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ISOLATION OF ENDOPHYTIC BACTERIA FROM GREEN GRAM AND STUDY ON THEIR PLANT GROWTH PROMOTING ACTIVITIES

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ABSTRACT: A total of twenty five endophytic bacteria were isolated from green gram *Vigna radiata* (L.) plant. Four isolates which have shown better response for plant growth promoting effects on green gram plants were used as Plant Growth Promoting Bacteria (PGPB) in this study. It has been detected that up to 40% of the genomic 16S rRNA of isolated bacteria belongs to the *Azotobacter* genus. The result for phosphate solubilizing activities in NPRIB broth and Pikovskaya broth by UV-VIS Spectrophotometer (830 nm) showed that isolate FR was the best candidate in solubilizing of phosphorous showing 49.817Ppm with NPRIB broth and 53.534Ppm with Pikovskaya broth,respectively. Of all studied endophytes, isolates NL and NR have shown high nitrogenase activities and the activities being 2570.40 and 2108.00 n mole of C_2H_4 $ml^{-1}h^{-1}$ respectively. These strains will be selected for further use. The IAA production of all isolates was identified by FTIR spectroscopy and Spectrum for Crude IAA Extract of isolate FL is the best candidate for IAA production. The insoluble Potassium (K^+) content in mica sample and the soluble K^+ content by the inoculated samples were analyzed the atomic absorption spectrophotometric (*AAS*) method and isolates FL, FR, and NR were found to be good at decomposing insoluble potassium. These results indicated that the selected all four endophytic microbes could be excellent candidates for use as biofertilizer.

Key words: Endophytic bacteria, Green gram, Plant growth

INTRODUCTION

The genus *Vigna* comprises 100 to 150 species. Of these, green gram or mungbean and black gram or urdbean are the most widely cultivated. Both of these species were classified in the genus *Phaseolus* until (Wilczek, 1954) created the new name *Vigna radiata* (L.) Wilczek for *Phaseolus aureus* (L.), greengram and (Hepper, 1956) gave the new name *Vigna mungo* (L.) Hepper for *Phaseolus mungo* (L.), blackgram. Methods of differentiation between the genera *Phaseolus* and *Vigna* have been reviewed by (Evans, 1975).

Green grams are very nutritious for human consumption. The dry beans are a good source of vitamins and minerals and contain around 20% protein. Around the world they are consumed directly and used in various dishes including curries, soups, breads, sweets, noodles, and solids (Anonymous, 2008).The agriculture and food sector are expected to move toward environmentally sustainable development, while increasing its productivity and simultaneously protecting the natural resource base for future generations. A renewed interest in the internal colonization of healthy plants by (nonrhizobial) bacteria has arisen as their potential for exploitation in agriculture becomes apparent (Fahey.et.al, 1991), (Kloepper.et.al, 1992), (Turner.et.al, 1993). Bacterial endophytes are consistently reported present in the root, stem, leaf, fruit, and tuber tissues of a wide range of agricultural, horticultural, and forest species.

The extensive and frequent occurrence of such populations seems to indicate that (1) healthy plants carry populations of endophytic bacteria, and (2) the Plant Kingdom represents a vast and relatively unexplored ecological niche for these organisms. Even so, the occurrence of communities of endophytic bacteria, although regularly identified, has received relatively little attention (Chanway, 1995). Recently, agronomists have begun to reexamine the possible roles of endophytic bacteria in the growth and health of plants; not only when devising new systems of crop production, but also in the interpretation of results from field experiments (Hallman.et.al, 1997). From the nearly 300,000 plant species in the globe, each one hosts several to hundreds of endophytes (Tan and Zou, 2001), creating an enormous biodiversity. Most healthy naturally propagated plants grown in field or potting soils are colonized by communities of endophytic bacteria, embracing a wide variety of species and genera. These bacteria form nonpathogenic relationships with their hosts: some beneficial, some neutral, and some detrimental. Such associations can increase plant growth and hasten development or improve resistance to environmental stress. Endophytic bacteria have been implicated in supplying biologically fixed nitrogen and these associations can increase the nitrogen economy of a crop, reducing the requirement for nitrogen fertilizers. On the other hand, endophytic bacterial ammonifiers and nitrifiers are responsible for the conversion of organic nitrogen compounds into inorganic forms (NH_4^+ and NO_3^-) which are available for plants. Endophytic bacteria can also enhance the solubility of insoluble minerals such as phosphorous and potassium. The availability of sulfur, iron and manganese are also affected by redox reactions carried out by endophytic rhizosphere bacteria. Likewise, chelating agents can control the availability of micronutrients and participate in mechanisms of biocontrol of plant pathogens. Due to these and other benefits on plant growth, some endophytic bacteria have been called Plant Growth Promoting Rhizobacteria (PGPR).

In this study, a total of twenty five endophytic bacteria were isolated from green gram *Vigna radiata* (L.) plant. Four *Azotobacter spp*; which have shown better response for plant growth promoting effects on green gram plants were used as Plant Growth Promoting Bacteria (PGPB). The capability of colonizing internal host tissues has made endophytes valuable for agriculture as a tool to improve crop performance. This study aims to show natural associations between bacterial endophytes and their hosts, and discusses how such relationships can be employed in agricultural crop production.

