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Exploring the Probiotic and Plant Growth Promoting Potential of Endophytic Bacteria Isolated from the Vegetable Crop Bhendi *Abelmoschus esculent*us

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ABSTRACT

Endophytes are microbes that reside within the plant tissues and facilitate the growth of the host plant. In the present study, occurrence of endophytic bacteria in the most common vegetable plant of India, Bhendi (*Abelmoschus esculentus*) was screened and analysed for its plant growth promoting (PGP) potential. Presence of a rod shaped bacterium within the tissues of the host plant was observed. The isolate was identified as *Bacillus firmus* by its biochemical profile. Phosphate solubilization and IAA production by the isolate at 60 h of incubation was found to be 37.3 μg ml^{-1} and 39.47 μg ml^{-1} respectively. *B. firmus* was found to produce trihydroxamate type siderophore with hexadentate nature. The isolate exhibited extracellular pectinolytic, proteolytic, cellulolytic, chitinolytic enzyme activity. Further, the isolate exhibited probiotic properties such as tolerance to acid (pH2), bile salt (2 %), gastric juice, auto-aggregation potential, antibiotic resistance and the absence of haemolytic activity. Seed biopriming and scanning electron microscope (SEM) imaging confirmed the endophytic and plant growth promoting potential of *B. firmus*. The results of this study illustrate the possible utilization of the endophytic bacterium *B. firmus* as a bioinoculant for plant growth promotion.

***Key words**: Probiotic, Plant growth promotion, Siderophore, Bacillus firmus*

Introduction

Agriculture renders basic sustenance to all living beings (Viana *et al.*, 2022). Sustainable agricultural growth is essential for eradication of poverty, hunger and under-nourishment. With the invention of Green Revolution Technologies, India has become a strongest testimony of how R & D could transform a food-deficit and food-importing economy into a food self-sufficient economy. Of the various crops, cultivation of vegetables provide good income to the cultivars with increased productivity, short maturity cycle and high market value (Dastagiri *et al.*, 2013). According to Indian Horticultural statistics 2018, India is the second largest producer of vegetables with the productivity of 17.7mt/ha that contributes 14% of the total world production. Bhendi (72%), tomato (12%), egg plant (9%) and potato (7%) are the four major vegetables contributing 58.6% of total vegetable production in our country (Vanitha *et al.*, 2013). Vegetables are the vital source of proteins, vitamins, minerals, dietary fibers, micronutrients and phytochemicals in our daily diet. Vegetable cultivation has been dwindled drastically in the recent years due to infestation of pests, soil borne pathogens, uneven seed germination and subsequent poor

crop establishment. To overcome the low productivity, chemical pesticides and fertilizers have been applied indiscriminately. Injudicious application of chemical pesticides and fertilizers not only culminated in environmental degradation and crop failure but also have an impact on the quality of the produce.

Biopriming of seeds with endophytic microbesis considered as an alternative way of reducing the use of chemicals in agriculture (Sutariati *et al*., 2020). Plant associated bacteria especially endophytes offer an added advantage in plant growth promotion and disease prevention (Bennet *et al*., 2009). Bacon and White (2000) defined endophytes as "microbes that colonize internal tissues of plants without causing any immediate, overt negative effects". The intimate association of bacterial endophytes with the internal tissues of the plant offers a unique opportunity for their potential application in plant growth promotion, protection and disease control. Endophytes are the members of common soil microflora that enter the plant tissue primarily through the root zone (Lodewyckx *et al*., 2002). Endophytic microorganisms facilitate the growth of the host plant by producing a plethora of substances that impart beneficial effect on the humans. Therefore, some of the endophytes are defined as plant probiotics (Del Piano *et al*., 2006). Generally, probiotics are "Live microorganisms which when administered in adequate quantity confer health benefits" (Sanders, 2003). Murugappan *et al*. (2013) enlisted the prospects of using endophytes as potential probiotics. Based on the above facts, the present study was intended to evaluate the efficiency of endophytic bacteria that reside within the vegetable crop bhendi (*Abelmoschus esculentus*) for plant growth promotion and probiotic properties.

Materials and Methods

Vegetable crop bhendi (*Abelmoschus esculentus*) was selected for this study based on its marketability, value and frequent use in kitchen. Further the plants are grown in kitchen garden and the produce is consumed with or without cooking. Three different agricultural fields near the foothills of Alagarkovil hillock, Madurai District, Tamil Nadu, India were selected for this study. The sites were selected after knowing the cropping history and farm procedure followed in the field for the last three years.

