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Evaluation of endophytic bacteria from some rice varieties for plant growth promoting activities

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Abstract

Eighteen endophytic bacteria were isolated from different rice varieties by using Jensen's nitrogen free medium. According to morphological and biochemical tests, isolates S1, R1, R2, R5 and R6 may be *Rhizobium* spp. and the remaining isolates may be *Azospirillum* spp. Fourteen out of isolates had plant growth hormone (Indole Acetic Acid) producing activity. R7 was the best Indole Acetic Acid producer among the isolated strains at two days incubation period in Jensen's nitrogen free medium with L-tryptophan 0.5mg /ml at 37˚C and the concentration produced was 120.55 ppm. In nitrogen free semi-solid agar medium containing trace amount of bromothymol blue as indicator, all isolates showed nitrogen fixing activity during seven days incubation period. All isolates except S6 could solubilize phosphate on Pikovskaia's medium supplemented with (0.5% $Ca_3(PO_4)_2$). S5 was the best P- solubilizer at 5 days incubation period both in plate screening showing zone diameter 45mm and 539.78 ppm quantitatively. Fourteen isolates except (LS, R2, S4, S8) could also decompose potassium (ranging from 2-30mm in diameter) on K- decomposing medium with 0.12% K-mica. R3 was the best K-decomposer by plate screening (30mm in diameter) and 19.84 ppm quantitatively. S3 and R3 had good antagonistic activities against *Rhizotonia solani* and LS and R5 to *Phythium* spp. and S7 to *Fusarium oxysporum* respectively.

Keywords: Endophytic bacteria, Jensen's nitrogen free medium, Indole Acetic Acid producer, Nitrogen fixing activity, P-solubilizer, K-decomposer.

INTRODUCTION

Plants are constantly involved in interactions with a wide range of bacteria. These plant associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species [1].

Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth by production of plant hormones [2], nitrogen fixation [3] and induction of resistance to plant pathogens [4-6], enhanced nutrient uptake, enhanced grain quality and increased drought tolerance. Some endophytic microorganisms are able to produce compounds of biotechnological value as antibiotics and antitumor drugs. It is well established that plant bacterial endophytes are to be found in most healthy plant tissues [6-8]. This particular host endophyte interaction has been variously defined as altruism, commensalisms, mutualistic, symbiosis or passivity to pathogenicity. Whatever the specific relationship(s) involved, internal plant colonization by bacteria constitutes a vast and, as yet, little mapped ecological niche.

There is much debate as to how to define an endophyte. For example, [6] suggested that bacteria and fungi that are isolated from surface sterilized plant tissues, and that do no apparent harm to the plant, could be considered as endophytes. Endophytic microorganisms have been defined as those that reside at some phase of their life cycle within living plant tissues [9, 10]. Endophytes have been found in all plants that have been examined [11]. These microorganisms include both commensal species, which have no direct effect on the host plant, and mutualistic symbionts, which could be used in the biological control of pathogens or plant growth promotion. The intimate relationship between endophytic bacteria and their host involves co-evolutionary processes, and may influence the physiological mechanisms of plants [12].

It has also been shown that in some cases endophytes can accelerate seed emergence and promote plant establishment under adverse conditions [13] as well as increase plant growth and hasten plant development [14]. On the other hand, the effects of endophytes may also be deleterious, possibly contributing to disease status [15,16], or some endophytes may interact with other endophytic populations [15]. Causes of these contrasting outcomes are mostly unclear, but they are very likely affected by the complex dynamics of interactions among endophytes, which are in turn affected by environmental conditions, plant species, and soil type.

MATERIALS AND METHODS

Sampling

The healthy and disease symptomless rice plants were collected from different rice *viz.* (Shwe thwe yin, Ma jan taw, Ma naw thukha, Paw san baykyar and Sin thwe latt) from different places (around Mandalay Technological University and Kyaukse, Myanmar).

