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SCREENING OF ENDOPHYTE BACTERIA FOR BIOPROSPECTING POTENTIAL FROM *SYZYGIUM CUMINI* (JAMUN) AND *OCIMUM TENUIFLORUM* (TULSI)

Tanvi Taneja, Indu Sharma, Himani Garg, and Raj Singh*
Department of Bio-Sciences and Technology, Maharishi Markandeshwar Deemed to be University
Mullana-Ambala-133207 (Haryana), India.
*Corresponding author: dr.rajsingh09@gmail.com
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ABSTRACT

Endophytic microorganisms can promote plant growth through a number of mechanisms, including as the production of plant hormones and anti-microbial substances, as well as the addition of nutrients to soils, such as inorganic phosphate. This study aimed to evaluate the ability of endophytic bacteria isolated from Jamun (*Syzygium cumini*) and Tulsi (*Ocimum tenuiflorum*) leaves and stems to produce substances that aid in plant growth, such as indole-3-acetic acid, phosphate solubilization ability, nitrogen fixation, and ammonia synthesis, among others. It was found that out of five bacteria isolated, 4 isolates were gram negative B_1, B_2, B_3 and B_5. However B_4 isolate was gram positive. One bacterial isolate B_4 was found indole-3-acetic acid positive. All bacteria were producing ammonia and all isolates were found catalase test positive. Four bacterial isolates B_1, B_2, $B_3$and B_5 produced HCN. Three bacteria B_1, B_3 and B_5 had phosphate solubilizing activity and two B_1 and B_5 had nitrogen fixation activity.
Keywords **:** Endophytes, plant growth promoters, gram staining, nitrogen fixing bacteria, HCN production

Introduction

Endophytes are endosymbionts, generally bacteria that spend the most of their life inside plants without ever causing illness. Endophytes have been primarily detected in all plant species tested to date, and they are known to occur worldwide. The majority of the endophyte to plant time ratios, however, are not fully known (Azevedo, 2000). It is also known that endophytes are found in fractions and algae. Almost all of the plants that have been investigated have endophytic bacteria, which infiltrate the host tissues and can form a variety of interactions, including symbionts, mutualists, commensals, and trophobias. Endophytes can be used to create a variety of biological enzymes, including cellulose, gelatinase, xylanase, amylase, and tyrosinase. (Gómez, 2018). Endophytic bacteria reside in plant tissues but provide no real harm or advantage other than to be there (Kobayashi and Palumbo 2000).

According to Hallmann *et al*. (1997), a bacterial endophyte can be removed from inner plant tissue or separated from surface-disinfected plant tissue. Endophytes are any microorganisms that remain inside the plant regardless of the specific tissue colonized. The majority of plant species' roots, leaves, and stems, as well as some of their flowers, fruits, and seeds, have all been found to contain endophytic bacteria (Lodewyckx *et al*., 2002). Endophytic bacteria may accompany certain metabolic properties, such as fostering plant development, eradicating soil-borne diseases, or assisting the host plant in reducing stress responses to external stressors (Mastretta *et al*., 2006; Taghavi *et al*., 2007; Ryan *et al*., 2008). The majority of endophytes seem to come from the rhizosphere or phylosphere, however others can spread through seeds. Endophytic bacteria are biological pest controllers and aid in the growth and performance of plants. Endophytes can also be advantageous to their hosts since they produce a number of organic products that are useful in industry, agriculture, and medicine.Endophytes and host plants typically have a symbiotic connection in which they get nutrients from plants (Zou *et al*., 2001). Additionally, it has been demonstrated to have the capacity to eliminate soil contaminations by enhancing phyto-mediation and to contribute to enhancing soil fertility through phosphate solubility and nitrogen fixation (Berg and Hallmann, 2006). Increasing interest can be seen in increasingpotential biopharmaceutical applications of the endophytes to improve plant purification and sustainable Production of non-food crops in the production of biomass and biofuels.

According to Hallmann *et al*. (1997), Bacteria that survive on non-pathogenic bacteria are known as endophytes. This definition of an endophyte includes any bacterium that can be recovered from the plant's interior or from surface-infested plant tissue and does not obviously affect the plant. Endophytic bacteria are those that reside in plant tissues and cause serious problems or have other benefits over non-living organisms. The endophytes' secondary metabolites are used in agriculture, medicine, fuel, and other industries. Endophytic bacteria create organic medicines that will soon be used in the pharmaceutical business. Bacterial endophytes can be isolated from disinfected plant surface tissue or plant

internal tissue. Numerous plant species have produced Gram (+) and Gram (-) bacterial endophytes in a variety of tissue types (Germaine, 2007). In addition, several bacterial species of a single plant were isolated. Endophytes penetrate mainly through the root region into the tissue of the plant; However, plant airborne species, such as flowers, stems and barrels, can also be used in the entrance. In particular, bacteria come into tissue through seedlings, secondary roots, stomach or leaf damage.

