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Application of Endophytic Bacteria in Plant Growth Enhancement

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Abstract
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. Most endophytes appear to originate from the rhizosphere or phyllo sphere; however, some may be transmitted through the seed. 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 the present study the leaves and roots of medicinal plants were considered for the isolation of endophytic bacteria, namely, *Catharathus roseus, Murraya koenigii* and *Nerium oleander*. These bacterial isolates were tested for their ability to promote plant growth which was checked in *Vigna radiata* seeds and the potential of these isolates to increase the fertility of the soil indirectly was determined.

Keywords
Endophytic bacteria, *Vigna radiata*, Protein, Carbohydrates.

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INTRODUCTION:
Endophytic bacteria are ubiquitous with a rich biodiversity and unexplored biosynthetic potential[1,2]. By occupying the localized point of entry or by spreading within the plant, they produce an array of bioactive metabolites and hydrolytic enzymes to survive in the unique chemical environment of the host plant[3]. It has direct growth promotion effects through the production of phytohormones, N2 fixation, synthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase, phosphate solubilization and also through the production of antimicrobial metabolites or siderophores to inhibit pathogenic microorganisms [4,5,6].

Research has been conducted on the plant growth-promoting abilities of various endophytic bacteria. They differ from biocontrol strains in that they do not necessarily inhibit pathogens but increase plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients. Endophytes also promote plant growth by a number of similar mechanisms. These include phosphate solubilization activity [7,8], indole acetic acid production[9] and the production of a siderophore [10]. Endophytic organ, SMS can also supply essential vitamins to plants [12]. The recent areas where these plant growth-promoting bacterial endophytes are being used are in the developing areas of forest

regeneration and phytoremediation of contaminated soils.

As the plant growth promoting properties of endophytic bacteria can vary, it is important to study such properties from microbial populations associated with economically important and physiologically unique plants. In the current study, endophytic bacterial isolates from roots of *(Catharathus roseus, Murraya koenigii* and *Nerium oleander) M. koenigi* and *N. oleander and leaf sample of C. roseus* were investigated for plant growth promoting potential. Seven bacterial strains were isolated and identified.

MATERIALS AND METHOD:

Isolation of endophytic bacteria: A total of 12 colonies were isolated from the above 3 medicinal plants and depending upon the population and different morphological appearance of the colonies, 7 isolates were chosen for further study. Therefore, totally 7 colonies (2 from root and 5 from leaves) as shown in Table 1 were obtained for the study.

Table 1. Isolates obtained from 3 different medicinal plants

Plant sample	Leaf	Root
C.roseus	3 isolates	1 isolate
M.koenigii	1 isolate	1 isolate
N.oleander	1 isolate	-

Identification of Bacteria

Morphological Test: The identification of bacteria was carried out by morphological studies.

Biochemical Test: Indole test, Methyl red, Voges-Proskauer, Citrate utilization, H2S production, starch hydrolysis urease production and nitrate reduction was done.

MALDI-TOF-MS analysis: The organisms were further confirmed using MALDI-TOF-MS analysis.

Determination of Protein and Carbohydrate content in *Vigna radiata:* Bacterial isolates were grown in nutrient broth at 37 °C. The culture was centrifuged and the pellet was washed free of any medium. The pellet was resuspended in sterile distilled water. The culture was added in different concentrations (5ml,10ml,15ml) to 50gms of sterile garden soil. *Vigna radiata* (*V. radiata*) were sterilized using bavistin and sowed in the inoculated garden soil. The seeds were kept at room temperature and watered daily. After 7 days, the grown plantlets were removed, washed to remove the traces of soil.

The physical appearance of the plantlets was considered by measuring the shoot and root length with an inch tape and noted the values over a period of 7 days. The biochemical composition was determined by quantifying the protein levels of the plantlets were checked using Folin Lowry method of protein estimation analysis respectively. The amount of total carbohydrates present in the plantlets was determined by the anthrone method. Untreated garden soil was used as blank for growing *V. radiata*[11].

Estimation of protein by Lowry's method:

The blue colour developed by the detection of the phosphomolybdic. Photosynthesis compound in the Folin Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the Biuret reaction of the protein with alkaline cupric tartarate are measured at 660nm using colorimeter.

Materials required:

Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide.

Reagent B: 0.5% copper sulphate in 1% potassium sodium tartarate.

Reagent C: alkaline copper solution: mix 50ml of reagent A and 1ml of reagent B prior to use.

Reagent D: folin-ciocalteau reagent.

Preparation of stock standard solution: 100mg of Bovine serum albumin was weighed and dissolved in distilled water and the volume was made upto 100ml in the standard flask. 1ml of the solution contains 1mg protein.