Isolation of Endophytic Bacteria

Endophytic bacteria used in this study were isolated from root and stem tissues of healthy and disease symptomless rice plants which were collected from various places. They were washed with tap water to remove soil and other impurities and then roots and stems were cut into 2-3 cm pieces with sterile surgical blade.

These pieces were dipped and shaked in 96% ethanol for 10 seconds and then they were rinsed in sterile distilled water for three times.

After that they were dipped and shaked for 10 seconds in 0.1 % mercuric chloride and rinsed with sterile distilled water for three times. Then, these sterilized pieces were crushed with sterile motor and pestle.

Finally, the Jensen's Nitrogen free agar medium was prepared and it was poured into the sterilized petridish and divided into three parts; one part for control without inoculation, one for rolling of sterilized tissue pieces for checking efficient sterilization and one for inoculation of crushed sample. These were incubated at 37˚C for three to five days.

Screening for Indole Acetic Acid (IAA) Producing Activity

Indole acetic acid (IAA) productions of the isolated strains were determined by colorimetrically using Salkowski's reagent (50ml of 35% perchloric acid: 1ml of 0.5 N $FeCl_3$). To measure IAA qualitatively, isolated strains were aseptically cultured in Jensen's nitrogen free broth containing 0.5 mg L-tryptophan per ml and then incubated at 37ºC for 7 days.

After harvesting, these were centrifuged at 10,000 rpm for 20 mins. One millilitre of supernatant was mixed with two ml of Salkowski's reagent and one drop of orthophosphoric acid and incubated at room temperature for 25-30 min. Development of pink colour showed IAA production.

Quantitative Analysis on IAA Production by UV-Vis Spectrophotometer

Isolated endophytic bacteria were inoculated in Jensen's nitrogen free broth containing 0.5 mg L-tryptophan/ml and then incubated at 37°C for 1 to 10 days. After harvesting, these were centrifuged at 10,000 rpm for 20 mins. One ml of supernatant was mixed with 2 ml of Salkowski's reagent and one drop of orthophosphoric acid and incubated at room temperature for 25-30 min.

The absorbance of developed pink colour was measured at 530nm and then IAA concentrations are calculated by using IAA standard curve (generated by plotting absorbance on Y-axis and authentic IAA concentration (ppm) on X-axis).

Screening of Nitrogen-Fixing Activity

All isolates were inoculated in nitrogen free semi-solid agar medium containing trace amount of BTB as indicator.

Determination of Phosphate Solubilizing Activity by Plate screening method

A total of 18 bacterial isolates were tested on solid media to select the most efficient strain. The Pikovskaia's medium was used as a basal medium and tricalcium phosphate was used as substrate in this study. After autoclaving, the medium was adjusted to pH 8.6. All isolated endophytic bacteria were inoculated on this medium and incubated at 37°C for 5 days. After that, the solubilizing activity was measured by diameter of halo zone (millimetre) around the colony.

Quantitative Determination of Phosphate Solubilizing Activity by Spectrophotometric Method

The best five phosphorus solubilizing strains out of eighteen isolates (SR, S_5, R_6, S_7, R_8) determined by plate screening method were further evaluated quantitatively for their P- solubilizing ability in Pikovskaia's liquid medium. After autoclaving the medium, it was left to be cool and 0.5 % tricalcium phosphate was added as substrate to avoid its denaturation by heat. These five strains were inoculated in their respective broth and incubated at 37˚C and 150 rpm. Uninoculated medium was used as the control.

Ten-milliliter samples were withdrawn every 24 hours for 1 week and then each sample was passed through the cation exchange resin. Sodium molybdate solution and hydrazine sulphate solution were used to form blue color complex and P- solubilizing activities of these samples were measured at 830nm by UV-Vis spectrophotometric method. The intensity of the blue color is propotional to the amount of phosphate initially corporated in the heteropoly acid.

Determination of Potassium Decomposing Activity by Plate screening method

The potassium decomposing activity of the isolates was also determined on K decomposing medium with 0.12% insoluble potassium mica. The mica sample was washed to remove soluble potassium (K) and dried at 40^{o}C in microwave oven.

