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In Vitro Capability of Rice Endophytic *Streptomyces* spp. in Producing Indole Acetic Acid and Fixing Nitrogen

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ABSTRACT

Biological role of endophytic *Streptomyces* spp. which live inside rice plant tissue [illegible] promote rice plant growth. The research aimed to examine the capability of rice endophytic *Streptomyces* spp. in producing indole acetic acid (IAA) and fixing nitrogen. In vitro production of IAA from ten selected endophytic *Streptomyces* [illegible] isolated from local rice varieties and agricultural soil was examined. The tested isolates were grown in a liquid medium of International Streptomyces Project (ISP) [illegible] with the addition of 0.2 ml 0.2% L-tryptophan. All tested isolates produced indole acetic acid with various concentrations ranging about [illegible] ppm. Three isolates [illegible] capable of producing high indole acetic acid were [illegible] ([illegible] ppm), [illegible] ([illegible] ppm), and PS4-16 (93.4 ppm). Meanwhile, based on in vitro assay, the tested isolates have the ability to grow in N-free medium, [illegible] and produce ammonium with various activity. The [illegible] was considered as the most potential N2 fixing bacteria based on its ability to reduce [illegible] and [illegible] produced ammonia 2.144 ppm for 12 days in [illegible] medium. The colonization of the potential *Streptomyces* spp. on root of rice plant was observed under the light and scanning electron microscope. The observed isolates were able to penetrate and colonize the root of rice, confirming them as endophytes. The results indicated the endophytic *Streptomyces* spp. had promising role as plant growth promoter of rice plant.

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INTRODUCTION

Plant growth hormone such as Indole Acetic Acid (IAA) plays important role in plant growth acceleration. IAA functions to stimulate plant cell renewal, regulation of apical dominance and stimulate the formation of lateral roots and adventitious [28]. Some non actinomycetes bacteria were capable of producing IAA, e.g. *Pseudomonas putida* [26], *Rhizobium* strains from indigofera [35], *Azotobacter* [16] and Arthrobacter [9]. Some endophytic bacteria belonged to non actinomycetes which were isolated from rice plant were able to fix N_2 and produced IAA [33], thus could enhanced the growth of the rice plant [1]. Endophytic actinomycetes were known to harbour rice plant cell. However, the roles of endophytic actinomycetes, especially *Streptomyces* spp. in rice plants remain unclear. From our previous study (unpublished), it is known that endophytic actinomycetes, especially *Streptomyces* spp. were abundantly found on various local rice plant varieties in Indonesia. However, their role in increasing rice plant growth, e.g. through the production of IAA has not been done.

Nitrogen is considered as the nutrient that most frequently limits rice productivity. Meanwhile, the population growth in Indonesia is increasing which will directly affect in increasing rice demand as their staple food. For that reason, strong efforts should be conducted to increase rice productivity. Nitrogen (N)-fixing microorganisms are one of an important soil component caused by their ability to enhance available N for plant. The ability of microorganisms to provide available N by reducing atmospheric nitrogen to ammonium are catalyzed by nitrogenase enzyme. Microorganisms that have the nitrogenase enzyme can be derived from the archaea and bacteria, including actinomycetes [31]. Actinomycetes are the most important microbes as they have an ability to produce various metabolite compounds. Amongts 22,500 biological active compounds that are