Functions of *Azotobacter*: *Azotobacter* naturally fixes atmospheric nitrogen in the rhizosphere. There are different strains of *Azotobacter* each has varied chemical, biological and other characters. However, some strains have higher nitrogen fixing ability than others. *Azotobacter* uses carbon for its metabolism from simple or compound substances of carbonaceous in nature. Besides carbon, *Azotobacter* also requires calcium for nitrogen fixation. Besides, nitrogen fixation, *Azotobacter* also produces, Thiomin, Riboflavin, Nicotin, indol acitic acid and giberalin. When *Azotobacter* is applied to seeds, seed germination is improved to a considerable extent, so also it controls plant diseases due to above substances produced by *Azotobacter*.

MATERIALS AND METHODS

Endophytic Bacterial Growth Media

The calculated amount of ingredients for nitrogen free medium Modified Döbereiner medium (MDM medium, 1 L) was weighed as medium Composition (Agar: 15g, $CaCO_3$: 1.0g, K_2HPO_4: 1.0g, MgSO4: 0.2g, NaCl: 0.2g, $FeSO_4.7H_2O$: 0.1g, Na_2MoO_4: 5.0mg, Glucose Solution: 50ml) at pH 7.0$\pm$0.2 at 250C for this endophytic bacteria. The plates were incubated at 26 ± 2°C for 6-7 days for growth of endophytes to occur.

Isolation of Endophytes and Effectiveness of Surface Sterilization

It is important to validate the effectiveness of surface sterilization (Schulz B et.al., 1993). Four strains of endophytic bacteria used in this study were obtained from root and leaves tissues of mungbean plant by sterilizing the surfaces of the tissues with different concentrations of cocorex (for e.g., 10%, 15%, 20%, etc), 70%ethanol, antibiotics and antifungal agents.

Firstly, the surface sterilized samples were washed in sterile distilled water thrice and then soaked in 5ml sterile water and stirred for 1min. An aliquot of 0.5-ml suspension was then inoculated on to MDM agar plates, incubated at 26 ± 2°C, and observed for microbial growth. Then, the isolated endophytes cultures that developed on MDM-Agar medium were submerged in 70% (v/v) ethanol for 5min and sodium hypochlorite solution (0.9% w/v, available chlorine, cocorex was used in this study) and antifungal agents for 20min, then reinoculated on to MDM-agar medium, incubated at 26 ± 2°C for 7days and any growth of endophytes recorded.

Test for the Characterization of Bacteria

A total of twenty five strains were identified tentatively on the basis of their colonial, morphological and microscopic characteristics and some criterion tests such as Motility, Fluorescein production, growth on other N_2 free media, Methyl red, Voges-Proskauer, H_2S production, Citrate utilization, Starch hydrolysis, Gelatin liquefication, Catase , Oxidase, Urease, Nitrate reductase and fermentation with Dextrose, Mannitol , L(-) Rhmnose and Triple Sugar Iron as carbon sources. The observations obtained indicated that, they were endophytic *Azotobacter* sp.

Screening and Determination of Phosphorous Solubilizing Activity

The phosphate solubilizing activities of the isolates were qualitatively tested in Pikovskaya medium. All *Azotobacter* strains were cultured on Pikovskaya's medium supplemented with 0.5% insoluble phosphate and incubated at 30ºC for 5 days. After that, the solubilizing activity was measured by diameter of clear zone around the colony. Tricalcium phosphate, rock phosphate and calcium phosphate were used as substrates.

The phosphate solubilizing activities of the isolates were quantitatively tested by UV-VIS spectrophotometer.

*(a)Preparation of sample solution***:** Bacterial isolates were further evaluated for their Phosphorous solubilizing activity in Pikovaskia liquid medium. Phosphate solubilization in liquid media was quantified in a flask (100ml) and incubated at 37ºC and 150rpm. Uninoculated medium served as the control. Ten-milliliter samples were withdrawn every 24 hours for 1 week and P solubilizing activities of these samples were measured by spectrophotometric method. Sodium molybdate solution and hydrazine sulphate solution were used to form blue color complex and measured at 830nm. The intensity of the blue color is propotiobal to the amount of phosphate initially corporate in the heteropoly acid.

*(b)Preparation of molybdate solution***:** 12.5 g of Analytical Grade sodium molybdate ($Na_2MoO_4.2H_2O$) were dissolved in 10N sulphuric acid.

*(c)Preparation of standard phosphorous solution***:** 0.2197 g of Analytical Grade potassium dihydrogen phosphate (KH_2PO_4) were dissolved and diluted to 1 liter with deionised water. The concentration of solution was 500ppm. The required concentrations were made up by serial dilution method.

*(d)Construction the calibration curve***:** By using spectrophotometer, 2ml sodium molybdate solution was added into the conical flasks containing 10 ml of various concentrations of soluble KH_2PO_4 obtained from serial dilution, diluted to 100ml with demonized water and mixed well. The flasks were immersed in a boiling water bath for 10minutes, removed and cooled rapidly. The flask was shaken and adjusted the volume and measured the optical density (OD) at 830nm against blank solution. The calibration curve was constructed by using absorbance versus various dilution of standard phosphate solution, containing various concentrations of phosphate.