All isolated strains were cultured on K decomposing medium supplemented with insoluble potassium mica and incubated at 37˚C for 5 days. The decomposing activity was measured by diameter of clear zone around the colony after this incubation period.

Quantitative Determination of K- decomposing Activity

The mica sample was supplemented after cooling the sterilized K-decomposing broth, and inoculated the best two potassium decomposing bacteria, S_3 and R_3, and incubated on shaker at 37˚C and 150 rpm. The samples were withdrawn every day for one week and the insoluble mica was removed by centrifugation at 6000 rpm for 10 minutes and then the supernatants were analyzed by AAS (Atomic Absorption Spectrometry) method.

Study on the Antagonistic Activity of Isolated Endophytes

Isolated strains were screened for their antagonistic activities against common soil borne plant pathogenic fungi like *Fusarium oxysporum*, *Pythium* spp. and *Rhizoctonia soloni*. This was carried out by hyphal growth inhibition assay. Czapeak's medium was used for screening of antifungal activity. Inhibition of hyphal growth was observed.

Tests for Characterization of Isolated Endophytic Bacteria

These strains were identified on the basis of their colonial morphology, microscopic characteristics and some biochemical tests etc.

RESULTS AND DISCUSSIONS

Isolation and Identification of Rice Endophytic Bacteria

Eighteen endophytic bacteria were isolated from different rice viz. (Shwe thwe yin, Ma jan taw, Ma naw thukha, Paw san bay kyar and Sin thwe latt) by using Jensen's nitrogen free medium.

Colonial morphologies of all isolates were round raised, mucoid and transparent on Jensen's nitrogen free medium. All isolates were gram-negative and cell diameters are in the range of 0.5-1.5 x 2-4 µm rods. According to morphological and biochemical tests, isolates S_1, R_1, R_2, R_5 and R_6 may be *Rhizobium* spp. and the remaining isolates may be *Azospirillum* spp.

Although *Rhizobium* association had been extensively explored in the root nodules of legumes where they fix atmospheric nitrogen, recent studies also suggest that *Rhizobium* can exhibit plant growth promoting (PGP) activities with non-legumes such as rice [17].

Azospirillum spp have broad host range and these two species can promote plant growth via secretion of plant growth hormones (IAA, GA), vitamins, solubilization of insoluble phosphates, decomposition of potassium, biological nitrogen fixation, induced resistance to some plant pathogens via secretion of antimicrobial compounds that may hinder colonization of hosts by phytopathogens, thereby suppressing the diseases they cause, enhancement in stress resistance to abiotic factors [18] such as salt tolerance, drought resistance and production of siderophores under iron limiting condition.

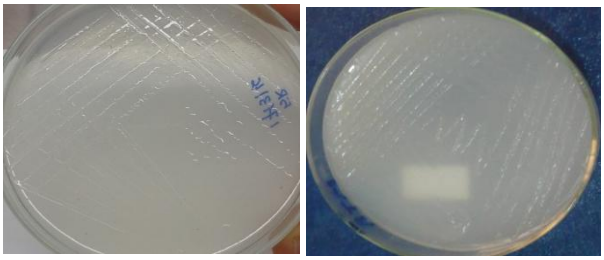

Figure 1: Growth of Endophytic Bacteria on Jensen's medium (*Azospirillum* spp.) and on YEM Medium (*Rhizobium* spp.)

