Diversity of Bacterial Endophytes from Various Agricultural Crops

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***Abstract*: Endophytes are endosymbionts residing in the internal tissues of host plants without causing any apparent damage to the host. Total 50 bacterial endophytes were isolated from leaves of various crop plants. Some Gram positive and some Gram negative bacteria were isolated where Gram positive bacteria were predominantly observed. Conformation of isolates was done on the basis of biochemical characteristics and on Enzyme study such as Catalase, Amylase, Gelatinase, Protease, Lipase, Chitinase. These isolates were characterized for plant growth promoting traits as Phosphate Solubilization, Production of Ammonia, IAA, Production of Phenolic acid, Hydrogen Cyanide production and Root formation of carrot slices, mataki and chana.**

***Keywords*: Endophytic bacteria, Gram positive bacteria, IAA, Root formation.**

INTRODUCTION

The term endophyte (Gr. endo, with in; phyton, plant) was first coined by (De Bary, 1866).Plants are naturally associated with mutualistic microbes that include endophytes. Endophytes have been defined as diverse microbes, most commonly fungi and bacteria (Wilson, 1995; Strobel and Daisy, 2003), which spend the entire or part of their life cycle living in internal plant tissues causing no apparent or immediate disease symptoms (Hallmann *et al.*, 1997, Bacon and White, 2000; Long *et al*., 2008).

Endophytes play many important beneficial roles in the metabolism and physiology of the host plant *via* direct and indirect way. Direct way includes productions of phytohormones, phosphorous solubilisation, nitrogen fixation, and siderophore production as an iron chelators etc., while indirect way includes suppression of plant diseases by elevating plant resistance mechanisms or by producing various enzymes or metabolites (Bakker and Schippers, 1987; DeFago *et al*., 1990; Kachhap *et al*., 2014).

The study of endophytic bacteria has evoked great interest due to their beneficial properties like fixing nitrogen, producing auxins and protecting host plants from plant pathogens. Endophytic bacteria may play a significant role in protection against plant pathogens and in the overall productivity of an agricultural ecosystem (Hallmann *et al*. 1997; Sturz*et al.*, 2000). They can also produce unique secondary metabolites that can be exploited in pharmaceutical, agricultural and other industries.

They help to improve crops yields, by stimulating plants growth and immune response, excluding plant pathogens by niche competition, as well as activity participating in phenylpropanoid metabolism and antitoxidant activities (Pandey *et al*., 2018). Among plant microbiota, endophytic bacteria can be found in most plant species and be recovered from roots, leaves, steam and a few from flowers, fruits, and seeds (Lodewyckx *et al*., 2002). Leaf endophytic bacteria are the collection of selective phyllosphere bacteria and they reside in the leaves and maintain endophytic relationship with the host plant as well other microbe lives within the leaf. Every microbe within the leaf may have some beneficial function in terms of plant protection as well as to provide to nutrients to the plants (Neilands, 1981; Lindow *et al*. 2003).

Plants need phosphate and potassium they are major essential macronutrients, but soluble phosphate and potassium concentrations in soil for plant intake are usually very low. Phosphorus is one of the most essential elements for the growth and development of plants. The most efficient phosphate solubiliting Gram-positive bacteria (PSB) belong to the genera Bacillus. Besides, it has been reported that *B. amyloliquefasciens, B.megaterium, Bacillus* sp., exhibit phosphorus, potassium, and zinc solubilization (Verma *et al.*, 2015). Endophytes are known to supply nutrients to plant by fixing atmospheric nitrogen and solubilizing ion. This ultimately leads to increase in plant immune system as well as protects plants from infection by plant pathogens studies have also shown role of endophytes in removal of soil contaminants (Barac *et al*., 2004; Doty *et al*., 2009).

The endophytic bacteria are group of microorganism for promoting plant growth and have three metabolic pathways that result in plant development. They are phytostimulation, biofertilization and biological control are interrelated in the physiological processes of the plant (Gaiero JR *et al.* 2013).

Therefore, the aim of this study is to isolate and characterize some endophytic bacteria and study their ability for plant growth promoting traits *viz.*, phosphate solubilization, IAA, hydrogen cyanide production, ammonium production and production of phenolic compound; and preliminarily screening for Chitinase, Amylase, Catalase, Protease, Gelatinase and Lipase and the root formation.

