

Potency of Endophyte Bacterium Isolated from *Shorea selanica* on Producing IAA Hormone and Supporting the Growth of Soybean

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Abstract

Growth of soybean plants was supported by the presence of nitrogen fixing bacteria. Besides nitrogen, other elements such as phosphate, potassium and growth hormones are also required. Endophytic bacteria associated with *Shorea selanica* were isolated and tested for their ability to produce indole acetic acid (IAA) hormone and exhibit stress tolerance. Colorimetric analysis showed that isolate SSBt2 produced the highest IAA (43.01 µg/ml) in culture supplemented with L-tryptophan. Isolate SSBt2 grew well in the some stress tests, except on heat and oxidative stress. The highest IAA producing strain was selected for determining its capability and compatibility to support the growth of soybean plants in glass house experiment. The results indicated that the endophytic bacteria isolated from *S. selanica* are compatible to support the growth of soybean. SSBt2 was identified as *Enterobacter hormaechei* based on 16S rRNA gene analysis.

Keywords: endophytes bacteria, Indole Acetic Acid, soybean, stress test

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Introduction

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant without harming the host plant. Bacterial endophytes can be isolated from surface-disinfected plant tissue or extracted from internal tissue (Zinniel *et al.*, 2002). Several endophytic bacterial strains have been shown to have beneficial effects on their host plants by production of plant growth enhancing chemicals such as indole acetic acid or cytokinins and protection against biotic and abiotic stresses (Khan *et al.*, 2012). They can promote the growth of many field crops and increase nutrient uptake by producing plant growth-promoting substances and by fixing nitrogen from the atmosphere (Lacava *et al.*, 2011).

Plant hormones regulate cellular and physiology process such as cell division, cell enlargement, bud dormancy, flowering, fruit ripening, seed dormancy and germination. Auxin, one of plant hormone stimulates differentiation of phloem and xylem, root initiation on stem cutting and development of branch roots (Kelen *et al.*, 2004). Indole acetic acid (IAA) is an endogenous auxin hormone that is synthesized in stem and roots.

Numerous endophytes are actively involved in the synthesis of auxins in pure culture and plants. Therefore, screening of endophytes for their potential of auxins production could provide information for effective plant growth hormone. It is necessary to study the application of IAA-producing bacteria that isolated from forest plant to stimulate plant growth. *Shorea selanica* is a Dipterocarp family that recommended by Indonesian government for rehabilitation and management of natural and industry forest (Soekotjo, 2007). In this study, we focus on the culturable bacterial endophytes of *S. selanica* and their possible contribution in the growth of another plant species.

Materials and Methods

Screening for IAA Production. Eight endophyte bacteria of *S. selanica* from collection of Plant Symbiotic Microbe Laboratory, Research Center for Biotechnology LIPI were screened for IAA production. The production of IAA was determined by the method Dey *et al.* (2004). Bacterial isolates were grown on 10 ml of

Nutrient Broth (NB) medium containing 0.5 mM L-tryptophan. These cultures were incubated at 28°C with shaking at 150 rpm for 24 hours and then harvested by centrifugation at 10,000 rpm for 10 minutes. Two ml of the supernatant was mixed with 2 ml of reagent Salkowsky. Optical Density (OD) was read at λ 530 nm after 60 min. The appearance of pink color indicated IAA production. The level of IAA produced was estimated, compared with the IAA standard.

Molecular Identification of Endophyte Bacteria. Endophyte bacterium that produced the highest of IAA was identified by 16S rRNA gene sequence. Single colony of bacterium was picked using sterile toothpicks and dipped it into PCR tube. A 49 μ l of PCR mixture was added into PCR tube and pipette up and down to mix. The PCR mixture was contained 25 μ l of ready mix, 25 μ l of dNTP, 2 μ l of each oligonucleotide primer 9F (5' GAG TTT GAT CCT GGC TCAG 3'), 1541R (5' AAG GAG GTG ATC CAG CC 3') and 20 μ l of sterile distilled water. Initially denaturation accomplished at 96°C for 5 min. Thirty cycles of amplification consisted of denaturation at 96°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. An elongation phase was performed at 72°C for 7 min. 3 μ l of amplified reaction mixture was analyzed by agarose (1 % w/v) gel electrophoresis in TAE buffer. After run at 100 V for 30 min, the gel was stained with Atlas sight DNA for 30 min and was observed by UV transilluminator. The 16S rRNA gene sequence obtained was compared to BLAST analysis.