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produced by microbes, 45% of that active compound comes from actinomycetes, 38 % from fungi, and 17% from unicellular bacteria [1]. Actinomycetes which have been successfully isolated from soil, rhizosphere, and phylosphere were found to be very potential as anti-pathogens for plant. Some actinomycetes which have been isolated from the rhizosphere of white clover (*Trifolium repens*) was capable of solubilizing phosphate, producing siderophores, and fixing nitrogen [5]. Endophytic actinomycetes have been reported to have potency in fixing nitrogen beside its benefit capability as a biocontrol agents, plant growth promoters, and enzyme producer [12, 17]. The nitrogen fixation ability can be shown by actinomycetes that was able to reduce acetylene into ethylene which commonly used as an indicator of nitrogenase activity [31]. Previous study proved that the used of endophytic actinomycetes was able to stimulate the growth of rice plants. Actinomycetes have been reported to be able to produce siderophores, HCN, chitinase, able to solubilize phosphate and inhibit pathogenic microorganisms such as *Xanthomonas oryzae* pv. *oryzae* that caused of leaf blight disease in rice plants [13]. The biological functions of endophytic actinomycetes in plants were presumably also associated with their presence and diversity in the host plant tissue [10]. Therefore, assessment of the roles of endophytic *Streptomyces* spp. in producing IAA and fixing nitrogen as well as their colonization are required. The output of this work is to elucidate the biological role of endophytic actinomycetes in promoting rice plant growth.

MATERIALS AND METHODS

Subculturing Streptomyces spp:

Streptomyces spp. used for this study were AB131-1, AB131-2, AB131-3, Impara 6A, A Fat, Membramo A which were isolated from various Indonesian rice variety plants, and four isolates of PS4-16, LSW-05, LBR-02, and SSW-02 were isolated from soils. The tested isolates have been reported to have the capability to solubilize phosphate, and to have antimicrobial activity [14]. *Streptomyces* spp. were subcultured on Yeast Extract Starch Agar (YSA) medium containing 15 g soluble starch, 4 g yeast extract, 15 g bacto agar, 0.5 g K_2HPO_4 and 0.5 g MgSO4·7 H_2O in 1 L, then incubated in room temperature for 14 days.

Capability of Streptomyces spp. in Producing IAA:

There was ten of endophytic *Streptomyces* spp. e.g. AB131-1, AB131-2, AB131-3, PS4-16, 6A Impara, A-Fat, Membramo A, LSW-05, LBR-02 and SSW-02 which were grown on ISP no 2 media. The culture was incubated at room temperature for 5 days, and then was spectrophotometrically measured for IAA production by the method of Salkowski [26]. As many as 2 disc isolates (5 mm disc) was transferred to 19.8 ml of ISP no. 2 liquid media, which was then added with 0.2 ml of 0.2% L-tryptophan, and incubated on a shaking incubator with a speed of 125 rpm at room temperature for 10 days. Cultures were obtained and centrifuged at 11,000 rpm at 4°C for 15 min (Beckman Coultier, Avanti® J-E). A total of 1 ml of the supernatant was taken and mixed with 4 ml of Salkowski reagent, and kept in a dark room for 30 minutes. IAA formation was indicated by color changes to pink. Absorbance measured at a wavelength of 530 nm [18] using a Spectronic 20 Baush and Lomb with two replication. The absorbance of IAA was read by subtracting the sample absorbance value plus the value of Salkowski reagent Optical Density (OD). Absorbance was corrected and incorporated into the standard curve IAA equation.

Mesurement of produced IAA was made on day-5, 10, 15 and 20. Isolates were grown on ISP no 2 medium at room temperature for 5 days, then as many as 4 disc isolates (5 mm per disc) was transferred to 39.6 ml ISP no 2 liquid medium, added 0.4 ml of 0.2% L-tryptophan and incubated on a shaking incubator with a speed of 125 rpm at room temperature. Cultures were then centrifuged at 4500 rpm at 4 ° C for 30 min. An amount of 1 ml supernatant was mixed with 4 ml of Salkowski reagent, and kept in a dark room for 30 minutes. Absorbance was measured at a wavelength of 530 nm. IAA measurement was performed in conjunction with measurement of cell biomass with two replications.