Method

Collection of leaf sample

Healthy leaf sample were collected from legumes and non-legumes plants such as Cotton, Tur, Soyabean, Jower, Mung, Urad, Wheat, Chana, Mango, Chiku, Sitafal, Lemon, Guava, Papaya, Brinjal, Cabbage, Tomato, Lady Finger, Palak, Methi, Pudina, Tulsi, Neem, Ashoka, Kaner, Zandu, Shevanti, Kunda, Mogra and Hibiscus were obtained from various different places. The collected leaf were placed in clean plastic bags, brought to the laboratory and subjected to isolate endophytic bacteria.

Surface sterilization

Leaf sample was washed under running tap water for 1-2 min followed by 1 min wash with 75 % alcohol to remove the soil particles completely. Then the leaf sample was washed with sterile 1% (v/v) sodium hypocholoride embedded with 0.05% (v/v). Finally washed with phosphate buffer (pH 7.0) (Musson *et al*., 1995).

Isolation of Endophytic Bacteria

After surface sterilization, leaf sample cut into 5 mm square pieces with sterile blade and macerated separately in phosphate buffer of pH 7.0. Each of the leaf pieces embedded carefully on Nutrient Agar media and R2A Agar media. In each plate four leaf pieces were placed. Incubate at 37 ^{0}C for 24 hr.(Musson *et al.,* 1995).After incubation, suspension of bacteria which grown around the leaf sample was prepared. Further, isolation and purification of endophytic bacteria were carried out by using quadrant streak method on Nutrient Agar media (Sanders *et al.*, 2012). Incubate at 37^{0}C for 24 h. Then each of purified colonies than transferred to nutrient agar slants and were stored at 4^{0}C.

Identification

The pure cultures of organism were obtained. The identification is based on Morphological and Biochemical studies. The process of Gram Staining was done.

The identification was done on the basis of Bergey's Manual of Determinative Bacteriology.

Biochemical Studies

Enzyme study like Catalase, Amylase, Gelatinase, Protease, Lipase and Chitinase were studied IMViC test were performed sugar test were done using Glucose, Lactose and Mannitol (Cappuccino and Sherman, 1992).

Plant growth promoting traits

The selected bacterial strains were screened for PGP traits using various assays such as Production of Ammonia, Phosphate Solubilization, Indol Acidic Acid Production, Hydrogen Cyanide Production and Production of Phenolic Compound.

Phosphate Solubilization

Phosphate solubilizing ability of bacteria was determined on Pikovskaya agar medium. The isolates were spotted onto Pikovskaya agar and incubated at 28 ^{0}C for 3 days. The presence of halo zone around the bacterial colony was considered as indicator for positive ability to inorganic phosphate solubilization (Nguyeu *et al*., 1992).

Indole Acetic Acid (IAA) production The endophytic bacteria isolates was grown in Nutrient broth embedded with 0.2 ml of 1% L- trypotophan for 48 hrs. Further, 1.0 ml supernatant of each endophytic bacterial isolates mixed with 1.0 ml of Salkowski's reagent to develop the pink colour for the positive result of IAA production ad measured at 530 nm. The absorbance of the samples obtained was plotted against a standard to determine the concentration of IAA produced (Loper and Scroth, 1986).

Ammonia Production

Endophytic isolate inoculated into 10 ml of sterile Peptone Water Broth and incubated at 36^{0}C for 48-72 h. Nesseler's reagent (0.5 ml) was added in each tube. Colour change from brown to yellow indicates positive test and measured on spectrophotometrically at 450 nm. Concentration of Ammonia produced was estimated against standard curve of Ammonium Nitrate in the range of 0.1-1.0 µg/ml (Cappuccino and Sherman, 1992).

Hydrogen Cyanide Production

The selected isolates were grown in Nutrient Agar supplement with Glycine (4.4g/l). A Whatman filter paper No.1 soaked in 0.5% (w/v) Picric acid solution then placed on the surface of plate. Incubate at 37 ^{0}C for 5-7 days and the production of HCN was determined by the change of filter paper from yellow to red-brown (Lorck *et al*., 1948).