Stress Test. To determine if the *S. selanica* endophytes that produced high level of IAA exhibited increased tolerance to stress, the endophytes were exposed to harsh condition, including heat and cold shock, UV irradiation, osmotic and heat shock and oxidative stress. SSBt2, the highest IAA producing endophyte was used in this study. SLBT1 that did not produce any IAA was used as a negative control. Cells were grown aerobically at 30°C in NB media. After 24 hours, the cells were exposed to different stress test. UV irradiation of cell suspensions (10 ml) was performed by exposing the cells to UV light for 2 h. For osmotic shock, the cells were incubated with 3 M NaCl for up to 2 h at 30°C. For acid stress,

cells were grown at pH 3.0 and 4.0 for 2 h at 30°C. For oxidative stress, cells were treated with 3% H₂O₂ and grown for 2 h at 30°C. For heat shock, cells were exposed to 75°C for 5 min by immersion of cultures in a water bath. For cold treatment, cultures were incubated at 8°C for 6 days. After that 50 μ l of the diluted treated cell were dropped on NA plates and incubated at 30°C for 24 hr. The presence of colonies indicated cell tolerant to stress condition.

Effect of IAA Producing Endophytes on the Growth of Soybeans. Another experiment was done to see if IAA producing *S. selanica* endophytes can support growth of another plant species. We chose soybean which more require nitrogen fixation bacteria to support its growth. Seedlings of soybeans were soaked in 10 ml of bacterial suspension for 30 minutes. Seedlings were planted into polybag containing 10 kg of soil, then added 1 ml of bacterial suspension in each holes. Each polybag consist of two seedlings. Isolate SSBt2 which produces the highest of IAA was grown overnight in NB medium. *Rhizobium* BTCC B64 (Rh B64) and mixtures Rh B64 and SSBt2 were used as a comparison. Rh B64 was grown in YMB medium for 7 days. For control, seedlings were fertilized with NPK (0.8 g/polybag) (CN) and without fertilizer (CO). Inoculants treatments were fertilized with NPK (0.4 g/polibag). Chemical fertilization was done 15 and 45 days after planting. Each treatment had 3 replicates. The growth was observed by measuring height of plant at month 1 and 2. Plants were harvested 90 days after planting. The upper and lower plant of soybeans were separated and their weight were measured. The upper and lower plants were dried in an oven 70°C for 24 h to constant weight and dry weight per plant was measured. Numbers of pods and seed weight per two plants were also observed.

Results

Eight bacterial endophytes of *S. selanica* were selected in its ability to produce the IAA hormone. The results on the production of growth promoting hormone indicated that only three isolates were able to produce IAA. SSBt2 produced the highest amount of IAA (43.01 μ g/ml), followed by SSBt1 and SSKK6 (39.89

and 5.06 µg/ml), whereas the other isolates could not produce IAA (Table 1). The 16S rRNA gene sequence from isolate SSBt2, showed 99% similarity with *Enterobacter hormaechei* sequences from GenBank (Table 2).

Result of stress test on IAA producing bacteria showed that isolate SSBt2 survived in the some stress tests, except on heat and oxidative stress (Table 3). Number of colony of isolate SSBt2 was stable at 10⁸ cfu, but declined at heat shock and oxidative stress. Isolate SLBt1 that did not produce any IAA and was used as negative control, was also resistant to stress test although number of colony tend to decline. Even on oxidative

stress, number of colony isolate SLBt1 was only 10¹ cfu.

Table 1. Production of IAA by endophytes bacteria of *S. selanica*

Code of isolates	Production of IAA (µg/ml)
SSBt1	39.89041
SSBt2	43.00685
SSKK2	-0.4863
SSKK4	-2.69863
SSKK6	5.061644
SSDt1	-3.59589
SLBt1	-3.4863
SLBt2	-5.31507

Table 2. BLAST analysis of SSBt2 based on 16S rRNA gene sequence

Description	Total score	Query coverage	E Value	Max ident
<i>Enterobacter hormaechei</i> strain TMPSB-T10	2710	99%	0.0	99%
Uncultured bacterium clone WH051-BH4	2704	99%	0.0	99%
Uncultured bacterium clone JSC2-D5	2700	99%	0.0	99%
<i>Enterobacter hormaechei</i> subsp. oharae	2700	99%	0.0	99%
<i>Enterobacter</i> sp. CTSP23	2693	99%	0.0	99%