Isolation of endophytic bacteria

Healthy 45 days old bhendi (*Abelmoschus esculentus*) plants without the symptoms of disease or damage were selected for analyses. Plants were uprooted from the field and the adhering soil particles were removed by washing with water. Stem sections (1.0 cm thickness) were taken 5 cm above the soil line using sterile knife. Leaves, stems and roots of the plant were cut into small discs (0.5 cm diam) using sterile cork borerand surface sterilized following the five step procedure described by Tiwari *et al*. (2010); 3 min wash in 5 % $NaClO_3$, followed by a 10 min wash in 2.5 % $Na_2S_2O_3$, a brief wash in 75 % ethanol, then a 10 min wash in 10 % $NaHCO_3$ and finally a rinse in sterile water. Aliquots (100 µl) from the final wash were transferred to nutrient agar plates as a sterility check. Plant tissue samples were discarded if bacterial growth was detected after 48 h of incubation at 30 °C. Surface-sterilized tissues were air dried and triturated with phosphate buffered saline. Macerated tissues were serially diluted and spread onto the nutrient agar medium free of inorganic nitrogen, as the natural endophytic environment is very low in nitrogen (Tejera *et al*., 2006). The plates were incubated for 48 h at 30 °C.

Identification of endophytic bacteria

Endophytic bacteria isolated from the plant tissues were identified by conventional physico chemical characteristics as described by Bergey's Manual of Systemic Bacteriology (Brenner *et al*., 2005).

Plant growth promotion (PGP) traits

Phosphate solubilization and IAA production

Phosphate solubilizing ability of the endophytic isolate was determined qualitatively and quantitatively by stannous chloride method (Gaur, 1990) using Pikovskaya medium with 0.5% tricalcium phosphate [Ca3(PO4)2]. The change in pH of the medium following tri- calcium phosphate (TCP) solubilization was also recorded. Indole acetic acid (IAA) production by endophytic bacteria was determined following the method described by Patten and Glick (2002) with slight modifications.

HCN and Ammonia Production

The isolate was screened for hydrogen cyanide production following the method of Bakker and Shipper (1987). Ammonia producing ability of the isolate

was determined by adding Nessler's reagent (0.5 ml) to the bacterial culture in peptone water (10 ml) at mid log phase. Development of brown colour indicates ammonia production.

Screening for enzyme activity

Cellulase production by the isolates was detected following the method of Essghaier *et al.* (2009) using carboxy methyl cellulose (CMC) agar. Pectinase enzyme production by the isolates was determined using Hankin's medium (Aneja, 2006) using Hankin's medium. Chitinase enzyme production by the isolates was determined following the method of Renwick *et al*. (1991) using minimal medium supplemented with colloidal chitin (0.2%). Protease production by the isolates was determined as per the procedure described by Kamensky *et al*. (2003) using skimmed milk agar.

Siderophore production

Siderophore producing ability ofthe isolate was determined by FeCl3 test and chrome azurol sulphonate (CAS) agar plate method (Schwyn and Neilands, 1987). The amount of siderophore in the culture supernatant was quantified by Chrome azurol sulphonate shuttle assay. Furthermore, the culture supernatant was subjected to Csaky, Arnow and Vogel's assay to ascertain the nature of siderophore produced. Siderophore was further distinguished into mono-, di- or trihydroxamates based on the absorption maxima and electrophoretic mobility (Jalal and Helm, 1990). The iron-binding property of siderophore was determined by the colour reaction at different pH values.

Screening for probiotic properties

Probiotic nature of the isolates was elucidated by a battery of assays.

Acid, bile salt and gastric juice tolerance

A probiotic microorganism must overcome the chemical stress in the gastrointestinal tract. Therefore, in this study acid and bile salt tolerance property of the isolate was determined following the procedure described by Erkkila and Petaja, (2000) and Ahire *et al*. (2011) respectively. Tolerance of the isolates to gastric juice was determined as described by Pedersen *et al*. (2004) with slight modifications.