Capability of Streptomyces spp. in Fixing Nitrogen:

Growth Capability on Free Nitrogen Medium. To select endophytic actinomycetes for potential biological nitrogen fixation, isolates were cultured on free nitrogen medium containing 1 g K_2HPO_4, 3 g KH_2PO_4, 0.065 g $MgSO_4$, 0.01 g $FeCl_3·6H_2O$, 0.07 g $CaCl_2·2\ H_2O$, 5 g dekstrosa, 240 µg $Na_2MoO_4·2H_2O$, 3 µg H_3BO_4, 1.83 µg $MnSO_4·H_2O$, 290 µg $ZnSO_4·7H_2O$, 130 µg $CuSO_4·5H_2O$ dan 120 µg $CoCl_2·6H_2O$ in 1 L medium [27].

Nitrogen Fixation Measurement using Acetylene Reduction Method. The capability of strains to fix dinitrogen was tested by the activity of nitrogenase, which converts acetylene into ethylene in the presence of an external energy source. 2 disc (Φ 5 mm) endophytic actinomycetes cultures that grown on YSA medium for 5 days inoculated in 20 ml free nitrogen broth medium in 50 ml vial and incubated for 15 days. After growth time, vial closed by *serum stopper* and 10% (3 ml) from headspace injected by pure C_2H_2. Culture incubated at 30°C for 2 hours and the headspace concentrations of ethylene analysed by Hitachi gas chromatograph porapak-N column with operational conditions: $T_{injector}$ 110°C; T_{column}60°C; $T_{detector}$ 110°C; flow rate of fluoride as a carrier

gas 45 ml/min. Stoichiometric decrease in acetylene concentration with concomitant production of ethylene was indicative of nitrogenase activity.

Ammonium Production Measurement using Nesslerization Method [22]. Two discs (ϕ 5 mm) of 5-days endophytic actinomycetes culture inoculated in 20 ml free nitrogen broth medium and shaking-incubated at 13.09 x g in a room temperature for 15 days. After incubation, 0.125 ml Nessler reagent was added to 5 ml endophytic actinomycetes culture. The yellow until brown colour produced by the reaction of Nesslerization reagent and ammonium ions indicated a positive results and measured using spectrophotometer at λ = 500 nm. Uninoculated free nitrogen medium was used as a blank for the colorimetric assay and 0-5 ppm NH_3-N used for making a ammonium curve standard.

Collonization of endophytic Streptomyces spp. on root of rice plant:

Staining Method and observation under light microscope. Observations were made on day 15 after inoculation with reducing tetrazolium method [25]. Roots of rice plant which were inoculated with *Streptomyces* spp. were rinsed in sterile distilled water, soaked in 1% chloramine T for an hour, rinsed in sterile water, and re-soaked in tetrazolium phosphate buffer for one night. The cross sectional root was placed next to the glass object that has spilled 50% glycerol and observed under a light microscope with a magnification of 40x10.

Sample preparation for SEM observation. Root samples were soaked in phosphate buffer for 2 hours, followed by agitation in Ultrasonic cleaner for 5 minutes. Root sample was then immersed in a solution of 2.5% glutaraldehyde for up to 2 days. Samples were further fixed with 2% Tannat acid for 6 hours and washed with caccodylate 4 times for 5 minutes. The samples were fixed and dehydrated in 50% alcohol for 5 minutes 4 times, soaked in alcohol 70, 80, and 95% each for 20 minutes, and finally immersed in absolute alcohol for 10 minutes 2 times. The sample was drying by soaking it in tert butanol for 10 minutes 2 times, frozen in the freezer, then put in a freeze dryer for the drying process and observed by SEM JSM-5000.

Result:

Streptomyces spp. Producing IAA:

All tested isolates were able to produce IAA with various concentrations ranging about 11-99 ppm (Fig. 1). Three selected endophytic *Streptomyces* spp. namely AB131-1, AB131-2 dan PS4-16 produced IAA at 99.2 ppm, 99.2 ppm and 93.4 ppm, respectively.