Table 3. Resistance of endophytes isolates to various stress condition

Stress condition	SSBt2 (IAA producer) (cfu/ml)	SLBt1 (non IAA producer) (cfu/ml)
Normal condition	10 ⁸	10 ⁸
UV exposure	10 ⁸	10 ⁶
Heat shock	10 ²	10 ³
Cold shock	10 ⁸	10 ⁷
pH 3	10 ⁸	10 ⁴
pH 4	10 ⁸	10 ⁵
NaCl (3 M)	10 ⁶	10 ⁵
H ₂ O ₂ (3%)	10 ²	10 ¹

The endophyte treatment was also promoted a growth response resulting in increased yields. Soybean plant showed positive response on endophyte bacterial inoculation. The inoculation treatment by Rh B64 showed similar response to plants growth at month 1, but gave higher response compared to control (CO and CN) at month 2 (Figure 1). The other treatments of single inoculation by SSBt2 and double inoculation by Rh B64 and SSBt2 also showed similar result as with Rh B64 treatments (Figure 1).

Measurement of plant height showed that all treatments gave similar response at month 1, but isolate SSBt2 provided the highest response on plant growth compared the other treatments at month 2, followed by Rh B64 treatment (Figure 2A). Effect of double inoculation to support plant height was almost

similar with control. Inoculation treatment was not significantly difference on plant height at month-1, but affected significantly at month-2.

Single and combined inoculation also increased weight of plant. Single inoculation of Rh B64 provided the biggest response on dry weight of upper plant, whereas isolate SSBt2 (IAA producer) was on dry weight of lower plant compared to control (Figure 2B). Double inoculation (Rh B64 + SSBt2) showed lower yields than single inoculations. Single inoculation (Rh B64) affect significant on dry weight of upper plant compared to control (CO), but was not significant difference to other treatments. Inoculation SSBt2 affect significant on dry weight of lower plant compared to control (CO and CN), but was not significant difference to another inoculation treatments.

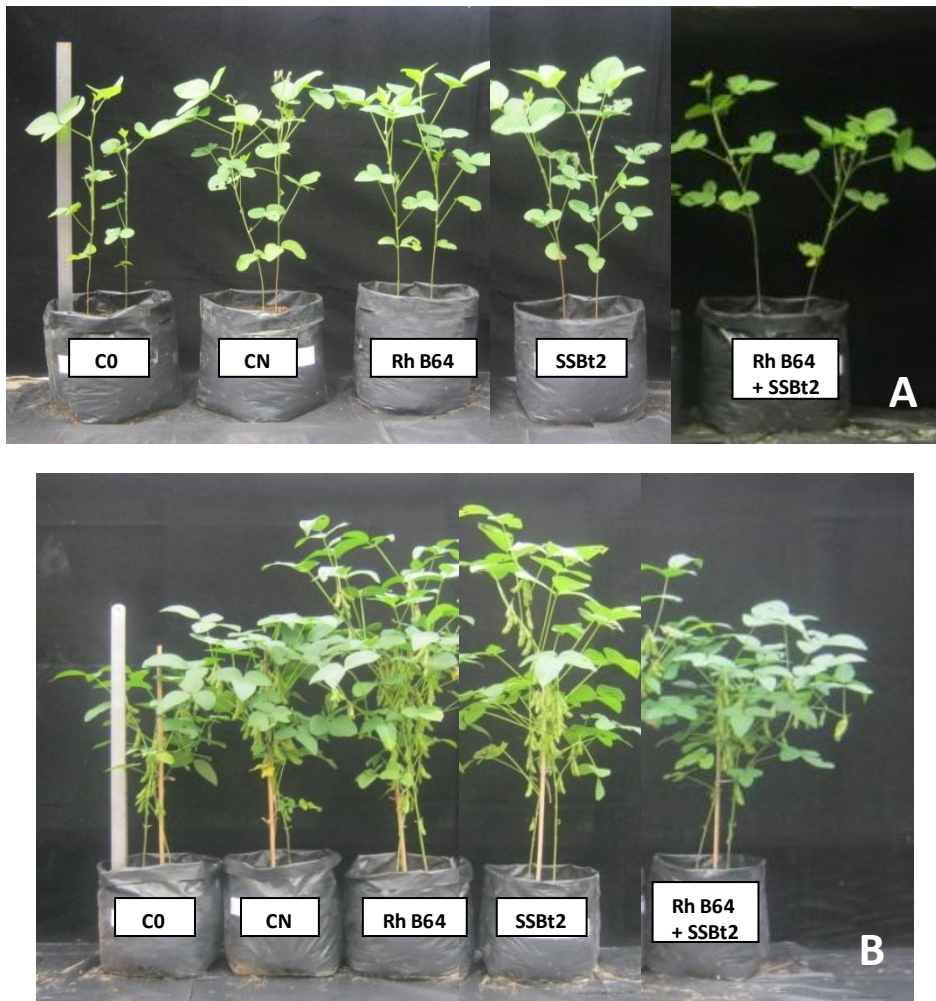


Figure 1. Effect of single inoculation (Rh B64), (SSBt2), and double inoculation (Rh B64 + SSBt2) on growth of soybean plant from one (A) to two months (B) after planting compared to CO (without fertilizer) and CN (with chemical fertilizer)