Production of Phenolic Compound

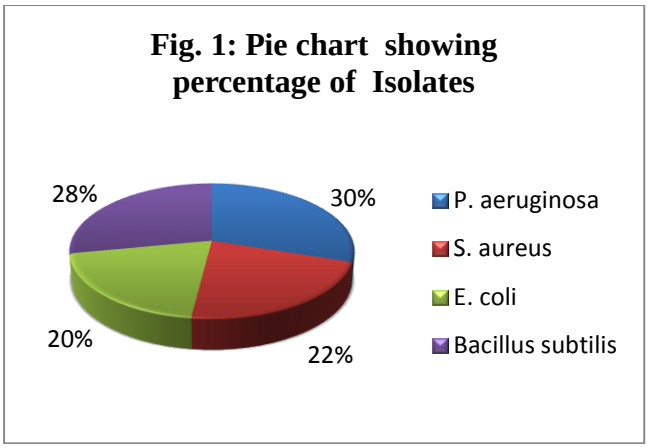
The isolates were grown in nutrient broth 10 ml each test tube incubated at 37^{0}C for 24 hr., after incubation centrifuge for 10 min. Then 10% Lead Acetate was added to the supernatant of centrifuged culture media, white precipitate was obtained confirming the presence of Phenolic Compounds (Tamiselvi *et al*., 2012).

Effect of Endophytic Bacteria on Root Formation

An experiment was carried out to test if endophytic bacteria can induce root formation. Small pieces of carrot, matki and chana with the same size were prepared, surface sterilized by submerging in 70% ethanol for 3 min, rinsing 3 times with 0.5% NaClO for 7 min. Finally, the sample was washed with sterile-distilled water three times. Sample were transferred to sterile tubes containing 5 ml MS growth media and 80 µl of the endophytic bacteria (OD_{600} ∓0.5) was added. The tubes were incubated at 25^{0}C for four weeks with a 16 h photoperiod. Observations were recorded on wet weight of the roots. Cutting samples placed in tubes containing media without endophytic bacteria were used as control (Etminani *et al.*, 2018).

Results and Discussion

A total 50 endophytic bacteria were isolated from 30 different leaves samples. Out of which 15 isolates of *P. aeruginosa*, 10 isolates of *E. coli*, 11 isolates of *S. aureus*, and 14 are of *Bacillus subtilis* (Fig.1).



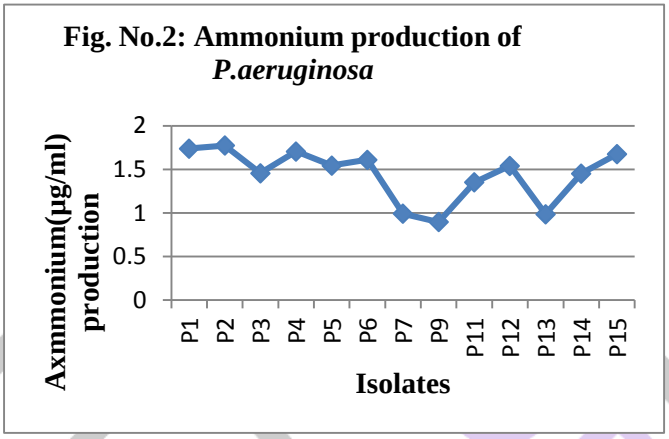


Table 1: Plant Growth Promoting Traits of Endophyte *Pseudomonas aeruginosa*

Isolates	Phosphate solubilization	IAA	HCN	Production of Phenolic compound
P1	+	+	-	+
P2	+++	+	-	++
P3	+	-	-	-
P4	+	+	-	+
P5	-	+	+	+
P6	+	+	-	+
P7	-	++	-	++
P8	+	-	-	+
P9	+	+	+	+
P10	+	+	-	+
P11	++	+	-	+
P12	-	+	-	+
P13	+	+++	-	+
P14	+	+	-	+
P15	++	+	-	+

Table No. 2: Plant growth promoting traits of Endophytic *E. coli*.