*(e)Determination of phosphorous solubilizing effect in broth culture***:** Total P accumulation in cultures of different bacterial species grown insoluble mineral phosphate ($Ca_3(PO_4)_2$) was determined as follows. The optical densities (OD) were measured at 830nm against the reagent blank. When the measured value was substituted in the equation obtained from the calibration curve, the amount of P-solubilized in the broth is obtained. The value obtained from the measurement of the control was then similarly calculated and subtracted from that of the value from the culture. This resulted in the value of P solubilized only by the bacterium.

Screening and Determination of Nitrogen Fixation Activity

Nitrogen fixation activities of the isolates were measured by Ammonia test kit and estimated fixing amount by endophytic organisms.

Acetylene Reduction Test: The discovery that the nitrogenase enzyme responsible for N2-fixation also reduced C2H2 (acetylene) to C2H4 (ethylene) (Dilworth, 1966) provided a useful assay for the quantification of the N2-fixation process. All twenty five isolates were grown in separate conical flasks and incubated at 30ºC for 7 days. These flasks are then kept on shaker for about 72-50 hours so as to obtain full growth of bacteria in the medium of which 10-15 ml., of both is transferred to a bottle, to this bottle 10 ml of acetylene gas is added and bottle is closed with cork borer and allowed to stand in the shed for 2-4 hours to have reaction of enzyme nitrogenase with acetylene gas. During this period, acetylene is converted to ethylene. Percentage of both the gases is measured chromatographically. The strain which has more nitrogenase enzyme forms more ethylene gas.

Screening and Quantitative Measurement of Potassium Decomposing Activity

The potassium (K^+) decomposing activity of the isolates was also determined on Nitrogen Free Medium placing insoluble Potassium Mica. The insoluble K^+ content in mica sample was analyzed by AAS method that was carried out by department of atomic energy (Pyin Oo Lwin, Ministry of Science and Technology, Myanmar). The mica sample was washed to remove soluble potassium (K^+) and dried at 40ºC in microwave oven and then mica samples were supplemented in bacterial culture to determine the K^+ decomposing activity.

Quantitative measurement of K^+ decomposing activity: Nitrogen free media were prepared and sterilized at 121ºCfor 15 minutes at 15 psi pressure. After sterilization, the mica sample was supplemented, inoculated bacteria and cultivated at 28ºC in 90rpm. After 7 day culture, the insoluble mica was removed by centrifugation at 3000rpm and then the supernatants were dried and analyzed the soluble K content by AAS method that was carried out by department of atomic energy (Pyin Oo Lwin, MOST, Myanmar).

Screening of Indole Acetic Acid (IAA) Productive Activity

Indoleacetic acid (IAA) productions of the isolates were determined by calorimetrically using ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$). To measure IAA, the major auxin involved in many of the physiological processes in plants, *Azotobacte*r isolates were aspetically cultured in Luria ' s broth containing 100 mg tryptophan per one litre . These were then incubated on the rotary shaker at 200 rpm, at 30ºC under dark conditions for 72 hrs. After harvesting, these were centrifuged at 10,000 rpm at 10ºC for 10 min. One ml of supernatant was mixed with 2 ml of Salkowski's reagent (20 ml of 35% percholic acid: 2 ml of 0.5 N $FeCl_3$) and one drop of 93 % orthophosphoric acid and incubated at room temperature for 15-30 min. Development of pink color showed IAA production.

Study on Antagonism of Endophytes

For evaluation of the antagonistic potential of isolates against common soil borne plant pathogenic fungi such as *Fusarium oxysporum*, *Pythium* sp., *Rhizoctonia soloni*, the dual culture antagonistic bioassay has been conducted in this study. All three plant pathogenic fungi were obtained from **BIOTEC** culture collection LAB, Thailand Science Park. Potato Dextrose Agar has been used. Inhibition zone formation was studied.

Study on Drug Sensitivity Pattern of Endophytes

Different antibiotics were used as markers for strain selection and as well as to test intrinsic antibiotic activities of the isolates. Four kinds of antibiotics such as Ampicillin, Kanamycin, Tetracycline and Gentamycin were used in this study.

Study on the Plant Growth Promoting Effect and Endophytic Activity on Green Gram

Each endophytic isolate was cultivated in nitrogen free media. After seven days incubation, the culture broths in pot and petridish cultivation of mungbean to study the potential effects of plant growth regulators.

For the endophytic activity, the sterilized soil was used and mungbean was cultivated in glass house. After seven days cultivation, various parts of plant tissues were carried out surface sterilization, crushing and microscopic determination. The plant growth of individual endophytic strain was recorded photographically. Plant growth parameters with endophytes in pot experiment were studied.

Isolation of Endophytic Genomic DNA

In this work, four isolates of endophytic *Azotobacter*, showing Plant Growth Promoting activities were identified. 10 ml bacterial culture each was divided into four microcentrifuge tubes and centrifuged at 5,000 rpm at room temperature for 5 min. The supernatant was discarded and the cell pellet was crushed in liquid nitrogen to fine powder using an acrylic shaft. Then, 800 µl of extraction buffer (2.5% CTAB , 100mM TrisHCl, 20mMEDTA,1.4 mM NaCl , 1% Polyvinyl pyrotidone and 2%β- mercaptoethanol) was added into each tube and incubated in water-bath at 65°C for one hour. 400µl of chloroform: isoamyl alcohol (24:1) was added into each tube and shaken gently for 10 min till the suspension is formed. Then, these were centrifuged at 10,000 rpm at 4 °C for 10 min. The upper layer was transferred into fresh tubes and 400µl of cold ethanol was added and kept at – 20° C for overnight. The tubes were centrifuged at 10,000 rpm, 4 °C for 10 min and DNA pellet was washed with 400 µl of 70 % ethanol. The DNA pellet was then dried under vacuum and dissolved in 50µl of sterile water and stored at 4 °C. DNA quality and quantity was compared with known standard genomic DNA on 1 % agarose gel in TAE buffer.