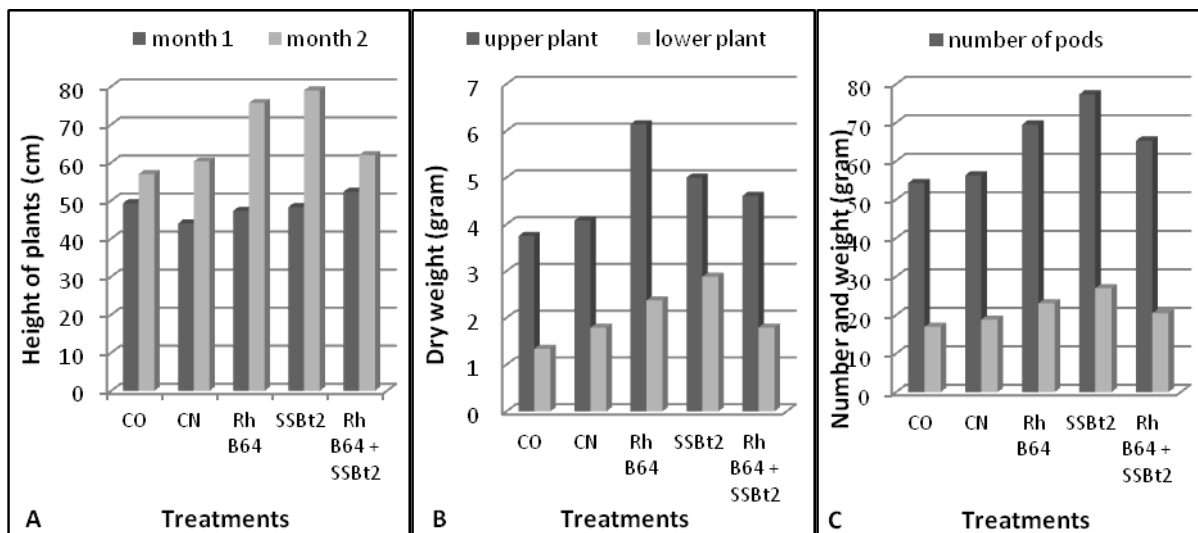


Figure 2. Effect of bacteria inoculation on height of soybean plant from one to two months after planting compared to CO (without fertilizer) and CN (with chemical fertilizer) (A); Dry weight of upper and lower per two plants inoculated by bacteria compared to control (B); Number of pods and seed weight per two soybeans plants inoculated by bacteria compared to control (C)

Number of pods and seed weight increased for both single and combined inoculation treatments. The highest values of number of pods and seed weight per two plants were obtained on SSBt2 treatment compared to all treatments, followed by Rh B64 and double inoculation (Figure 2C). Inoculation Rh B64 affects significant on number of pods and seed weight compared to CO and CN, but not significant difference to another inoculants treatments. Inoculation SSBt2 only affect on number of pods and seed weight compared to CO, but was not significant to CN, whereas double inoculation was not significant to all treatments.

Discussion

In this study, IAA producing isolates indicated that isolates were able to synthesis tryptophan in media cultures. Tryptophan functions as a physiological precursor for auxin biosynthesis in plants and microorganism (Vitorino *et al.*, 2012). This precursor contains active compounds that stimulate the growth of microbes in producing secondary metabolites. Difference of producing IAA concentration may be due to capability or mechanism of bacteria differ on utilize tryptophan for IAA production. The difference is influenced by indolepyruvate decarboxylase enzyme activity. Production of IAA varies greatly among different species and is also depending on the activity and the number of cells, nutrition availability and tryptophan substrate in the media (Sharma *et al.*, 2012).

Some of endophytes can synthesize IAA, which is involved in plant stem and root growth regulation. The capacity to synthesize IAA production is widespread among soil and plant associated bacteria (Verma *et al.*, 2001). Endophytes affect plants differently, but overall they do make differences in the root system development, improving the general health and providing stress tolerance.