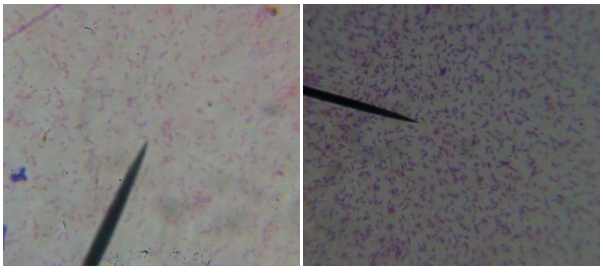

Figure 2: Microscopic Morphology of Some Isolated Endophytes

Table 1: Biochemical Characteristics of Isolated Endophytic Bacteria

Isolated Bacteria Biochemical characteristics	R_3, R_7, R_8, S_2, S_3, S_4, S_5, S_6, S_7, S_8, LS, SS, SR	S_1, R_1, R_2, R_5, R_6
Gram reaction	-	-
Colonial morphology	Small, white, dense colonies on semi-solid NFb agar medium	Circular, convex, mucous, opaque or transparent on YEM
Microscopic morphology	Clump shape, slightly curved and straight rod, single and pairs (0.6–1.7 x 2.1–3.8µm)	Small rod, x, y shape (0.5–1 x 1.2–3 µm)
Motility	+	+
Gelatin agar	-	-
Starch hydrolysis	-	-
Catalase	+	+
Citrate utilization	+	-
Carbon sources utilization		
Mannitol	+	+
Glucose	+	+
Sucrose	+	+

Screening on Indole Acetic Acid (IAA) Producing Activity

Four isolates, R_2, SS, SR, LS couldn't produce IAA by screening them with IAA detecting reagent (Salkowski's reagent) after 7 days incubation.

Thus, fourteen out of isolates had plant growth hormone, IAA, producing activity determined by the development of pink colour.

Figure 3: Screening on IAA Producing Activities of Fourteen Isolates

Quantitative Determination of IAA Producing Activity of Isolated Endophytic Bacteria

Among fourteen isolates, R_7 was the best IAA producer and the concentration produced was 120.55 ppm in two days incubation period. S_6 produced 104.58 ppm and R8 produced 95.55 ppm. R_6 had the lowest

IAA producing activity among fourteen isolates, and it produced 6.35 ppm in 2 days incubation period.

The highest concentration of IAA was detected in two days incubation period and in the following days, these IAA hormones may change to other compounds and so these cannot be detected by the IAA detecting reagent (Salkowski's reagent).

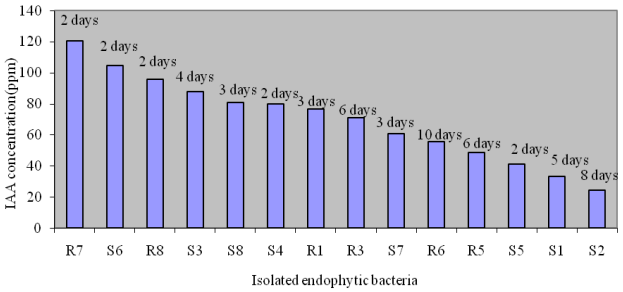

Figure 4: The Best Incubation Periods for High IAA Productivity of Isolated Endophytic Bacteria (0.5mg L- tryptophan/ml)

Nitrogen Fixation Activity of Isolated Endophytic Bacteria

In nitrogen free semi-solid agar medium containing trace amount of bromothymol blue as indicator, all isolates showed nitrogen fixing activity after three days incubation period.

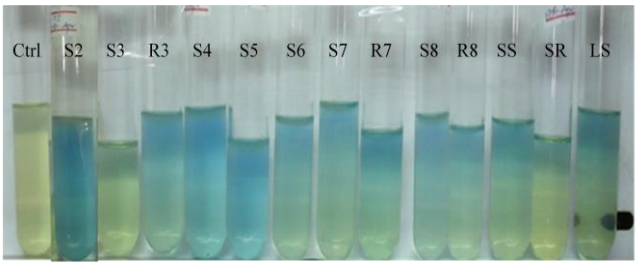

Figure 5: Nitrogen-Fixing Activity of Thirteen *Azospirillum* spp. In Semi-solid Nitrogen Free Broth during Seven Days Incubation Period