Preparation of working standard solution: 10ml of the stock solution was diluted to 100ml with distilled water in a standard flask. 1ml of this solution contains 100µg protein. Into the series of test tubes 0.2,0.4,0.6,0.8 and 1ml of the working standard solution was pipetted out. Into the other test tubes 0.4ml of the test sample was taken. The volume in all the tubes were made up to 1ml with distilled water and one with only water which serve as the blank. 5ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10minutes. Then 0.1ml of reagent D was added and incubated at room temperature in dark for 30minutes. The blue colour developed was read at 660nm using colorimeter. The amount of protein present in the given sample was calculated using the

standard graph drawn by taking the O.D value in % axis and concentration of protein in X-axis.

Estimation of carbohydrate by anthrone method:

Sugars in the presence of concentrated H_2SO_4 get dehydrated and produce furfural or 5-hydroxy methyl furfural which then reacts with anthrone to produce a green coloured compound with an absorbants maximum at 630nm.

Materials required:

Anthrone reagent: 0.2% anthrone was dissolved in ice cold concentrated sulphuric acid. Prepared fresh before use.

Stock standard solution: 100mg of glucose was dissolved in 100ml of water in a standard flask.

Working standard solution: 10ml of the stock solution was diluted to 100ml.1ml of this solution contains 100µg of glucose.

Procedure:

Into the series of test tubes 0.2,0.4,0.6,0.8 and 1ml of the working standard solution was pipetted out. Into the other test tubes 0.4ml of the test sample was taken The volume in all the tubes were made up to 1ml with distilled water and one with only water which serve as the blank. 4ml of anthrone reagent was added to each tube including the blank, mixed well and kept in boiling water bath for 8minutes.after cooling to room temperature a dark green colour developed were read at 630nm against blank. The amount of carbohydrate present in the given sample was calculated using the standard graph drawn by taking the O.D value in Y-axis and concentration of carbohydrate in X-axis.

RESULTS AND DISCUSSION:

All the 7 bacterial isolates were selected to check the enhancement of plant growth physically and biochemically. V. *radiata* was treated for 24hrs in the presence of endophytic isolates culture medium with different concentration in the soil and were sowed into the soil. After 7 days of incubation the plant growth was observed. Among the 7 isolates, the isolates EB2, EB 4, EB5 and EB7 showed better growth after the 7 days of incubation. Hence it has been selected for further biochemical analysis - protein and carbohydrate estimations. The germination rate of root length and shoot length of the plants were measured and results are shown in Table 5.

Table 5: Measurement of root length and shoot length of *Vigna Radiata* on 7th day

Organism	Root length (cm)			Shoot length(cm)		
	5ml	10ml	15ml	5ml	10ml	15ml
Control	3.5			9.5		
B.flexus (*C.roseus)*	6	7	8	20	25	27
B.Cereus (C.roseus)	3	5	8	17	19	24
B.Cereus (M.koenigii)	4	5	6.5	10	12	17
S.Warneri (*N.oleander*)	3	4	10	7	11	17

As shown in Table 5 the root length of *V. radiata* in the presence of the 4 bacterial isolates varied with change in concentrations (5ml, 10ml and 15ml). In the presence of the isolate, *B.flexus,* the root length of the plantlets were measured as 6cm in 5ml,7cm in 10ml and 8cm in 15ml concentration and its shoot length was measured and found to be 20cm in 5ml, 25cm in 10ml, 27cm in 15ml followed by the presence of isolate *B.cereus* which showed a measure of root length like 3cm in 5ml,5cm in 10ml and 8cm in 15ml concentrations and its shoot length were 17cm in5ml,19cm in 10ml,24cm in 15ml . However, the plantlets in the presence of isolates *B.cereus* isolated from *M.koenigii* shows the measurement of 4cm in 5ml of isolate, 5cm in 10ml and 6.5cm in 15ml which indicates that the root length incrementally increases with the rising concentration of the inoculums. Similar trend was observed in shoot length measurement as 10cm growth in the presence of 5ml of inoculum,12cm in 10ml and 17cm in 15ml concentrations in the case of *B.cereus*

In the presence of *S.warneri.* the root length was found to be 3cm in 5ml of isolate, 4cm in 10ml and 10cm in 15ml and its shoot length includes 7cm in 5ml,11cm in 10ml and 17cm in 15ml concentration of isolates. These results clearly indicate the escalation in shoot and root length with increase in the concentration of the endophytes. Similarly, in the absence of the isolates the control plantlet was measured and it was found to 3.5cm as is root length. A study was done in lichen associating bacteria which was checked in *Zea mays* seeds where 3 different species were used and the root length and shoot length was measured[12]. The isolates were *Azotobacter chroococcum* which showed 24.4cm in

root and 24.7cm in shoot, *Bacillus cereus* showed 23.7cm in root and 25cm in shoot followed by *Burkholderia gathei* showed 25cm in root and 26.5cm in shoot; whereas the control shows root length of 20.5cm and shoot length of 23.5cm[13].