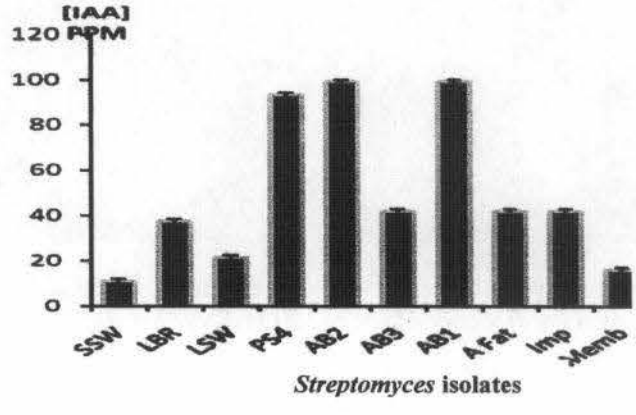

Fig. 1: Capability of endophytic *Streptomyces* spp. in producing IAA when grown for 10 days in ISP no 2 liquid culture media.

Effect of Culture age of Streptomyces spp. on the Production of IAA:

The highest concentration of IAA was generally produced by 15 days old culture. This phenomenon occurred for the three tested isolates (Fig. 2a). At 15 days old culture, AB131-1, AB131-2 and PS4-16 produced similar trend in IAA production as previously mention, where each of the isolate produced 105 ppm, 99 ppm and 82 ppm respectively (Fig. 2b). The results were in line with their biomass production, where at 15 days old culture, AB131-1 yielded 0.46 g, AB131-2 was 0.45 g and PS4-16 produced 0.41 g. It seemed that increasing biomass was followed by increasing in IAA production. At 20 days old culture, the biomass production tended to decrease, presumably because of some nutrition factor limitation.

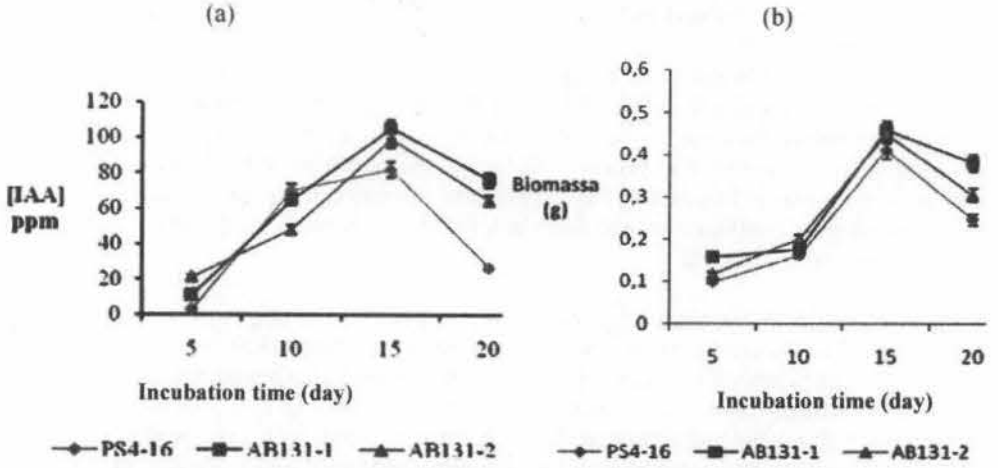

Fig. 2: Relationship between (a) IAA production and (b) biomass production by endophytic *Streptomyces* spp. grown for 20 days in ISP no 2 liquid medium.

Growth Capability on N-free Medium:

Ten of endophytic *Streptomyces* spp. isolates could be grown on free nitrogen medium with vary of their growth rate. Although they tended to have slower growth rate compared with their growth rate in sufficient nitrogen medium (Fig. 3).The result clearly indicated that nitrogen is a essential nutrient for microbes. Nitrogen was used to synthesize macromolecules, such as amino acid required for cell protein arrangements (White 2000). Capability of the tested *Streptomyces* spp. to grow on free nitrogen medium indicated its capability in fixing N_2 from atmosphere.