Antibiotic susceptibility and haemolytic assay

Susceptibility of the isolates to different antibiotics (Amoxicillin, Ampicillin, Kanamycin and Penicillin) was determined withthe commercially available antibiotic discs (Hi Media, Mumbai) using Muller Hinton agar medium. The haemolytic ability of the isolate was examined on nutrient agar plate supplemented with sheep blood (5 %) after 24 h of incubation at 30 °C.

Auto aggregation assay

Auto-aggregation is an important property of a probiotic microbes and it was assessed following the method of Del Re *et al*. (2000) with certain modifications. The isolate was grown over night at 30 °C in nutrient broth. The cells were pelleted, washed and dissolved in PBS (pH 7.3) to get an absorbance of 0.5 at 600 nm. The bacterial suspension was incubated at room temperature and absorbance of upper suspension was measured at different time intervals.

Auto"aggregation % was expressed as : A0" (At / A0)X100

Where, At represents the absorbance at different time intervals and A0 the absorbance at t=0 h. Three replicates were maintained for all the experiments in this study unless other- wise represented.

Biopriming of seeds

To confirm the endophytic nature and growth-promoting characteristics of the isolate, plant inoculation assay was performed following the procedure of Tiwari *et al*. (2010). Seeds of bendi were collected from Tamil Nadu Agricultural University, ACRI, Madurai. The collected seeds were surface sterilized with 5 % sodium hypochlorite for 3 min and rinsed 5 times with sterilized distilled water. After surface sterilization the seeds were immersed in endophytic bacterial cell suspension (107 CFU per ml of phosphate buffer pH 7.0) containing 1% Carboxymethylcellulose (CMC) as adhesive in the ration of 2:1 for I h. 10 g of surface sterilized seeds were mixed with 10 ml of CMC slurry to coat the endophytic bacteria on seed surface. Seeds soaked only with CMC slurry were used as control. Treated and control seeds were air dried and planted singly in plastic bag (10×15 cm) containing sterile soil (500 g). Five replicates were maintained. The plants were grown in a greenhouse at 37°C with 16 h light: 8 h darkness photo- period. The plants were watered daily with sterile deionised water. After 45 days, the plants were uprooted and growth parameters such as shoot and root length was calculated. The presence of endophytes within the leaf tissues was estab-

lished by scanning electron microscopic (SEM) imaging (HITACHI S-3000H).

Results and Discussion

A gram negative rod shaped bacterium was isolated from the surface disinfected, macerated tissues of the stem, leaves and produce of healthy 45 days old vegetable plant *Abelmoschus esculentus*. The isolate responded positively for catalase, oxidase, starch hydrolysis test and negative for hydrogen sulphide, methyl red, citrate utilization,vogesproskauer tests (Table 1). Biochemical profilesand endospore forming capability facilitate the taxonomic identification of theisolate as *Bacillus firmus*. Strobel *et al.* (2004) postulated that each and every individual plant host one or more endophytes. Colonization of endophytes was analysed only in a very few vegetable plants. Occurrence of *Bacillus* species within the tissues of various plant was illustrated by earlier workers (Afzal *et al*., 2019)

Plant growth promotion assay

Plant growth promotion traits like the production of indole acetic acid, siderophore, HCN, ammoniaand phosphate solubilization confers the benefit of *B. firmus*. Phosphate solubilization and IAA production by *B. firmus* was determined qualitatively and quantitatively. *B. firmus* was found to solubilize the inorganic phosphate in the Pikovskaya medium with the concomitant decline in the pH (Fig. 1). The level of soluble phosphate in the medium was found to be 37.33 μgml^{-1} at 60 h of incubation (Table 2). Solubilization of insoluble phosphateillustrate that the isolate can be deployed as a bioinoculants to minimize the fertilizer application. The reduction in pH of the medium may be due to the synthesis of low molecular weight organic acids by the isolate (Kohler *et al.*, 2009). Reduction in pH of the medium is suppose to facilitate phosphate solubilization Presence of phosphate solubilizing endophytic bacteria in the tissues of different plants (wheat, pea, canola, barley, and corn) was earlier reported by Laslo *et al.* (2012).