It was observed that endophytic bacterial strains significantly promoted growth of seedling *V. radiata* under different concentrations of inoculum. The inoculation resulted in early seedling growth and development of the plant. Soil conditions influenced growth promotion by bacterial strains namely, *B.flexus, B.cereus* and *S.warneri* which had more effect on plant growth parameters when compared to control plant which was devoid to isolates. When the concentration of cultures increased, the growth of the plants also sequentially increased indicating that the isolates play a major role in the enhancement of the plant growth.

Biochemical Observations: Protein content of Plants in the Presence of Isolates

The protein analysis of the treated and untreated plantlets showed a difference in the protein content. In the presence of isolates, EB 2 showed protein content in 0.3mg/ml in 5ml,0.7mg/ml in 10ml and 5.2 mg/ml in 15ml concentration followed by the plants grown in the presence of isolate EB 4 showed 1.7mg/ml in 5ml, 2.2mg/ml in 10ml and 4.1 mg/ml in 15ml concentration followed by the presence of isolate EB 5 showed 0.7mg/ml in 5ml, 4.1mg/ml in 10ml and 6.0 mg/ml in 15ml concentration. The isolate EB7 showed 0.1mg/ml in 5ml,3.0mg/ml in 10ml and 6.1 mg/ml in 15ml concentration of protein as shown in Table 6. Maximum protein concentration was observed when the plantlets were grown at the concentration of 15ml.

Present work is in concordance with a study done to estimate the total protein content present in the *Vigna radiata* seedlings and it showed maximum protein concentration when the plantlets were grown in the presence of the isolate *Bacillus pumilus* (1.02 mg/ml) followed by the plants grown in the presence of isolate *Coccobacilli* (1mg/ml)[14]. Another study also provides information about the protein estimation done in *AdhathodavasicaNees* (ashoka) plant sample and it was observed that the control of the untreated plantlet showed protein content of 2.5mg/ml while the treated plantlet showed 7.2mg/ml [15].

Carbohydrate content in *V. radiata* plantlets in the presence of isolates:

The carbohydrate analysis of the treated and untreated plantlets showed a difference in the carbohydrate content. In the presence of isolates EB 2 showed protein content in 1.7mg/ml in 5ml of isolate, 2.0mg/ml in 10ml and 9.0 mg/ml in 15ml concentration, followed by the plants grown in the presence of isolate EB 4 which showed 1.7mg/ml in 5ml, 3.2mg/ml in 10ml and 5.8 mg/ml in 15ml concentration, In the presence of isolate EB 5, it showed 2.5mg/ml in 5ml, 5.0mg/ml in 10ml and 6.0 mg/ml in 15ml concentration and the isolate EB7 showed 3.1mg/ml in 5ml,6.2mg/ml in 10ml and 7.5 mg/ml in 15ml concentration of protein as shown in Table 6. Maximum carbohydrate concentration was seen when the plantlets were grown in the presence of highest concentration (15ml) of bacterial culture. The present work shows similar trends with another study where they have estimated carbohydrate in *Vigna radiata* seedlings and it showed maximum carbohydrate concentration when the plantlets were grown in the presence of the isolate *Bacillus pumilus* (0.164 mg/ml) and *Trichoderma harzianum* (0.124 mg/ml) [16]. Another study also provides information about the carbohydrate estimation done in *Codiaeuvariegatumpictum (Croton)* plant sample and it was observed that the control of the untreated plantlet showed total carbohydrate content of 1.20mg/ml while the treated plantlet showed 4.50 mg/ml[17]. Hence, from the present study it is proved that in the presence of endophytic bacterial culture the plantlets showed higher concentration of protein and carbohydrate content when compared to the control plants.

Table. 6 Protein content and carbohydrate content in treated and untreated *V. radiata* plantlets

Organism	Concentration (ml)	Amount of protein (mg/ml)	Amount of carbohydrate (mg/ml)
B.flexus EB 2 (*C.roseus* leaf)	5	0.3	1.7
	10	0.7	2.0
	15	5.2	9
B.cereus EB4(*C.roseus* leaf)	5	1.7	1.7
	10	2.2	3.2
	15	4.1	8.5
B.cereus EB5(*M.koenigii* root)	5	0.7	2.5
	10	4.1	5.0

	15	6.2	6.0
S.warneri EB 7(*N.oleander* leaf)	5	0.1	3.1
	10	3.0	6.2
	15	6.0	7.5

CONCLUSION:

The results of the present study indicated that endophytic bacteria has good plant growth stimulating activity than normal bacteria when compared to control plantlets which were devoid of the isolates. Instead of using synthetic bio fertilizer, the endophytic bacterial content can be suggested to be applied in agricultural field in order to promote growth of plants and indirectly increase the fertility of the soil. They also play a vital role in the nutrition of the plant which is evident in the content of proteins and carbohydrates that grew in the presence of the bacterial isolates compared to the control plants that were not sprinkled with isolates.

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