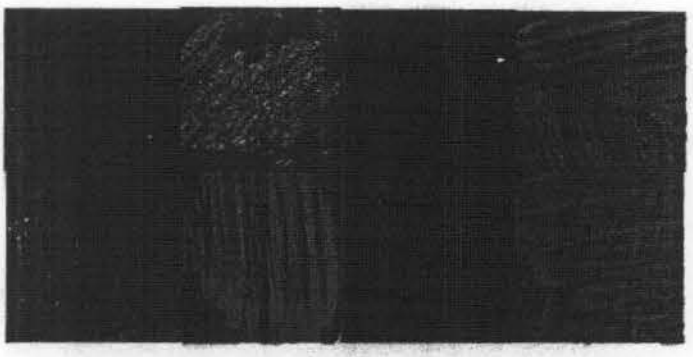

Fig. 3: Growth of *Streptomyces* spp. colony on free nitrogen medium (left) and on YSA medium (right) for 15 days at room temperature: a) Membramo A; b) AB131-2; c) SSW-02; d) Impara 6A.

Nitrogen Fixation Activity of Streptomyces spp. based on Acetylene Reduction Capability:

N_2 fixation activity was assessed by acetylene reduction method. Results showed that ten endophytic *Streptomyces* spp. could reduce acetylene to ethylene due to nitrogenase activity (Table 1). SSW-02 had the highest nitrogenase activity at 2.1750 nmol ethylene/hours, with specific activity was at 1.55 nmol ethylene/hours per mg cell, compared with the other tested isolates.

Table 1: Capability of *Streptomyces* spp. s in reducing acetylene.

Code of isolates	Ethylene (C_2H_4) measured (nmol/hour)	Cell dry mass (mg)	Ethylene (C_2H_4) specific measured (nmol/hour.mg)
SSW-02	$2.1750^{a} \pm 0.8132$	1.4	1.55
Membramo A	$1.8000^{ab} \pm 0.0707$	2.2	0.82
LBR-02	$1.7000^{ab} \pm 0.3536$	4.6	0.37
PS4-16	$1.4500^{b} \pm 0.0707$	3.9	0.37
Impara 6-A	$1.2250^{b} \pm 0.0354$	6.9	0.18
AB131-1	$1.2250^{b} \pm 0.3182$	4.0	0.31
AB131-3	$1.1250^{b} \pm 0.2475$	8.5	0.13
LSW 05	$1.4250^{b} \pm 0.0354$	6.5	0.21
A Fat	$0.4000^{c} \pm 0.0000$	2.5	0.16
AB131-2	$0.3000^{c} \pm 0.0707$	1.4	0.21
Control	$0.0000^{c} \pm 0.0000$	0.0	0.00

Ammonium Production of Endophytic Streptomyces spp.:

Ammonium produced by each of *Streptomyces* spp. tested isolates can also be used as an indication for having nitrogen fixing capability Quantitative test of ammonium production results using Nesslerization (Fig. 4) confirmed that AB131-3 and SSW-02 had a higher ammonium production than the others, with 3.900 ppm and 2.144 ppm of ammonium content, respectively. AB131-3 produced the highest ammonium production (Fig. 4), but had a nitrogenase activity slightly lower than SSW-02 (Table 1), which may be due to some ammonification from decayed cells. The abundance of ammonium could repressed nitrogen fixation and lead to the death of microbes, if ammonium content exceeds its tolerance limit [34]. Based on acetylene reduction assay and ammonium production, the selected endophytic *Streptomyces* spp. were SSW-02 that had a highest N_2 fixing capability.