Isolates	Phosphate solubilization	IAA	HCN	Production of Phenolic compound
E1	+	+	-	+
E2	-	+	-	+
E3	-	-	-	+
E4	+	+	-	+
E5	+	+	-	+
E6	+	+	-	+
E7	++	+	+	+
E8	-	+	-	+
E9	+	+	-	+
E10	-	+	-	+

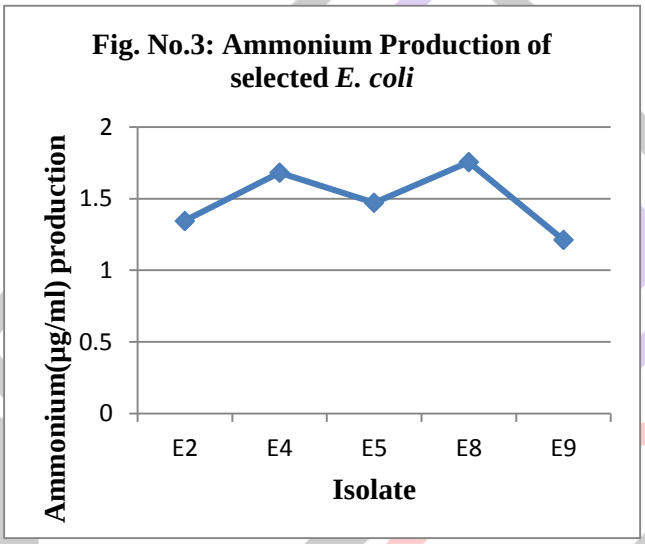


Table No. 3: Plant growth promoting traits of endophyte *S. aureus.*

Isolates	Phosphate solubilization	IAA	HCN	Production of Phenolic compound
S1	-	-	-	-
S2	-	+	-	-
S3	+++	+	-	-
S4	-	+	--	+
S5	-	-	-	-
S6	-	+	-	-
S7	+	-	-	+
S8	+	-	-	--
S9	++	+	-	-
S10	+	+	+	-
S11	-	+	-	-

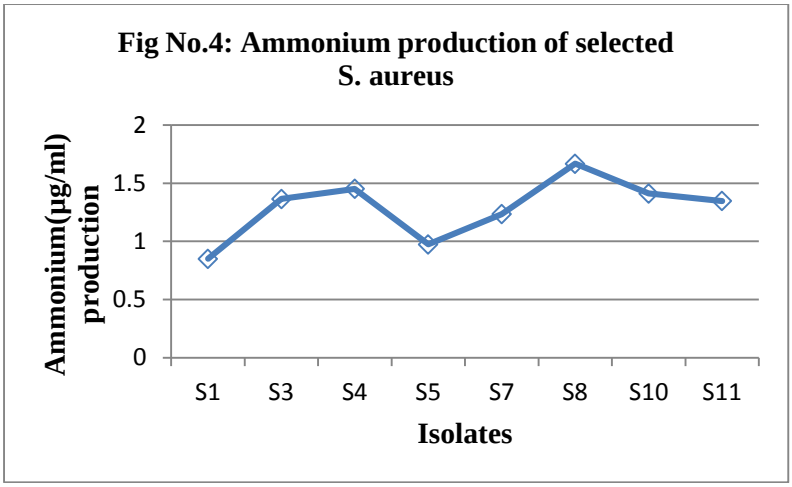


Table No. 4: Plant growth promoting traits of endophyte *Bacillus subtilies*

Isolates	Phosphate solubilization	IAA	HCN	Production of Phenolic compound
B1	+	+	+	+
B2	++	+	-	+
B3	+	+	-	+
B4	+	+	-	+
B5	+	++	+	+
B6	+	+	-	+
B7	+	-	-	+
B8	+	+	+	+
B9	-	+	-	+
B10	+	-	-	+
B11	+	-	-	+
B12	+	+	+	+
B13	-	+	+	+
B14	++	++	-	+