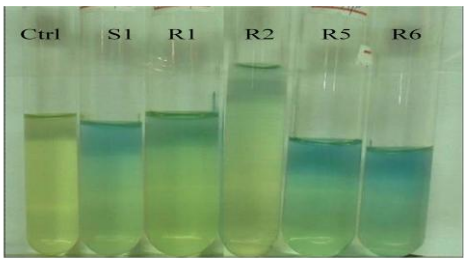

Figure 6: Nitrogen-Fixing Activity of Five Rhizobium spp. in Semi-solid Nitrogen Free Broth during Seven Days Incubation Period

Results for Phosphate Solubilizing Activity of Isolated Endophytic Bacteria

All isolates except S_6 could solubilize phosphate ranging from 3-45 mm in diameter on Pikovskaia's medium supplemented with (0.5% $Ca_3(PO4)_2$) and S_5 is the best P- solubilizer. In plate screening, halo zone of S_5 was 45 mm in diameter and also the best P solubilizer by spectrophotometric method, and solubilized P concentration was 539.78 ppm at 5 days incubation period. SR was second best P solubilizing strain and solubilized P concentration was 485.55 ppm and S_7 had the lowest P solubilizing activity among five strains.

Table 2: Soluble P Concentration of Five Isolates (ppm) in Pikovskaia's Medium by Spectrophotometric Method (at 830nm)

Isolates	Incubation Periods (Days)		
S_5	269.54	539.78	152.74
S_7	76.16	127.49	98.42
R_6	62.5	157.3	102.05
R_8	84.84	236.77	81.96
SR	121.28	485.55	252.57

Potassium Decomposing Activity of Isolated Endophytic Bacteria

Fourteen isolates except (LS, R_2, S_4, S_8) could decompose potassium (ranging from 2-30 mm in diameter) on K- decomposing medium with 0.12% K- mica and R_3 was the best K-decomposer by plate screening method and by AAS method (19.84 ppm).

Table 3: Soluble K Concentration (ppm) Decomposed by Two Endophytic Strains in 3, 5 and 7 Days Incubation Periods by AAS Method

Isolates	Incubation Period (Days)		
	3	5	7
S_3	3.36	14.91	18.73
R_3	3.28	17.46	19.84

Results for Antagonistic Activity

S_3 and R_3 had good antagonistic activities against *Rhizotonia solani* and LS and R_5 against *Phythium* spp. and S_7 to *Fusarium oxysporum* respectively. They couldn't be overwhelmed by these plant pathogens and could live with these pathogens. So, this fact can benefit the host plants they lived.

CONCLUSION

In this study, eighteen endophytic bacteria were isolated from stems and roots of different rice varieties from different places. According to the biochemical tests, five isolates were assumed to be *Rhizobium* spp. and thirteen isolates were *Azospirillum* spp. although species identification could not be done.

Most strains show high IAA producing activity at 2 days incubation period by UV-VIS spectrophotometer method (530nm) and isolate R_7 was found to be the best strain producing IAA, 120.55 ppm. In analyzing nitrogen fixation activity by ammonium test kit, S_5, S_7, S_8 isolates could fix nitrogen little and others couldn't fix. In nitrogen free semi-solid agar medium containing trace amount of bromothymol blue as indicator, all isolates showed nitrogen-fixing activity during seven days incubation period.

According to the results of phosphorous solubilizing activity by plate screening method and by UV-Vis spectrophotometric method (830 nm), isolate S_5 was found to be the best phosphate solubilizer. R_3 is the best K-decomposer by plate screening method and by AAS method. S_3 and R_3 have good antagonistic activities against *Rhizotonia solani* and LS and R_5 against *Phythium* spp. and S_7 to *Fusarium oxysporum* respectively. They couldn't be overwhelmed by these plant pathogens and could live with these pathogens. So, this fact can benefit the host plants.