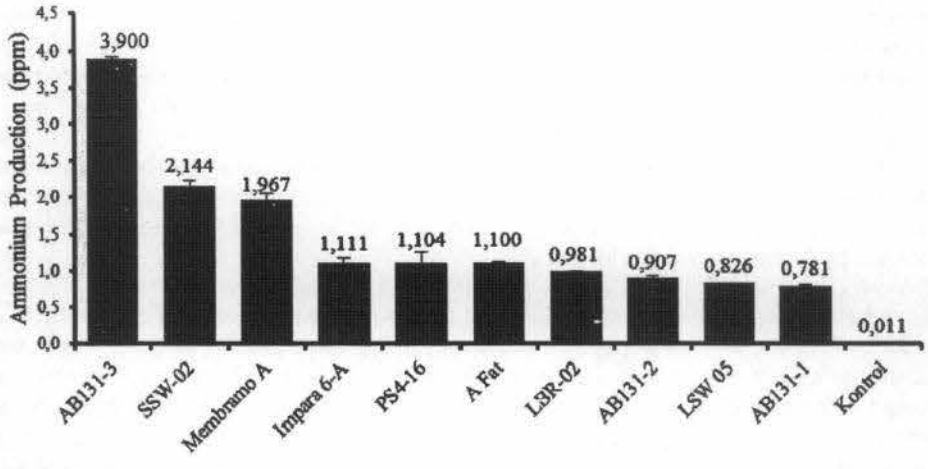

Fig. 4: Ability of *Streptomyces* spp. in producing ammonium.

Collonization of endophytic Streptomyces spp. on roots of rice plant:

Light microscopic observation of stained rice root showed that AB131-1 and PS4-16 able to collonize root tissue. In control root plant, there was no collonization observed (Fig. 5a), while on the treated root plant, the red colonies seen on the root tissue, indicating living cells of the tested endophytic *Streptomyces* spp. (Fig. 5b-c). The collonization of endophytic *Streptomyces* spp. on root tissue was confirmed with SEM observation. The result showed that no penetration phenomenon observed on the control root plant (Fig 5d). Meanwhile, the long-thick form mycelia that looked somewhat spiral, such as hooks, bulging, and jointed-hooks were clearly observed. These phenomenon indicated the penetration of endophytic *Streptomyces* spp. on the root rice surface (Fig.5 e-f).

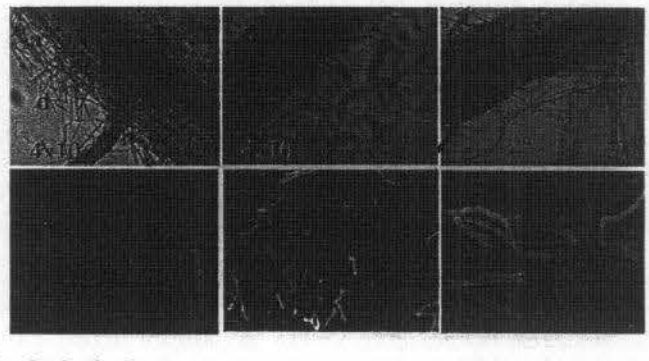

Fig. 5: Collonization of endophytic *Streptomyces* spp. on root tissue of rice plant. Light microscope observation: (a) no treatment, (b) AB131-1 (c) PS4-16; SEM observation: (d) no treatment (e) AB131-1, (f) PS4-16

Discussions:

A total of 10 isolates of endophytic *Streptomyces* spp. tested in this experiment capable of producing IAA at various concentrations ranging about 11-99 ppm, when grown in ISP 2 liquid medium with the addition of 0.2 ml of 0.2% L-tryptophan. Amongst them, AB131-1, AB131-2 and PS4-16 produced more than 99 ppm of IAA which is higher than the other tested isolates. The ability of endophytic actinomycetes and endophytic non-actinomycetes in producing IAA were also reported by other workers. Endophytic bacteria isolated from rice