According to our study, the effect of IAA treatments on bacterial cells showed that the cells were tolerant to a variety of stress condition. It accords with Bianco *et al.* (2006) which stated that IAA triggers an increased tolerance to several stress conditions (heat and cold shock, UV-irradiation, osmotic and acid shock and oxidative stress) and different toxic compounds (antibiotics, detergents and dyes).

Khan and Doty (2009) reported that strains of IAA production had more active metabolism resulting in tolerance to stress environment.

The increase in plant height of inoculated treatments is due to the stimulatory effects of microbe induced growth regulator IAA. Wall (2000) has suggested that plant hormones stimulate root development and consequently enhance absorption capacity of water and nutrients leading to plant growth (Camacho, 2001).

Based on BLAST, isolate SSBt2 isolated from *S. selanica* had 99% similarity with *Enterobacter hormaechei*. The presence of 99-100% similarity indicated that SSBt2 may have chromosome number, genom size and gene function same as with *E. hormaechei*. Drancourt *et al.* (2000) stated that identification at species level defined $\geq 99\%$ similarity with 16S rRNA gene sequence, identification at the genus level with $\geq 97\%$ similarity and to identify new genus determined lower than 97% similarity. *Enterobacter* bacteria were also found on *Shorea parviflora* and *Shorea stenoptera* with capability produce IAA hormone (Rahman *et al.*, 2010).

IAA production of SSBt2 (43.01 $\mu\text{g/ml}$) is lower than isolate *E. hormaechei* subsp. *verschuerenii* produced 152.63 $\mu\text{g/ml}$ and *Enterobacter* sp. produced 136.91 $\mu\text{g/ml}$ that isolated from rhizosphere of sugarcane (Inui-Kishi *et al.*, 2012). Another result showed that IAA production of SSBt2 is higher than *Enterobacter* sp. (0.08 $\mu\text{g/ml}$) isolated from leaf tissue of oil palm tree (Keyeo *et al.*, 2011).

In our study, endophyte of *S. selanica* which produced the highest IAA showed positive effect as a plant growth promoting when inoculated into another plant. Soybean seedlings inoculated with IAA-producing bacteria had root weight greater than the other treatments, while inoculation with nitrogen fixing bacteria affected to all parameters. The increase of plant height on inoculation treatments is due to the stimulatory effect of microbe induces growth regulators. Zaidi (2003) reported that seeds of soybean were inoculated with IAA-producing bacteria, the plant increased seed emergence. They also increased shoot and root length, dry weight, number of nodules and improved nutrient uptake. Soybean inoculated with IAA-producing bacteria resulted in synergistic effect on root growth and development and

increased yield. Phytohormone production of bacteria will stimulate root growth and proliferation, thus increasing the root area of plant in getting nutrition and uptake water more efficiently (Keyeo *et al.*, 2011).

In double inoculation between Rh B64 and SSBt2 showed lower result than single inoculation. The values were lower probably due to both of bacteria were different species and compete to get food and survival. In co-inoculation, values decreased slightly compared to single inoculation may be due to competition problems on the root surface for existence (Del Gallo & Fabbri, 1991).

Our result indicated that IAA-producing bacteria (SSBt2) have capacity to promote soybean growth. The growth parameters such as root dry matter showed the highest values on SSBt2 treatment. Better root development due to synergistic relationship of the inoculated bacteria. This capacity could be due to bacterial phytohormone biosynthesis during culture (Cassan *et al.*, 2009). Isolate SSBt2 is able to excrete plant growth regulator compounds into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues.

Application of exogenous IAA or inoculants of bacteria that produce high levels of IAA can stimulate primary root elongation. The bacterial IAA plays a major role in promotion of root elongation when a bacterium is associated with its host plant. IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. ACC deaminase, produced by many plant growth-promoting bacteria is involved in the stimulation of root elongation in seedlings (Patten & Glick, 2002).