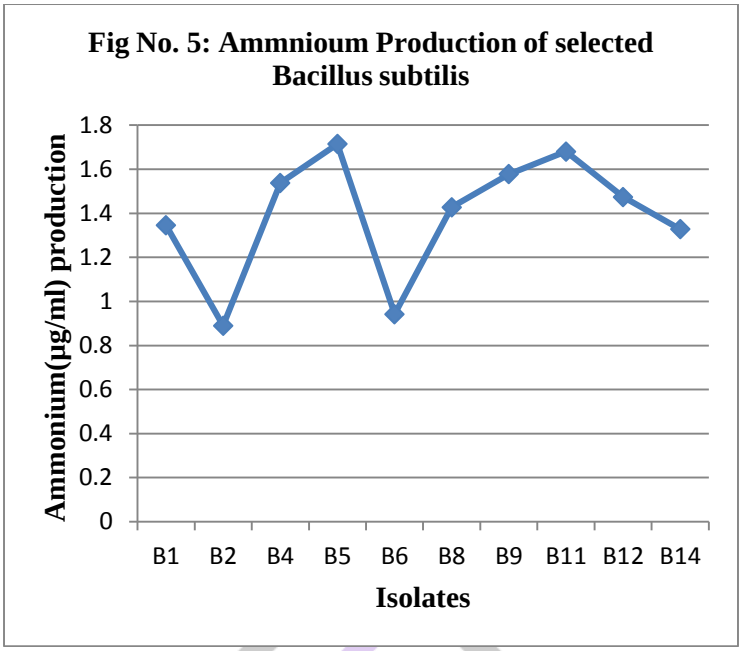


Table No. 5: Root colonization in plants inoculated with selected bacterial isolates

Sample	Isolate	Root length	Shoot length	Root fresh weight
Carrot	P2	2 cm	2.60 cm	0.10 g/tube
Chana	P11	3.10 cm	4 cm	0.15 g/tube
Mataki	P14	4 cm	4.50 c	0.20g/tube

The selected endophytic bacterial strains were morphologically characterized by Gram staining. It was observed that all the isolates were gram positive cocci in cluster and gram negative rods. The selected endophytic bacterial strains were screened for enzyme activity the results of which are tabulated.

On leaves of plants, limited sites are available where the pathogen can attack. Bacteria capable of multiplying within the leaf can compete with pathogens by producing enzymes or such chemical compounds as a metabolic product.

Microbes produce and excrete lytic enzymes such as chitinase, protease, gelatinase, and lipase, which can hydrolysepolymeric compounds *viz*., chitin, proteins and lipids. Secretion of these enzymes by different microbes results in the suppression of plant pathogenic activity directly. All endophytic bacteria show catalase positive. Similarly, also by Prasad *et al.*, (2014) show all gram positive bacteria catalase positive. In amylase activity except *E. coli* all the isolates shows the zone of hydrolysis. Though, *S. aureus* and *B. subtilis* show zone of liquification in gelatinase activity. Significantly, lipase activity was recorded with *P. aeruginosa*, *E. coli*, and *B. subtilis* as positive test. However, Prasad *et al.*, (2014) reported that from Avacado and Black grapes isolate were all Gram positive bacteria. Except A1 all other isolates were found to be lipase test. Also Mostly protease production was shown by 3 isolates, *E. coli*, *P. aeruginosa* and *B. subtilis.* Also by Prasad *et al.*, (2014) 2 *Bacillus sp.* shows protease positive. Among all endophytic bacteria only 2 bacteria *E. coli* and *B. subtilis* have zone of solubilization in chitin agar plate. According to Shaikh *et al*., (2017) *Pseudomonas sp*. showed all activities except chitinase production from *Gossypium hirsutum.* Hallmann *et al*., (1997) have reported that the *Pseudomonas* group (*Pseudomonas, Burkholderia*) and Enterobacteriacea (*Enterobacter, Klebsiella*) are the common taxa found in tomato, potato, cotton, soybean, rice, and maize. Similarly, according to Etminani *et al*., (2018) observed that *Pseudomonas protegens, Stenotrophomonas maltophilia* and *Bacillus anthracis* shows the colourless halo zone.

Phosphorus is one of the most essential elements for the growth and development of plants. From the 50 isolates (Table 5, 7, 9, 11) except P5, P7, P12, E2, E3, E8, E10, S1, S2, S4, S5, S6,S11, B2 and B14 other all the isolate shows phosphate solubilization. The highest production was seen in strains B2 and B14 *Bacillus subtilis*. Similarly, by Rodriguez and Fraga, (1999), strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. According to Ngamau *et al.*, (2012) *Pseudomonas, Serratia, Rahnella, Enterobacter, Yersinia, Yokenella and Ewingella Rahnella* showed the highest potential phosphate solubilizer from banana plants. By Abbas *et al*., (2018) *Citrobacter sp., Pantoea agglomerans, Pseudomonas oryzihabitans, Serrayia marcescens* and *Enterobacter aerogenes* shows phosphate solubilization.