Table 1. Biochemical and physiological response of endophytic bacterium *B. firmus*

Biochemical tests	Results
Colony morphology	Circular, convex with entire margin
Shape	Rods
Colour of the colony	Creamy white
Gram's staining	Gram negative
Motility	Motile
Endospore	+
Catalase	+
Oxidase	+
H_2S Production	-
Methyl Red Test	-
Voges - Proskauer Test	-
Citrate Utilization	-
Indole Production Test	-
Starch Hydrolysis	+
Nitrate reduction	+
Haemolytic Activity	-
Exoenzyme Activity	
Cellulase	+
Chitinase	+
Protease	+
Pectinase	+

+ Positive, - Negative

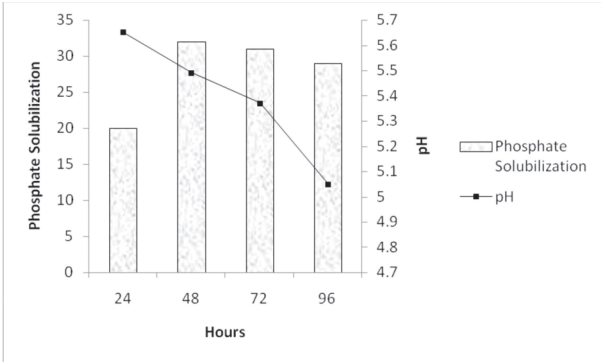

Fig. 1. Level of Phospahte Solubilization by *B. firmus* and pH variation in Pikovskaya medium.

Biosynthesis of 3-indole acetic acid (IAA) by the isolate in Luria-Bertani broth was estimated to be 39.47 μgml^{-1} at 60 h of incubation (Table 2). Tejesvi and Pirttila (2011) reported that biosynthesis of IAA by the endophytic microbes enhance the nutrient uptake and plant growth promotion. Nimnoi and Pongsilp (2009) demonstrated that bacterisation of the seeds with IAA producing endophytic bacteria enhanced root and shoot development of *Raphamus sativus* and *Brassicaoleracea* by fivefold. Dias *et al*. (2009) reported that *B. subtilis* isolated from the stems of strawberry plant produced 55 µg/mL of IAA after 48 hrs of incubation.

Production of the iron chelator siderophores by *B. firmus* was confirmed with the positive results of FeCl3 test, CAS assay and development of orange

halos in CAS agar plate. Maximum siderophore production was observed only at 60 h of incubation and it was found to be 42.31 ± 0.06 % (Table 2). Siderophore mediated iron sequestration, growth promotion and pathogen inhibition by endophytic bacteria in the plant *Oryza sativa* was reported by Ngalimat *et al*. (2021). Cell free culture supernatant exhibits an absorbance maximum at 409 nm indicating the hydroxamate nature of the siderophore produced and it was confirmed by the positive Csaky test (Table 3). A narrow shift in λ-max (up to 8 nm) with different pH and electrophoretic mobility results indicate trihydroxamate nature of the siderophore produced. The binding property of the ligand with Fe_3^+ was determined by the retention of colour by siderophore-metal complex. In the present study, the colour of the ferric hydroxamate remained red over a wide pH range (4–9) indicating a hexadentate nature (Table 4). Baakza *et al*. (2004) reported that hexadentate trihydroxamates exhibits strong binding affinity towards ferric iron than bidentate.

Table 2. Plant growth promotion traits of *B. firmus*

PGP traits	**Units**
Phosphate Solubilization (μgml^{-1})	37.33±0.12
IAA Synthesis (μgml^{-1})	39.47±0.59
Siderophore Production (%)	42.31±0.06

*60h of incubation

To better understand the mode of action involved in the penetration of the host tissues and biocontrol activity against pathogenic bacteria, extracellular enzyme synthesizing capability of the endophytic bacterium *B. firmus* was analysed. Development of clear zones around the bacterial colonies on colloidal chitin agar (4.0 mm), carboxy methyl cellulose agar (9.0 mm) and skim milk agar (6.0 mm) plates indicate extracellular hydrolytic enzyme activity (results not shown). Jha and Kumar (2007) reported that the cell wall hydrolyzing enzyme mediated endophytic colonization of *Klebsiella oxytoca* GR-3 in *Typha australis*. In addition, the endophytic isolate *B. firmus* exhibited other PGP traits such as ammonia and HCN production. The above results suggest that multiple mechanisms such as production of exoenzymes, competition for Fe *via* siderophore production, ammonia, HCN production and their synergistic effects may be responsible for the observed plant growth promotion activity of *B. firmus* in the vegetable crop bhendi *A. esculentus.*

Plant inoculation assay

Biopriming of *A.esculentus* seeds with endophytic bacterium *B.firmus* significantly enhance the shoot length and root length in comparison with the untreated control (Fig. 2). An increase in the number of

Table 3. Determination of the chemical nature of siderophore.

