$^{d}$ | 0.55±2.05$^{d}$ | 8.47±1.06$^{cb}$ | 7.08±1.67$^{d}$ |
| UPMB14 | 61.16±0.24$^{cb}$ | 2.13±0.03$^{b}$ | 1.93±0.015$^{b}$ | 41.54±0.009$^{c}$ | 0.80±0.26$^{c}$ | 8.56±0.44$^{b}$ | 7.24±0.52$^{c}$ |

Analyses are means of five replicates measurements ± standard deviation. Means within a column not sharing a common letter are significantly different at $p < 0.01$.

Results of this work have demonstrated that soybean inoculated with UPMB10 and UPMB12 as a PGPR had higher value for parameters such as root average diameter, root volume, root surface area, root dry weight, root colonization and population of bacteria in soil (Table 2). Hence, the inoculation process stimulated the root growth and development and there was an increase in root growth of PGPR-inoculated plants. This observation on increased root growth is supported by other researchers (Levanony and Bashan 1989, Bashan and Holguin 1997). Kouas *et al.* (2009) reported that PGPR stimulated root growth and dry weight in common bean cultivars.

**Table 3. Nodule dry weight, nodule number and nitrogen percentage of soybean inoculated with PGPR (UPMB10, UPMB11, UPMB12, UPMB13 and UPMB14).**

| Treatment | Nodule dry weight (g) | Nodule No. | N% in nodule | N% in leaf |
|---|---|---|---|---|
| Control | 0.24 ± 0.006$^{e}$ | 4.20 ± 1.65$^{f}$ | 4.39 ± 1.83$^{c}$ | 3.99 ± 0.45$^{e}$ |
| UPMB10 | 1.72 ± 0.65$^{a}$ | 17.8 ± 0.57$^{a}$ | 7.64 ± 7.4$^{a}$ | 6.59 ± 0.87$^{a}$ |
| UPMB11 | 0.43 ± 0.009$^{d}$ | 8.00 ± 0.51$^{e}$ | 6.88 ± 1.13$^{c}$ | 5.53 ± 2.26$^{d}$ |
| UPMB12 | 1.50 ± 0.41$^{b}$ | 14.20 ± 0.97$^{b}$ | 7.59 ± 0.92$^{a}$ | 6.30 ± 1.05$^{b}$ |
| UPMB13 | 0.55 ± 0.002$^{d}$ | 9.80 ± 1.49$^{d}$ | 6.83 ± 0.87$^{b}$ | 5.21 ± 0.85$^{c}$ |
| UPMB14 | 0.80 ± 0.12$^{c}$ | 11.80 ± 1.19$^{c}$ | 6.79 ± 2.26$^{b}$ | 5.77 ± 2.11$^{c}$ |

All analyses are means of five replicates measurements ± standard deviation. Means within not sharing a common letter are significantly different at $p \leq 0.01$.

Based on the results of the present study, significant differences was found for nodule number, dry weight and nitrogen percentage in nodules and shoot measured on all soybean inoculated with five isolates of PGPR (Table 3). Soybean inoculated with UPMB10 and UPMB12 increased nodules number, nodule dry weight, N% in nodule and shoot compared to soybean inoculated with UPMB14, UPMB13, UPMB 11 and control.

On the basis of the present findings, it could be concluded that UPMB10 and UPMB12 augmented the $N_2$-fixation efficiency and IAA production of soybean caused increased total N uptake, nodule number, dry weight, N% in nodules and shoots of soybean.

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