straw produced 8.3 ppm when grown in media in the presence of 1 mg L-tryptophan for 5-7 days [33]. *Streptomyces viridis* isolated from rhizosphere of medicinal plant produced 144 ppm IAA [18]. Moreover, *Kitasatospora* sp. produced IAA in the range of 0.8-1.8 ppm for 4-6 days of incubation by the addition of 0.5 g L-tryptophan [34]. The difference in IAA production of various bacteria may be influenced by the type of isolates and their ability to convert L-tryptophan contained in the media to IAA [26]. L-tryptophan is an amino acid that acts as a precursor for the formation of IAA. Availability of suitable precursors is one of the primary factors for secreting bioactive compounds from microbes. *Streptomyces violaceus, S. scabies, S. griseus, S. exfoliate, S. lividans* and *S. coelicolor* were capable of producing IAA when given L-tryptophan [23]. The highest IAA was yielded by 15 days old culture. The IAA production was gradually increasing at day 5 to day 10 then reaching the highest value at day 15 followed by slightly decreasing at day 20. The trend of IAA production correlates with the biomass production. This phenomenon indicates that biomass affects IAA production where the high biomass will yield high IAA. During cultivation period, the availability of nutrition will affect growth and IAA production [20]. IAA is a key hormone which function to stimulate plant growth. Thus the capability of endophytic microbes in synthesizing IAA is considered as one reason for the increase of plant growth [2]. IAA can be synthesized by microbes through its association with plants. Endogenous auxin IAA is a hormone that is synthesized in various parts of the plant and is commonly associated with the plants that are actively growing and developing as in all types of meristem, shoot tip, root tip and cambium [28]. Association of plants with microbes can affect the hormonal balance in plants [5]. Capability of producing IAA by the selected endophytic *Streptomyces* spp. thus can be used as the basis of screening the potential endophytic actinomycetes in promoting growth of rice plants.

Another very important for plant growth parameter is the availability of nitrogen. Nitrogen is a main nutrient that benefit for vegetative growth of rice plant [31]. Nitrogen is an essential constituent of amino acids, nucleic acids, nucleotides, and chlorophyll. It promotes rapid growth (increasing plant height and number of tillers) and increasing leaf size, and also grain protein content. Thus, N affects all parameters contributing to yield. Nitrogen is considered as the nutrient that most frequently limits rice productivity [32]. Most of nitrogen that absorbs by rice roots is translocated to rice leaves and gives affect in increased of total plant weight. Deficiency of nitrogen is the one of factors that inhibited plant growth. Nitrogen is required throughout the growth period, but the greatest requirement is between the early to mid-tillering and panicle initiation stages [8]. Previous research also found that some of *Pantoea agglomerans* isolate that harbour endophytic bacteria in stem of rice had vary capability of acetylene reduction from 0-5 nmol C_2H_4/culture.hour [27]. Some of endophytic enterobacteria in rice plant could reduced acetylene up to 26327 nmol C_2H_4/hour.mg protein by *Klebsiella pneumoniae* M5A1 and 2472 nmol C_2H_4/hour.mg protein by *K. oxytoca* [3]. More recent, *Stenotrophomonas maltophilia*, a nitrogen fixing bacteria that isolated from field soil in Myanmar, could accumulate maximum ammonium production to 2 ppm for 60 hours incubation [38]. Diazotroph endophytic bacteria were needed to improve plant growth productivity through their various biological role such as supplying nitrogen nutrient, phyto-hormone production, transform morphology and physiology of root, promote root growth and expand exploration of soil volume and give better effect in improving nutrient absorbing [4]. From this results, it is clearly shown that SSW-02 had a capability in fixing nitrogen and enhanced rice plant growth.

Microscopic observations of rice root treated with AB131-1 and PS4-16 and stained with tetrazolium indicates the collonization of those endophytic *Streptmyces* spp. Tetrazolium solution is used as an indicator to show the biological processes that occur in living cells. This compound is absorbed by the cell, and in the living cell tissue will react to the reduction in respiration. Dehydrogenase enzyme activity would release H^+ and reacts with tetrazolium to form a red formazan precipitate, stable and water insoluble [7]. Further observation using SEM showed the presence of mycelia shaped like a hook, length, slightly spiral, jointed-books and bulging which indicate penetration of *Streptomyces* spp. on root surface. The hyphae of *Streptomyces* sp. was observed in the cortical tissue of tomato roots [29]. While, *Streptomyces* aerial hyphae which looked long and somewhat spiral bulge was also observed on the surface of sterile wheat plant roots [6].