Biocontrol and Endophytes

Endophytic bacteria can reduce or even prevent the adverse effects of many pathogens. Because they may efficiently boost plant development and defend host plants from diseases. It has been demonstrated that endophytic bacteria are one of the most promising biocontrol agents. (Prasom *et al*., 2017). Bacterial endophytes appear to have beneficial impacts on their host plants through similar mechanisms to those mentioned in the associated rhizosphere bacteria. According to reports, some endophytic bacteria generate a condition that phenotypically resembles acquired systemic resistance and can be systemically caused by an ISR (SAR) (Azevedo, 1998). Some endophyte bacteria are thought to have traits that resemble acquired systemic resistance. When plants enable their effective defence system in response to primary pathogen infection, SAR occurs especially when this causes a hypersensitivity reaction bounded by the local necrotizing dry brown tissue (Araujo *et al*., 1999).

Natural Products from Endophytic Bacteria: -

Most of the endophytes are bacterial like *Pseudomonas*, *Burkholderia* and *Bacillus*. These families are known for their variety of secondary metabolites, including antibiotics, anti-cancer drugs, volatile organic compounds, antifungals, anti-viral drugs, insects, and immune suppressants. Although isolated from endophyte organisms produce a large variety of physiologically active chemicals, making them a respectable source of novel natural products. While research has mostly concentrated on the development of antimicrobial compounds for fungicides, a number of bacterial endophytes that are low in molecular weight and efficient against a variety of harmful microorganisms for people, animals, and plants have been discovered. It was found that the fluorescent element pseudomonades isolated on plants isolated from *Pseudomonas viridiflava* and several tissues of herbaceous tissue produced two new antimicrobial compounds, ecomycin. Ecomicinas represents the amount of new peptides and lipo is made up of a few unusual amino acids, such as homoserine and aspartic acid. These compounds have been found to prevent the human pathogens *Candida albicans* and *Cryptococcus neoformans*(Bacon, 1998).Applications for bacterial endophytes in pharmaceutical and drug discovery are numerous (Strobel 2006; Guo *et al*., 2008). As new bioactive agents and a possible source of natural products for use in oxidative stress, endophytes associated with ethnomedicinal plants are useful (Nongkhlaw and Joshi 2015). At incredibly low concentrations, they perform key functions in controlling how plants react to diverse stressors. Plant hormones are produced and modulated as a result of interactions between bacterial endophytes and plants (Lopez *et al*., 2008; Glick 2012). IAA is the auxins class of plant hormones' most prevalent naturally occurring member. Endophytic strains of *B. cereus, B. thuringiensis, Bacillus* sp.*, Bacillus pumilis, Pseudomonas putida, Clavibacter michiganensis,* and *Bacillus* sp. that were isolated from *C. longa* L. all produced IAA (Javid *et al*., 2011; Kumar *et al*., 2016). From the root of *Cassia tora*, *Pseudomonas, Agrobacterium*, and *Bacillus* were isolated. They generated phytohormones and solubilized tricalcium phosphate (Kumar *et al*., 2015).

Material and Method

Isolation of Endophytic Microorganisms Putative endophytic microorganisms were characterised as isolates that were obtained from surface sterilized plants. The surface of stem and leaves were sterilized by using the protocol (Strobel *et al*., 2003).Sub culturing and Streaking for isolation of pure culture done on nutrient agar medium, in which different codes were assigned to each bacterium isolated and again kept in incubator at suitable temperature for proper growth. After that some test apply on the isolated bacterium are: Phosphate Solubilisation Test and Nitrogen Fixation Test, Anti-microbial Test, Catalase Test, Screening of Hydrogen Cyanide Production (HCN), Screening for Ammonia production, Dye degradation activity and Indole - 3-Acetic Acid Production, Amylase, Protease, Cellulose production.