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References

Bianco, C., Imperlini, E., Calogero, R., Senatore, B., Amoresano, A., Carpentieri, A., Pucci, P., &

- Defez, R. (2006). Indole 3-acetic acid improves *Escherichia coli*'s defences to stress. *Archives of Microbiology*, 185, 373-382. doi:10.1007/s00203-006-0103-y.
- Camacho, M., Santamaria, C., Rodriguez-Navarro, F. T., & Daza, A. D. N. (2001). Co-inoculation with *Bacillus* species: Cect 450 improves nodulation in *Phaseolus vulgaris* L. *Canadian Journal of Microbiology*, 47, 1458-1462.
- Cassa'n, F., Perrig, D., Sgroya, V., Masciarelli, O., Penna, C., & Luna, V. (2009). *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination promote seed germination and early seedling grow in corn (*Zea mays* L.) and soybean (*Glycine max* L.). *European Journal of Soil Biology*, 45, 28-35.
- Del Gallo, M. & Fabbri, P. (1991). Effect of soil organic matter on chickpea inoculated with *Azospirillum brasilense* and *Rhizobium leguminosarum* bv. *ciceri*. *Plant Soil*, 137, 171-175.
- Dey, R., Pal, K. K., Bhat, D. M., & Chauhan, S. M. (2004). Growth promotion and yield enhancement of peanut (*Arachis hypogea* L.) by application of plant growth promotion rhizobacteria. *Microbiological Research*, 159, 371-394.
- Drancourt M., Bollet, C., Carlioz, A., Martelin, R., Gayrall, J. P., & Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacteria isolates. *Journal of Clinical Microbiology*, 38(10), 3623-3630.
- Inui-Kishi R. N., Kishi, L. T., Picchi, S. C., Barbosa, J. C., Lemos, M. T. O., Marcondes, J., & Lemos, E. G. M. (2012). Phosphorus solubilizing and IAA production activities in plant growth promoting rhizobacteria from brazilian soils under sugarcane Cultivation. *ARPN Journal of Engineering and Applied Sciences*, 7(11), 1446-1454.
- Kelen, M., Demiralay, E. Ç., Şen, S., & Özkan, G. (2004). Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99R (*Vitis berlandieri* × *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by reverse phase liquid chromatography. *Turkish Journal of Chemistry*, 28(5), 603-610.
- Keyeo, F., Ai'shah, O. N., & Amir, H. G. (2011). The Effects of nitrogen fixation activity and phytohormone production of diazotroph in promoting growth of rice seedlings. *Journal of Biotechnology*, 10(3), 267-273.
- Khan, Z. & Doty, S. (2009). Characterization of bacterial endophytes of sweet potato plants. *Plant and Soil*, doi: 10.1007/s11104-009-9908-1.
- Khan, Z., Guelich, G., Phan, H., Redman, R., & Doty, S. (2012). Bacterial and yeast endophytes

- from poplar and willow promote growth in crop plants and grasses. *International Scholarly Research Network*, 1-11
- Lacava, P. T., Araujo, W. L., & Azevedo, J. L. (2011). Evaluation of endophytic colonization of *citrus sinensis* and *catharanthus roseus* seedlings by endophytic bacteria. *Journal of Microbiology*, 45(1), 11-14.
- Petten, C. L. & Glick, B. R. (2002). Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. *Applied and Environmental Microbiology*, 68(8), 337955-3801.
- Rahman, A., Sitepu, I. R., Tang, S. Y., & Hashidoko, Y. (2010). Salkowski's reagen test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. *Bioscience, Biotechnology, and Biochemistry*, 74(11), 100361-100367.
- Sharma, B., Chandra, Subba, R., & Saha A. (2012). *In-vitro* solubilization of tricalcium phosphate and production of iaa by phosphate solubilizing bacteria isolated from tea rhizosphere of Darjeeling Himalaya. *Plant Sciences Feed*, 2(6), 96-99.
- Soekotjo. (2007). Komponen silvikultur intensif dalam rangka membangun hutan yang sehat, prospektif dan lestari. *Dirjen Bina Produksi Kehutanan, Departemen Kehutanan RI*. Jakarta.
- Verma, S. C., Ladha, J. K., & Tripathi, A. K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *Journal of Biotechnology*, 91, 127-141.
- Vitorino, L. C., Silva, F. G., Soares, M. A., Souchie, E. L., Costa, A. C., & Lima, W. C. (2012). Solubilization of iron phosphate and in vitro production of indole acetic acid by endophytic isolates of *Hyptis marruboides* Epling (*Lamiaceae*). *International Research Journal of Biotechnology*, 3(4), 47-54.
- Zaidi, S. F. A. (2003). Biocontrol of *Fusarium oxysporium* by plant growth promoting rhizobacteria in soybean. *Annals of Agricultural Research*, 24, 676-678.
- Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczarski, D., Higley, P., Ishimaru, C. A., Arunakumari, A., Barletta, R. G., & Vidaver, A. K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology*, 68(5), 2198-2208.