PCR (Polymerase Chain Reaction) Amplification of 16S ribosomal DNA (rDNA)

DNA Primers were specifically designed to anneal to highly conserved regions of the 16S rRNA of genus *Azotobacter*. The primers used for PCR amplification were 5′ CGCAACCCTTGTCCTTAGTT 3′ and 5′ TAGCGATTCCGACTTCACG 3′.The thermal circler was programmed as follows: 10 minutes at 94ºC; 25 cycles of 1 minute at 94ºC, 2 minutes at 59ºC, and 2 minutes at 72ºC; and 5 minutes at 72ºC. The amplification products were analyzed by electrophoresis with 2% (w/v) agarose gels in TAE buffer.

16S rDNA Sequencing and Comparative Sequence Analysis

PCR products are commonly purified to remove excess nucleotides and primers. The PCR products were purified by using a Wizard® SV Gel and PCR Clean-Up System kit (Promega). Up to 95% recovery is achieved depending upon the DNA fragment size. After the products have been analyzed with gel electrophoresis, pure DNA samples (10µl each) were sequenced. Sequencing was performed with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit and a 377 DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions. All sequences were subjected to the BLAST (Basic Local Alignment Search Tool) in the Gene Bank database to determine the most likely identities of the selected isolates.

RESULTS

More endophytes were found in roots compared to stems and leaves, implying that roots might provide a better niche for endophytic colonization. Thus, in my study, A total of twenty five endophytic bacteria were isolated from different parts of mung bean plants such as leaves and roots and four isolates which have shown better response for plant growth promoting effects were used as Plant Growth Promoting Bacteria (PGPB) marking as FR, FL, NR, and NL. Based on the morphology of the colony as well as the characteristics of bacteria according to biochemical testes, the observations obtained indicated that, they were endophytic genus of *Azotobacter*. SEM (Scanning Electron Microscopy) micrograph of endophytic microbes has indicated endophytic microbes have been naturally found in mungbean plant tissues. The Phosphate solubilizing activities in NBRIB and Pikovskaya broth by UV-VIS Spectrophotometer (830 nm) were shown in Table: 1. Acetylene reduction test was carried out to determine the nitrogenase enzymes of the isolates and isolates NL and NR have shown high nitrogenase activities. The activities being 2570.40 and 2108.00 n mole of C_2H_4 $ml^{-1}h^{-1}$ respectively have been determined.

Table: 1. Phosphate Solubilizing Activities in Pikovskaya Broth by UV-VIS spectrophotometer (830 nm)

Samples	NR	NL	FR	FL
Ppm(NBRIB)	13.736	47.021	49.817	2.463
Ppm(Pikovskaya)	16.868	29.161	53.534	49.072

Table: 2. Potassium Decomposing Activity of Isolate FL Measured by DAE

No	Z	Formula	Line	Net int.(KCps)	Calc.conc(%)	Stat.error
1	11	Na	Na KA1-HR-Min	0.01935	0.029	22.60%
2	13	Al	Al KA1-HR-Min	0.09264	0.02	14.90%
3	14	Si	Si KA1-HR-Min	0.4041	0.054	6.61%
4	15	P	P KA1-HR-Min	0.03753	0.003	27.80%
5	16	S	S KA1-HR-Min	14.92	0.622	1.04%
6	17	Cl	Cl KA1-HR-Min	0.5943	0.036	6.51%
7	19	K	K KA1-HR-Min	0.1076	0.004	18.00%
8	20	Ca	Ca KA1-HR-Min	26.77	0.849	0.78%
9	26	Fe	Fe KA1-HR-Min	2.429	0.0111	3.24%
10	42	Mo	Mo KA1-HR-Min	6.208	0.0037	4.74%
11	46	Pd	Pd KA1-HR-Min	0.161	0.004	19.80%

IAA production and its optical density was screened salkowski's reagent (20 ml of 35% percholic acid: 2 ml of 0.5 N $FeCl_3$) and one drop of 93 % orthophosphoric acid and the results were shown in Figure: 1. The qualitative determination of IAA production of selected endophyte was done by using one dimensional thin layer chromatography (TLC) and the chromatogram was recorded. Then the IAA production was identified by FTIR spectroscopy and the results were shown in Fig: 5 and Fig: 6. The antagonism of the isolates were shown in Fig: 2. The insoluble K^+ content in mica sample and the soluble K^+ concent by the inculated samples were analyzed by AAS method in department of atomic energy (DAE, Pyin Oo Lwin). Results were shown in Table 2, 3, 4 and 5. And the K^+ content of mica has been detected and the result was shown in Table: 6. The PCR products of the four selected endophytes were shown in Fig: 7. The products were approximately 210-240 bp in size. In figure it was shown that BLASTN results of four selected 16S rDNA sequences representing isolate FR (242bp), FL (210bp), NR (226bp) and NL (211bp) respectively. BLASTN results of 16S rDNA showed isolate FR belongs to genus *Azotobacter* sp., isolate FL belongs to genus *Azotobacter vinelandii*, and isolates NR and NL belong to genus *Azotobacter chroococcum*, respectively. Each culture broth of five days incubation period was applied by inoculating into sterilized soil and then mungbean seeds were cultivated. The photos of germinated plants of mungbean after three days and seven days cultivation were shown in Fig: 3 and 4. And the plant growth effect on green gram endophytic bacterial culture in germination test after five week application period was shown in table 7.