Identification of Endophytic Bacteria:

The approach outlined by Cruickshank *et al*. (1975) which comprises morphological, cultural, and biochemical testing, was followed for the identification of endophytic bacterial isolates. A staining technique called Gram staining, commonly known as the Gram method, is used to separate bacterial species into two major categories (gram-positive and gram-negative). The Endospore staining is a differential stain that illustrates the endosporebacteria. Endospores are formed from some bacterial strains such as *Bacillus*. Through the formation of spores, bacteria can survive under hostile conditions.

Methyl red (MR) test: The test is used for identification of bacteria that produce stable form of acids by mixing glucidicacid. Four "M" sections of the IMVC test The MR test is used to identify enteric bacteria according to the model of glucose metabolism. In this staining red colour immediately observe for positive result. After the staining some biochemical test are done.

Citrate test: recognizes the organism's ability to use citrate as its sole carbon source and for energy. Positive: In middle of the growth, even if the color is not changed, is considered positive. We would see a color change in the medium if the test organism forms acid or alkali during its growth. The usual color change that is observed is from green (neutral) to blue (alkaline).

Voges-Proskauer (VP): The test is a very significant test. This test is used for detection of acetoin in a bacterial broth in culture. The test is carried out by addition of alpha-naphthol and potassium hydroxide to the bacterial broth. Triple Sugar Iron (TSI) agar aggregates distinguish bacteria from their ability to reduce sulfur hydroxides, as well as glucose, lactose, and / or sucrose.

Hydroxysulfide (H_2S) test: can be made from the iron sulfate metabolism. H_2S forms a black precipitate at the ends of the slope.

Urease test: The test is also called as the CLO test (Campylobacter-like organisms test), is a fast test used for diagnosis of *Helicobacter pylori*. The test is based on H. pylori's ability to excrete enzyme urease. The enzyme urease is involved in the conversion of urea into ammonia and carbon dioxide. The goal is to find out if the microbe is fermenting carbohydrates (sugars) lactose as a carbon source.

Lactose Fermentation Test: In lactose fermentation test the MacConkey agar is a selective and helps in differentiation. It contains the indicator pH (neutral red), lactose, sapatosulas and crystal violet. Bile salts and crystalline viruses are selected from Gram (-) bacteria by inhibiting the growth of Gram (+) organisms. With a neutral red pH indicator, agar separates Gram (-) bacteria that can ferment lactose (Lac +) and those who cannot (Lac-). This medium is also called "Medium Indicator" and "Low Selective Medium".

Results

In the present study, a total of 5 bacterial endophytes were isolated from Tulsi and Jamun by using surface sterilization method (Figure 1).

Isolation of Endophytic Bacteria:

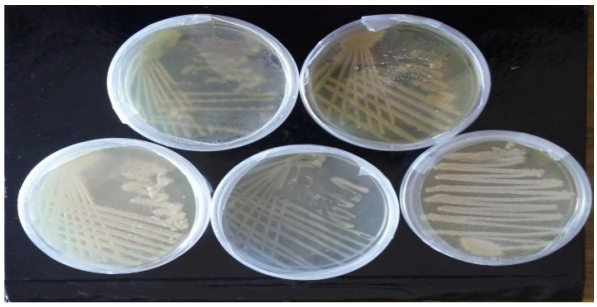


Fig. 1 : Pure culture of Bacterial isolates B1,B2,B3,B4 and B5.

Table 1: Biochemical Screening of Bacterial Endophytes

Sr. No	NFB Test	PSB Test	Antimicro-bial Test	Catalase Test	HCN Test	Ammonia Test	IAA Production Test
B1	+	+	-	+	+	+	-
B2	-	-	-	+	+	+	-
B3	-	+	-	+	+	+	-
B4	-	-	-	+	-	+	+
B5	+	+	-	+	+	+	-

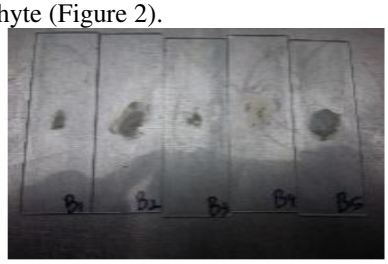
The NFB test was found +ve in bacterial isolates B1 and B5 and the PSB test was found +ve in bacterial isolates B1, B3 and B5. No anti-microbial activity was observed against all the test micro-organisms. Catalase test was found positive for all the bacterial isolates. HCN test was found +ve in bacterial isolates B1, B2, B3 and B5. Ammonia production test was found +ve for all bacterial isolates.IAA production test was found +ve for bacterial isolate B4 (Table 1).

After screening bacterial isolate B1 and B5 was observed as most efficient bacterial endophyte (Figure 2).