Isolate	Hydroxamate		Catecholate		Carboxylate		Nature
	Neilands Spectrophotometric assay	Tetrazolium salt test	Neilands Spectrophotometric assay	Arnow's test	Neilands Spectrophotometric assay	Vogel test	
B. firmus	409	+	465	+	-	-	Hydroxamate Catecholate

Table 4. Classification and ligand denticity of hydroxamate siderophore.

Isolate	λ_{max}(nm) of Siderophore		λ_{max} (nm)	Color of the	Inference ferrate	Color of the ferrate hydroxamate	Binding properties
	pH	λ_{max}(nm)	siderophore in electrophoresis				
B.firmus	4	414	6	Red	Trihydroaxamate	Pink purple	Hexadentate
	5	404					
	6	405					
	7	436					
	8	445					
	9	423					

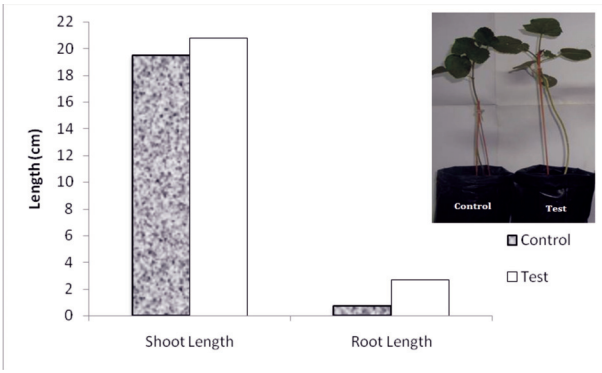

Fig. 2. Influence of biopriming of *A. esculentus* seeds with endophytic *B. firmus*.

leaves (20%), shoot length (27.17%) and root length (58%) was observed in the endophytic bacterium treated plants. SEM imaging illustrates that the root tissue of bhendi plant was found to be heavily colonized by endophytic bacteria compared to the stem. However, bacterial cells were found to colonize on the shoot surface, epidermal cells, intercellular spaces and vascular tissue. Surface furrows appeared on the emerging lateral roots are the sites where the bacteria spreads into the intercellular spaces and vascular bundles (Fig. 3).

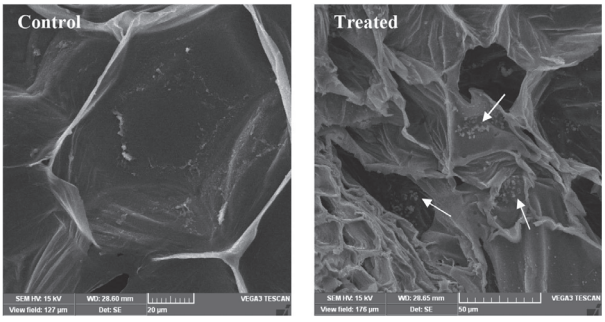

Fig. 3. Scanning Electron Microscopic (SEM) observation of *B. firmus* within the leaf tissues of Bhendi (*A. esculentus*)

Probiotic properties

The acid tolerance profile of endophytic *B. firmus* after 3h of exposure to pH 2 and pH 4 was found to be 46.83 and 87.65 respectively (results not shown). In addition to acid tolerance, the isolate was able to survive at 2-4% concentration of bile salt. There was no great variation in viable count of *B. firmus* at different bile concentration. The absence of haemolytic activity indicates a potential probiotic property of the isolate. The isolate was found to be susceptible to tetracycline, gentamycin, chloramphenicol, erythromycin but resistant to penicillin and streptomycin. The auto- aggregation percentage was found to be linearly associated with the incubation period with an aggregation percentage of 71.4 after 12 h of incubation.

Conclusion

In conclusion, to the best of our knowledge, this is the first to report the presence of growth promoting endophytic bacterium *B. firmus* within the tissues of the vegetable crop bhendi. It is still on a debate that whether plants benefit from an endophytic association or if the advantage is higher for the bacteria. However, it is evident from the results, the benefits conferred by endophytes are more conspicuous by plant growth promotion. Further research is needed to evaluate whether the isolate could have a positive impact on the growth and yield of bhendi in the field.

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