Indole acetic acid production by bacteria is a part of mechanism between plant-microbial interactions. IAA is an auxin that regulates the plant growth and its cell division. To determine the IAA production each isolate was grown in LB broth embedded with tryptophan because tryptophan is belived to be the primary precursor for the formation of IAA in microbes. After 72 hr. incubation, significant amount of IAA production among all the endophyte *P. aureuginosa* P13 and the highest amount in *Bacillus subtilis* strain B5 and B14 was observed. Other strains also produced IAA in small or negligible amount (Table 1, 2, 3, 4). Similarly by Prasad *et al.,* (2014) observed maximum production of IAA was exhibited by gram positive cocco bacilli from avocado. According to Abbas *et al.,* (2018) *Citrobacter sp.* and *Pantoea agglomerans* endophytic bacterial strains produced IAA. Kuffner *et al*., (2007) observed *Serratia* give the production of IAA.

HCN production was observed only by 9 isolates out of 50 isolates *P. aeruginosa* P5 and P9, were *Bacillus subtilis* B1, B5, B8, B12 and B13 gives the highest HCN production. Were *E. coli* E*7* and *S. aureus* S10 observed in a very negligible amount of production (Table 1, 2, 3, 4). Similarly, Etminani *et al.,* (2018) observed *Bacillus anthracis* produces the highest HCN production and also according to Prasad *et al.,* (2014) noticed that gram positive short rods show the production of HCN from black grapes.

Ammonia production is an important plant growth promoting trait, where microbes can able to breakdown complex nitrogenous materials and convert it into ammonia, which is taken up by plant as a nitrogen source. In our study, peptone was used as a nitrogenous compound which breakdown leads to formation of ammonia; it was detected by colour change using Nessler's reagent. In the present study, it was observed that some endophytic isolates showed ammonia production in the range of 0.850-1.800 (µg/ml) within 48 hr. (Fig. 2, 3, 4,5). Similarly, Shaikh *et al.,* (2017) observed that all endophytic bacteria isolate showed ammonia production in a range of 1.6-0.8 (µg/ml).

The production of phenolic compound was observed that except P3, S4 and S7 other all isolates shows production of phenolic compound (Table 1, 2, 3, 4). Similarly, Vichare *et al*., (2015) observed Rods in chain shows the highest production of phenolic production comparatively by Rods in chains and singles, Coccobacilli in clusters, Cocci in clusters and Short rods from *Ficus racemosa L*.

In this research 3 selected strains were able to produce different amounts of phytohormone, indole acetic acid. The highest production of indole acetic acid had the greatest influence on root formation of carrot slices under in vitro condition. This phytohormone has a role in plant growth, promotes primary and lateral root elongation, and increase yield. The bacteria *P. aeruginosa* had promoted root formation when inoculated on the MS medium containing carrot slices, mataki and chana. The plants parameters root length, shoot length and root fresh weight were obsereved (Table no. 5). The highest value of root fresh weight recorded as 0.20 g/tube of mataki. Similarly by, Eetminani *et al.,* (2018) reported that *Pseudomonas protegens* had the highest effect of root formation. Zaghloul *et. al.,* 2016 observed inside the roots of faba bean plant, have the highest value log CFU 6.4×10^5 of isolate TN10 and its root fresh weight is 0.10 g/tube. This study had shown that there is a great diversity of endophytic bacteria colonizing different plant structures, and various benefits are related to this interaction. These potential isolates could be used to make consortium as biofertilizer or bioprotectant to enhance plant productivity and protection. To our knowledge this is report which is concerned with the isolation of bacterial endophyte from this plant host. The endophytic isolates also showed production of some compounds which if further identified can be used for probable application so. Many such plants associated with endophytes are an untapped source of novel bioactive compounds which could have significant industrial, pharmaceutical and medical application. This existence of such microorganisms suggests that they can be utilized in controlling plant diseases or enhancing plant growth.

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