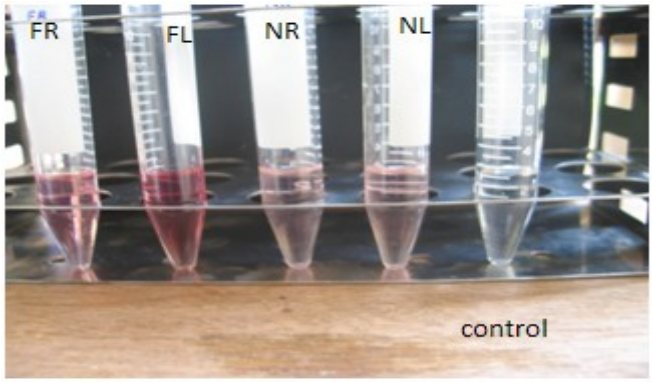

Figure: 1. Screeing of Isolates for IAA Productive Activity

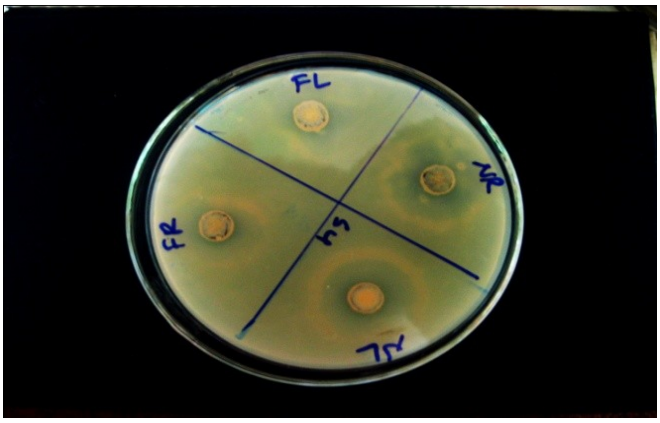

Figure: 2. Antagonastic Activity of Isolates against Common Soil Borne Pathogenic Fungi

Figure: 3. Comparative Study for the Response of Endophytes on Tested Plants after3 Days Application Period

NR NL FR FL

Figure: 4. Comparative Study for the Response of Endophytes on Tested Plants after 7 Days Application Period