B_1,B_3,B_5 in PSB

B_1& B_5 in NFB

Antimicrobial Test



Catalase Test

B_4IAA Test

Before After

B_1,B_2, B_3 Ammonia Test

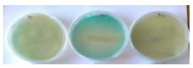
B_1,B_2,B_3,B_5 HCN Test

Fig. : 2 Biochemical Screening of Bacterial Endophyte

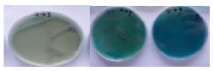
Table 2: Efficiency of dye degradation of Bacterial Endophytesto degrade the malachite green

Bacterial isolates	0.01%	0.03%	0.05%	0.09%	0.20%	0.22%
Bacterial isolate 1	+	+	-			
Bacterial isolate 2	+	+	-			
Bacterial isolate 3	+	+	+	+	-	-
Bacterial isolate 4	-					
Bacterial isolate 5	-					

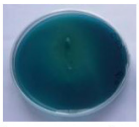
As observed in the table 2 the three bacterial isolates (B1, B2 and B3) were found positive at conc. 0.01 %. Three bacterial isolates (B1, B2 and B3) were found positive at conc. 0.03 %. and one bacterial isolate (B3) was found positive at conc. 0.05 and 0.09 %. None of the bacterial isolate was found positive at conc. 0.20 and 0.22 % (Figure 3).



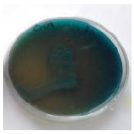
3 bacterial isolates were found degrading the dye (malachite green) at conc. 0.01%



Out of 3 previously screened endophyte bacterial isolates, 2 was found degrading the dye (malachite green) at conc. 0.02%



Out of 3 screened endophytes bacterial isolates, 1 was found degradingthe dye(malachite green) at conc. 0.05%



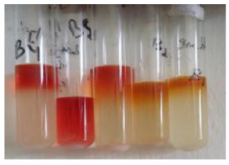
B3 was found degrading the dye (malachite green) at conc. 0.09%

No degradation of dye was found by both bacteria when the conc. of dye (malachite green) was at 0.20% and 0.22%

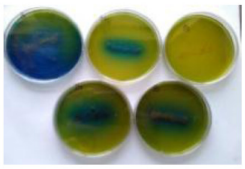
Fig. 3 : Efficiency of Dye Degradation

Identification of isolates of Endophytic Bacterial

For the biochemical and morphological characterization of bacterial isolates, different tests were performed (Anjum and Chandra 2015). The morphological tests namely Gram's staining, Endospore staining and Biochemical tests such as Catalase test, Indole acetic acid production test, MR test, VP test, citrate utilization test, H_2S test, lactose fermentation test and urease test were performed for identification of endophytic bacteria as shown in the figure 4. Bacterial isolates B3, B4, B5 changed its colour from yellow to dark orange indicating +ve result for methyl red test. Bacterial isolate B1, B2, B4 and B5produced blue colour around the colonies indicating positive result for citrate utilization. Bacterial isolate MIB1 changed its colour from yellow to pink and black butt and also produces gas indicating positive result for H2S test. Bacterial isolate MIB1 changed its colour from yellow to dark red indicating positive result for VP test. Bacterial isolates MIB1, MIB2 and MIB5 changed its colour from yellow to pink indicating positive result for urease test. Bacterial isolates were producing red colonies indicating positive result for lactose fermentation test.

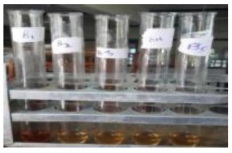


Methyl red Test

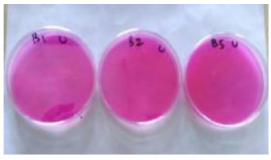


Citrate utilization

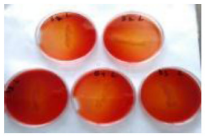
H2S test.



Vogespros kauer Test



Urease test



Lactose fermentation test

Fig. 4 : Identification of Endophytic Bacterial isolates

Table 3: Morphological and biochemical characteristics of bacterial isolates

Bacterial Isolates	B_1	B_2	B_3	B_4	B_5
Gram Staining	-	-	-	+	-
Endospore Staining	-	-	-	+	-
IAA TEST	-	-	-	+	-
Methyl red Test	-	-	+	+	+
Catalase Test	+	+	+	+	+
Citrate Utilization Test	+	+	-	+	+
H_2S Test	+	-	-	-	-
VP Test	+	-	+	-	-
Urease Test	+	+	-	-	+
Lactose Fermentation Test	+	+	+	+	+
Suspected Bacterial identification	***Proteus* sp.**	***Klebsiella* sp.**	***Shigella* sp.**	***Bacillus* sp.**	***Proteus sp.***