Conclusion:

Rice endophytic *Streptomyces* spp. were able to produce IAA and fixing nitrogen at various concentrations under in vitro assay. The three selected *Streptomyces* spp. isolates are AB131-1 (99.2 ppm), AB131-2 (99.2 ppm) and the PS4-16 (93.4 ppm). Endophytic actinomycetes, isolate SSW-02 was considered as the best isolate that capable to fixing N_2 based on its capability to reduce acetylene and produce ammonium. Microscopic observations on isolates AB131-1 and PS4-16 confirmed that the two isolates are endophytes which able to penetrate to the rice roots. These data indicate the potential of endophytic *Streptomyces* spp. in increasing the growth of rice plants.

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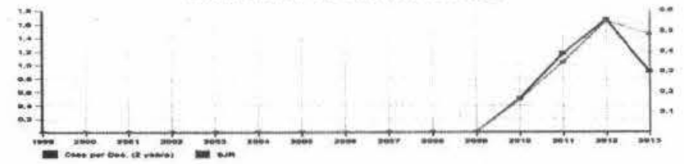
SJR indicator vs. Cites per Doc (2y)



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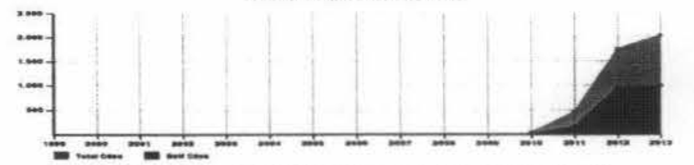
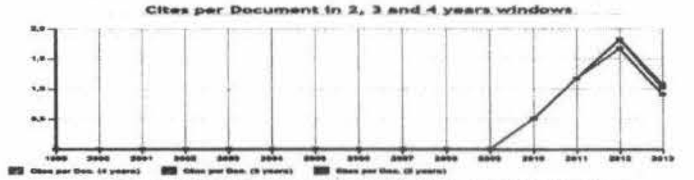


Evolution of the total number of citations and journal's self-citations received by a journal's published documents during the three previous years.

Cites per Document vs. External Cites per Document

Evolution of the number of total cites per document and external cites per document (i.e. journal self-citations removed) received by a journal's published documents during the three previous years.

Cites per Document in 2, 3 and 4 years windows

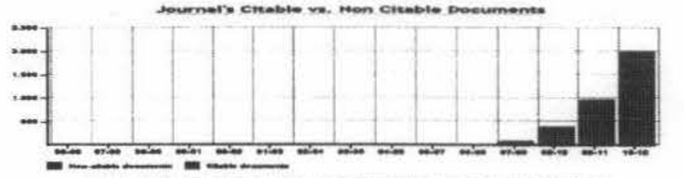


Evolution of Citations per Document to a journal's published documents during the two, three and four previous years. The two years line is equivalent to journal impact factor ™ (Thomson Reuters) metric.

International Collaboration

International Collaboration accounts for the articles that have been produced by researchers from several countries. The chart shows the ratio of a journal's documents signed by researchers from more than one country.

Journal's Citable vs. Non Citable Documents



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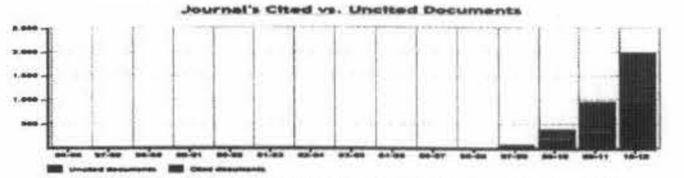
Journal's Cited vs. Uncited Documents



Ratio of a journal's items, grouped in three years windows, that have been cited at least once vs. those not cited during the following year.