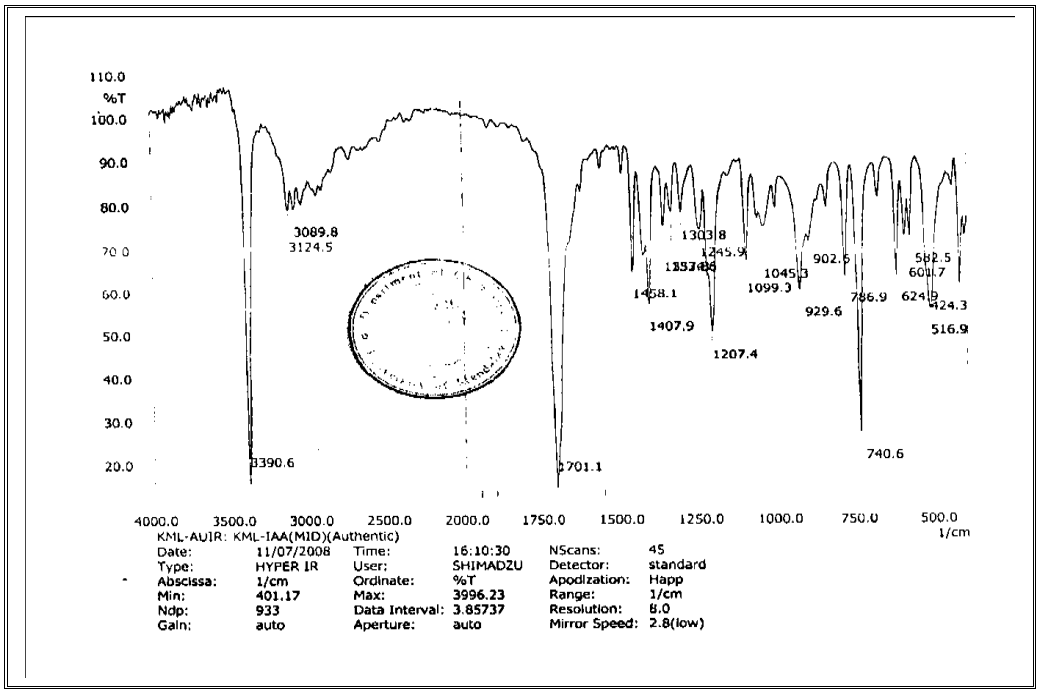

Figure: 5. FTIR Spectrum for IAA Authentic

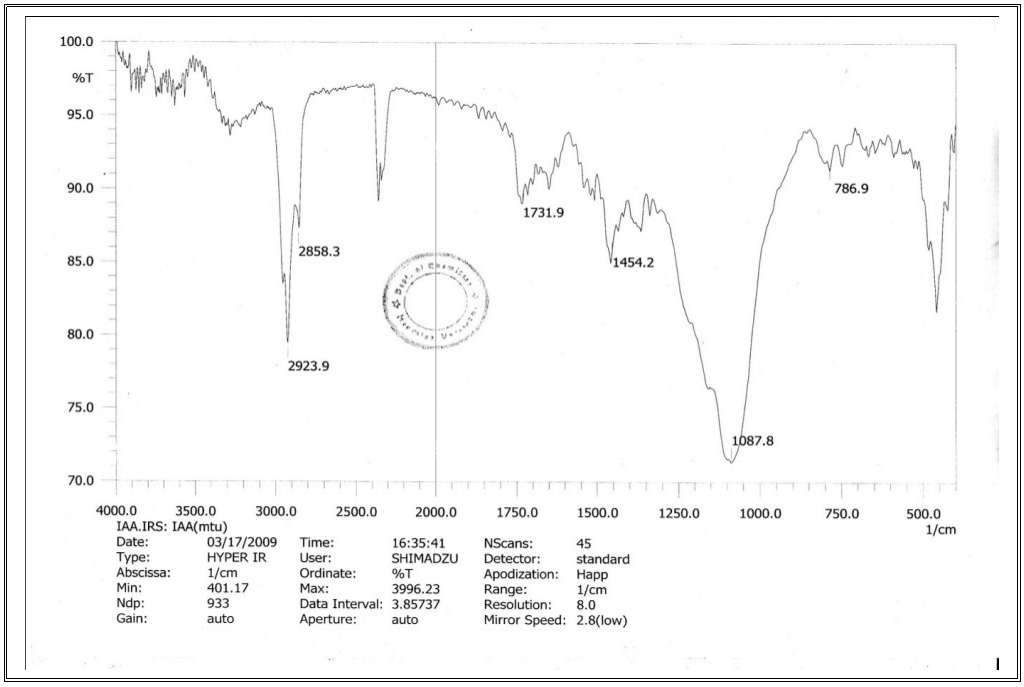

Figure: 6. FTIR Spectrum for Crude IAA Extract of Isolate FL

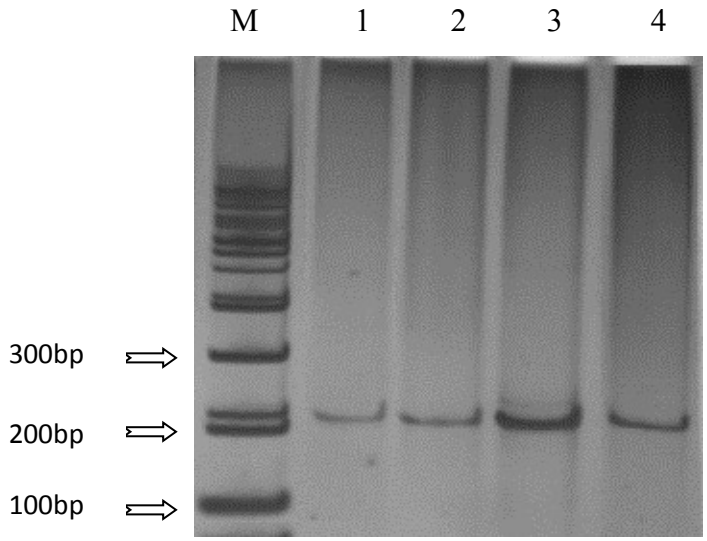

Figure.7. PCR patterns of selected endophytes. Lane1: FR, Lane2: FL, Lane3: NR, Lane 4: NL, and M: Molecular Weight Markers (100-bp DNA ladder)

Table: 3. Potassium Decomposing Activity of Isolate FR Measured by DAE

No	Z	Formula	Line	Net int.(KCps)	Calc.conc(%)	Stat.error
1	11	Na	Na KA1-HR-Min	0.03082	0.046	16.50%
2	12	Mg	Mg KA1-HR-Min	0.04257	0.011	17.40%
3	13	Al	Al KA1-HR-Min	0.05087	0.011	21.80%
4	14	Si	Si KA1-HR-Min	0.3682	0.05	6.99%
5	16	S	S KA1-HR-Min	21.92	0.918	0.86%
6	17	Cl	Cl KA1-HR-Min	0.6785	0.042	6.00%
7	19	K	K KA1-HR-Min	0.1042	0.004	17.10%
8	20	Ca	Ca KA1-HR-Min	33.6	1.09	0.69%
9	26	Fe	Fe KA1-HR-Min	2.264	0.0109	3.38%
10	42	Mo	Mo KA1-HR-Min	6.754	0.0042	4.16%
11	46	Pd	Pd KA1-HR-Min	0.1451	0.003	20.70%