B_1 were found positive for catalase, citrate utilization, lactose fermentation, H2S, Vogesproskauer and urease test and found negative for Gram's staining, endospore staining , methyl red and IAA test and were identified as *Proteus sp.*B_2 were found positive for catalase, urease, citrate utilization and lactose fermentation test and found negative for H2S, Vogesproskauer, Gram's staining, endospore staining , methyl red and IAA production test and were identified as *Klebsiella*.B_3 were found positive for catalase, lactose fermentation and methyl red test and found negative for Gram's staining, endospore staining, H_2S, IAA, citrate utilization, Vogesproskauer and urease test and were identified as *Shigella* sp.B_4 were found positive for catalase, citrate utilization, methyl red, Gram's staining, endospore staining, lactose fermentation and IAA production test and found negative for H_2S, urease and vogesproskauer test and were identified as *Bacillus* sp.B_5 were found positive for catalase, citrate utilization, methyl red, lactose fermentation and urease test and found negative for H_2S, Gram's staining, IAA production, endospore staining and vogesproskauer test and were identified as *Proteus sp.* (Table 3).

Discussion

In this investigation into endophytic microbes, the healthy stem and leaves of Jamun and Tulsi plants generated a total of 5 bacterial endophytes. From Tulsi plant 2 bacterial while from Jamun 3 bacterial endophytes were isolated. Same research was done by Abhram *et al.* (2010) in which they isolated 40 bacterial endophytes from Tulsi to check their bioprospecting potential.

5 bacterial endophytes were taken for screening for various plant growth promoting factors like phosphate *solubilisation* in which 3 isolates were positive, nitrogen fixation in which 2 isolates were positive, catalase production in which all the isolates were found positive, IAA production in which 1 isolate was positive, ammonia production in which all isolates were found positive, HCN production in which 3 isolates were found positive and for dye degradation out of 5 endophytic bacterial isolates 3 werefound degrading the dye (malachite green) at 0.01% conc., 3 were found degrading the dye at 0.02% conc., 1 was found degrading the dye at both 0.05% and 0.09% conc., and none of bacterial isolate was found for degrading the dye at 0.20% and 0.22% concentration. A research was done by Kumar *et al.* (2015) on french bean in which 30 bacterial endophytes were isolated out of which 5 isolates were positive for the phosphate solubilisation, 10 isolates were positive for Indole acetic acid (IAA) production, 20 isolates were positive for ammonia production, 25 isolates were found positive for both catalase and ammonia production. A research was done by Germaine (2007) on bacterial endophytes for degrading xenobiotic factors. Another research was done by Shah *et al.,* (2013) by using *Pseudomonas stutzeri*, they isolated bacterial and fungal endophytes and tested for microbial breakdown and decolorization of Reactive Black. Another research was done by Shraddha *et al.* (2011) for Lactase production, potential, and purification biotechnological applications from endophytes in which 31 isolates of 55 were found positive for the result.

Identification of isolated endophytic bacteria was done in which isolate B_1 was found as *Proteus* sp., B_2 was found as *Klebsiella,* B_3 found as *Shigella* sp., B_4 was found as *Bacillus* sp. and B_5 *Proteus* sp.

Abbreviations

IAA Test - Indole Acetic acid Test
H_2S Test - Hydrogen sulphide Test
VP Test - Vogesproskauer Test
NFB Test -Nitrogen Fixation Test
PSB Test - Phosphate Solubilisation Test
HCN Test -Screening of Hydrogen Cyanide Production (HCN)
IAA Production Test - Indole -3-Acetic Acid Production

Conclusion

Endophytes are endosymbionts that live their entire lives inside the body of the plant without showing any signs of illness. Through a number of mechanisms, including the synthesis of plant growth promoters, the stimulation of phosphate solubilization and nitrogen fixation, the production of IAA, etc., they are advantageous for the growth and development of plants. There are many papers related to the beneficial effects and screening of endophytes from plants like rice, maize, French bean, neem etc. but a few on Tulsi and Jamun. Five beneficial bacterial endophytic were isolated from tulsi and jamun plant. Isolated bacteria and fungus were screened for different plant growth promotion activities. Out of all isolates B1 and B5 were found as most efficient which solubilized insoluble phosphate, has great affinity for nitrogen fixation, produces catalase, ammonia production and also produced HCN.

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