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REFERENCES

1. Kobayashi, D. Y. and Palumbo, J. D.: Bacterial endophytes and their effects on plants and uses in agriculture, In: Bacon, C.W. and White, J. F. (Eds.) Microbial endophytes. Marcel Dekker, Inc., N.Y., New York, 2000, pp. 199-233.
2. Sturz, A. V., Christie, B. R., Matheson, B. G. and Nowak, J.: Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth, Biol. Fertil. Soils, 1997;25:13 – 19.
3. Kirchhorf, G., Reis, V. M., Baldani, J. I., Eckert, B., Döbereiner, J. and Hartmann, A.: Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants, Plant Soil 1997;194:45-55.
4. Chen, C., Bauske, E. M., Mussan, G., Rodriguez-Kabana, R. and Kloepper, J. W.: Biological control of Fusarium wilt on cotton by use of endophytic bacteria, Biol. Control, ;9955:83-91.
5. Liu, L., Kloepper, J. W. and Tuzun, S.: Induction of systemi resistance in cucumber against Fusarium wilt by plant growth-promoting rhizobacteria, PhytoPath, 1995;5:695-698.
6. Sturz, A. V.: The role of endophytic bacteria during seed piece decay and potato tuberization, Plant Soil, 1995;175:257-263.
7. Frommel, M. I., Nowak, J. and Lazorovits, G.: Treatment of potato tubers with a Pham Quang Hung et al. growth promoting Pseudomonas sp.: plant growth responses and bacterium distribution in the rhizosphere, Plant Soil, 1993;150:51-60.
8. McInroy, J. A. and Kloepper, J. W.: Survey of indigenous bacterial endophytes from cotton and sweet corn, Plant Soil, 1995;173:337- 342.
9. Carroll, G. C.: The Biology of Endophytism in Plants with Particular Reference to Woody Perennials. In: Microbiology of Phyllosphere (Fokkema, N., J. and van den Heuvel, J., eds.), Cambridge University Press, London, 1986;205-222.
10. Petrini, O.: Fungal Endophyte of Tree Leaves. In: Microbial Ecology of Leaves (Andrews J and Hirano SS, eds.), Spring-Verlag, New York, 1991;179-197.
11. Saikkonen, K., Faeth, S. H., Helander, M. and Sullivan T. J.: Fungal endophytes: a continuum of interactions with host plants, Ann. Rev. Ecol. Syst, 1998;29: 319-343.
12. Misaghi, I. J. and Donndelinger, C. R.: Endophytic bacteria in symptom-free cotton plants, Phytopathology, 1900;80: 808-811.
13. Chanway, C. P.: Inoculation of tree roots with plant growth promoting soil bacteria: an emerging technology for reforestation, Forest Sci, 1997;43: 99-112.
14. Ting, A. S. Y., Meon, S., Kadir, J. and Radu, S.: Endophytic microorganisms as potential growth promoters of banana, BioControl, 2008;53: 541-553.
15. Araújo, W. L., Marcon, J., Maccheroni, W. J. and Van Elsas, J. D.: Diversity of endophytic bacterial populations and their interaction with Xylella fastidiosa in citrus plants, Appl. Environ. Microbiol, 2002;68: 4906-4914.
16. Lacava, P. T., Araújo, W. L., Marcon, J. and Maccheroni, W. J.: Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria Xylella fastidiosa, causal agent of citrus-variegated chlorosis, Lett. Appl. Microbiol, 2004;39: 55-59.
17. Yanni, Y. G., Rizk, R. Y., Corich, V., Squartini, A., Ninke, K., Philip-Hollingsworth, S., Orgambide, G., Bruijn, F. D., Stoltzfus, R., Buckley, D., Schmidt, T., Mateos, P. F., Ladha, J. K. and Dazzo, F. B.: Natural endophytic association between Rhizobium leguminosarum bv. trifolii and rice roots and assessment of its potential to promote rice growth. Plant and Soil, 1997;194: 99-114.
18. Mayak, S., Tirosh, T. and Glick, B. R.: Plant growth promoting bacteria confer resistance in tomato plants to salt stress, Plant Physiology and Biochemistry, 2004;42: 565-572.