Table: 4. Potassium Decomposing Activity of Isolate NR Measured by DAE

No	Z	Formula	Line	Net int.(KCps)	Calc.conc(%)	Stat.error
1	11	Na	Na KA1-HR-Min	0.0218	0.032	20.50%
2	12	Mg	Mg KA1-HR-Min	0.03399	0.009	21.10%
3	13	Al	Al KA1-HR-Min	0.06823	0.015	15.35%
4	14	Si	Si KA1-HR-Min	0.548	0.0734	5.61%
5	16	S	S KA1-HR-Min	3.242	0.134	2.25%
6	17	Cl	Cl KA1-HR-Min	0.6981	0.041	5.99%
7	20	Ca	Ca KA1-HR-Min	7.062	0.214	1.52%
8	26	Fe	Fe KA1-HR-Min	0.7939	0.003	7.93%
9	42	Mo	Mo KA1-HR-Min	5.072	0.0027	5.47%
10	44	Ru	Ru KA1-HR-Min	0.09992	0.002	19.50%

Table: 5. Potassium Decomposing Activity of Isolate NL Measured by DAE

No	Z	Formula	Line	Net int.(KCps)	Calc.conc(%)	Stat.error
1	11	Na	Na KA1-HR-Min	0.01702	0.025	24.60%
2	13	Al	Al KA1-HR-Min	0.07314	0.016	14.80%
3	14	Si	Si KA1-HR-Min	0.5887	0.0789	5.40%
4	16	S	S KA1-HR-Min	6.09	0.252	1.63%
5	17	Cl	Cl KA1-HR-Min	0.4354	0.026	7.89%
6	19	K	K KA1-HR-Min	0.08115	0.003	20.80%
7	20	Ca	Ca KA1-HR-Min	11.46	0.35	1.19%
8	26	Fe	Fe KA1-HR-Min	2.221	0.0093	3.49%
9	42	Mo	Mo KA1-HR-Min	4.831	0.0027	5.73%

Table: 6. Potassium Content of Mica Measured by DAE

No	Z	Formula	Line	Net int.(KCps)	Calc.conc(%)	Stat.error
1	11	Na	Na KA1-HR-Min	0.257	0.211	5.14%
2	12	Mg	Mg KA1-HR-Min	1.263	0.237	2.36%
3	13	Al	Al KA1-HR-Min	11.04	2.17	1.23%
4	14	Si	Si KA1-HR-Min	50.09	8.574	0.57%
5	15	P	P KA1-HR-Min	0.1708	0.027	11.30%
6	16	S	S KA1-HR-Min	103.7	10.28	0.39%
7	19	K	K KA1-HR-Min	6.881	0.823	1.54%
8	20	Ca	Ca KA1-HR-Min	124.2	14.66	0.36%
9	22	Ti	Ti KA1-HR-Min	1.847	0.216	3.00%
10	24	Cr	Cr KA1-HR-Min	0.1654	0.011	15.20%
11	25	Mn	Mn KA1-HR-Min	1.446	0.0646	3.77%
12	26	Fe	Fe KA1-HR-Min	63.2	1.971	0.51%
13	30	Zn	Zn KA1-HR-Min	0.3136	0.004	18.40%
14	37	Rb	Rb KA1-HR-Min	1.041	0.011	10.70%
15	38	Sr	Sr KA1-HR-Min	19.35	0.229	1.08%
16	40	Zr	Zr KA1-HR-Min	3.959	0.0481	3.77%
17	56	Ba	Ba KA1-HR-Min	0.1403	0.074	13.00%

Table: 7. Plant Growth Effect on Green Gram Endophytic Bacterial Culture in Germination Test after Five Week Application Period

Treatment	Germination rate (%)	Weekly data analysis (numbers of leaves/ plant height(cm))			
		1st week	2nd week	3rd week	4th week
FR	97.14	2/6.44	4/9.708	6/14.71	6/14.62
FL	88.57	2/5.74	5/8.978	6/13.20	7/14.46
NR	100	2/6.54	5/9.415	6/14.40	7/15.23
NL	100	2/6.11	5/8.164	6/11.76	7/12.86
Control	82.86	2/5.326	4/8.396	5/12.83	7/13.96

DISCUSSION AND CONCLUSION

Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic. Endophytic bacteria can promote plant growth and yield and can act as biocontrol agents. Endophytes can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry. In addition, it has been shown that they have the potential to remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilization and nitrogen fixation. There is increasing interest in developing the potential biotechnological applications of endophytes for improving phytoremediation and the sustainable production of nonfood crops for biomass and biofuel production (Ajcann, 2007).

Baldani, et.al, states that the ability to colonize the root interior, to survive only poorly in the soil, and to fix nitrogen in association with these plants is a characteristic of all these bacteria (Baldani.et.al, 2007).

Then, Dobbelaere, et.al, states that some endophytic bacteria, such as the genres of *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Azorhizobium*, form symbiotic relationships with their host plants and fix atmospheric nitrogen into plant available forms (Dobbelaere.et.al, 2003). According to Bai, et.al, endophytic bacteria, such as *Rhizobium*, are more likely to be plant growth promoting bacteria than bacteria found only in the rhizosphere (Bai.et.al, 2003).

For this study all endophytic bacteria that have a positive effect on plant growth will be considered PGPB. There are many different ways in which PGPB can aid in improving the growth, health, and yield of plants. The growth enhancement is due to the different effects the bacterium has on the plant itself or in the rhizosphere. Many PGPB can fix atmospheric nitrogen, produce growth promoting substances, increase nutrient uptake, enhance stress resistance, increase the solubility of organic and inorganic phosphate, control pathogens, and reduce heavy metal toxicity (Dobbelaere, et.al, 2003); (Burd.et.al, 2000).

Therefore, the reason for the isolation and application of these microbes in this study is because of their abilities to the plant, i.e, endophytic microbes were the best for bacterial colonization and the investigation of entry site of colonization, location and the spreading of the bacteria in root tissues, and quantification of bacterial colonization in leaves in addition to roots than some other kinds of bacteria that can be used in biofertilizer. So, it can be assumed that these bacteria may be excellent candidates for use in application as foliar spray.

Evaluations of Strains from this Experiment

Thus, in my study, four endophytic bacteria which have shown better response for plant growth promotion on green gram plants were used as Plant Growth Promoting Bacteria (PGPB) and marked as FR, FL, NR, and NL. From this study , all isolated endophytic microbes were tested their potassium decomposing activities at department of atomic energy, and three of them such as samples FR(*Azotobacter*. sp.), FL (*Azotobacter vinelandii*)and NL (*Azotobacter chroococcum*)were found that they could decompose the insoluble K^+.

Then, isolates were screened their intrinsic antibiotic sensitivities by using different antibiotics such as Ampicillin, Kanamycin, Penecillin and Gentamycin with different concentrations. Results showed that all the isolates were shown to be resistant to two antibiotics. So, it can be concluded that all these bacteria have the genetic material such as plasmid that can promote plant growth and together with antibiotic resistant genes as well. So, these bacteria were resistant to antibiotics because of their genetic materials such as chromosome or may be plasmids. As a consequence, to apply these microbes to field trial, proliferation of their genetic material such as proliferation of plasmid copy numbers and insertion of plasmids into competent cells may be the best way. According to the experiment, all isolates were found that they have antagonism against target pathogenic bacteria.

Of all studied strains, Isolate NL and NR (*Azotobacter chroococcum*) have shown high nitrogenase activities by acetylene reduction assay and these strains will be selected for further use naturally. But there have also been reports of microbes that can grow in nitrogen-free medium yet were negative in the acetylene reduction assay (Sharon L. Doty, et al, 2009). One possible explanation is that the test conditions may not be optimized for these isolates even though other isolates showed acetylene-reducing activity under the same conditions (Sharon L. Doty, et al, 2009). Alternatively, the capacity to reduce acetylene might not be essential for a functional nitrogenase (Sharon L. Doty, et al, 2009). For example, Gadkari et al. showed that the nitrogenase of *Streptomyces thermoautotrophicus* did not reduce acetylene and was not inhibited by acetylene (Gadkari et al., 1992). The nitrogenase enzyme was purified from this organism and verified to be unable to reduce ethine or ethene (Ribbe et al., 1997). Furthermore, Brighnigna and colleagues demonstrated that some epiphytic isolates could grow in nitrogen-free medium yet they were acetylene reduction negative (Brighnigna et al., 1992). Ozawa et al. described the isolation of 42 endophytes from which the *nifH* gene fragment could be isolated and could grow on nitrogen-free medium yet were negative for the acetylene reduction assay (Ozawa et al., 2003). It has been assumed that this indirect assay for nitrogen fixation may not be an absolute determinant for nitrogen fixation (Sharon L. Doty, et al, 2009). Therefore, the identification of *nifH* gene fragment of nitrogen fixing endophytes is highly recommended for further study.

According to the results of phosphorous solubilizing activities on both Pikovskaya broth and NBRIB by UV-VIS spectrophotometer (830 nm), isolate FR (*Azotobacter*. sp.) was found to be the best endophyte though all four isolates appeared to produce IAA. According to the results of screening of IAA productive activity, isolate FL (*Azotobacter vinelandii*) was found to be the best strain in terms of color reaction, and it has also been identified by the FTIR spectroscopy. Then Spectrum for Crude IAA Extract of isolate FL indicated it was the best candidate for IAA production

In this experiment, endophytic microbes were applied into pot experiment to evaluate the response of these microbes on plant growth. According to this study, isolate NR (*Azotobacter chroococcum*) shows the better response than the other three isolates after two weeks application period. So, according to these results, isolate NR(*Azotobacter chroococcum*) was considered to produce some more plant growth promoters for the shoot elongation in addition to IAA production although isolate FL (*Azotobacter vinelandii*) was shown the best candidate to produce IAA. Then, all endophytic bacteria were found that they have a positive effect on plant growth and health.

Finally, the endophytic bacteria which gave the beneficial effects to the target plant could be isolated from living tissues of the mungbean plant. So, all these isolates which gave the beneficial effects to plant respectively could be used biofertilizer and foliar spray form as well because all endophytic microbes were the best for bacterial colonization and the investigation of entry site of colonization, location and the spreading of the bacteria in root tissues, and quantification of bacterial colonization in roots than some other kinds of bacteria that can be used in biofertilizer. The authors detected a high abundance; up to 40% of the genomic 16S rRNA of bacteria belonging to the *Azotobacter* genus, indicating that this genus may play an important role in mungbean. In our study of mungbean endophytes, *Azotobacter* were the most abundant, and all of these grew vigorously in nitrogen-free medium. Furthermore, it has been known that endophytic microbes have target specificity, i.e., isolated endophytes from mung bean will only give the beneficial effects to mung bean plant. So, other endophytic bacteria from different crops should be isolated to standardize the tested microbes. And application of these microbes into other crops in addition to mung bean is recommended as well.

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