Advances in Bioresearch
Adv. Biores., Special Issue 1: 2023: 285-297
©2023 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.S1.285297

Advances in Bioresearch

Plant growth-promoting attributes of Indian gooseberry (*Phyllanthus embilica* L.) endophytes

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ABSTRACT
Endophytes are plant symbionts which use plants as their habitat for their survival for a life span or during some phases of their life cycle. During the tenure of their growth and survival in plant parts, they offer a variety of benefits to their symbionts plant by promoting their growth and protecting them from pathogens. Medicinal plants like Phyllanthus emblica L. commonly known as Indian gooseberry (Amla) have made a huge impact on humankind by offering versatile health benefits; this plant is rarely searched for the presence of endophytes. Isolation and characterization of endophytes from Amla plants can open a new door of possibility as they may be helpful or may have some plant growth-promoting properties. This current study focuses on the isolation of endophytes from various plant parts of Phyllanthus emblica L. and their characterization and analysis for various plant growth-promoting traits. A total of 25 endophytic microbes were isolated from different plant parts of the Amla plant. Amongst them, 18 are bacterial isolates while 3 are actinomycetes and 4 are fungal isolates. From these 25 isolates, the current study is focused on bacterial isolates and actinomycetes. Amongst them isolates YR1, YR2, SN4, FN2, RN2, RN3 and LN2 are showing good plant growth-promoting activity, which can be furtherly checked for pot study.

Keywords: Indian gooseberry, Endophytes, Sustainable agriculture; Plant Growth Promoting Bacteria (PGPB); Medicinal plants; Biotic stress, Abiotic stress

Received 24.05.2023 Revised 20.06.2023 Accepted 24.07.2023

How to cite this article:
Richa S, Vidhi S, Shweta K, Nehal C, Tarun Y·Knowledge, Attitude, and Practice of Health Care Practitioners Regarding Micronutrient Recommendations. Adv. Biores., Special 1:2023: 285-297.

INTRODUCTION

Endophytes are indigenous microbes residing inside plant parts, they are microorganisms which can be isolated from disinfected (surface-disinfected) plant parts. Endophytes offer a variety of services for plant growth enhancement; their beneficial effects include nutrient availability, protection of plants from plant pathogens, nitrogen fixation, etc. Hunting and scientific analysis of such endophytes can open a door of possibilities for the isolation and detection of endophytes having the potential for plant growth stimulation [1].

Medicinal plants are randomly occurring in nature and have potently been used for a variety of illnesses by animals and humans for a long time. *Phyllanthus emblica* L. (Amla) are also one of them; Amla (Indian Gooseberry) is a member of the *Phyllanthaceae* family and it is used as a remedy. Importance of amla as a medicinal plant has been mentioned in Siddha, Unani Ayurveda and the naturopathy medicine system. Plant parts of amla are being used as medicine without any side effects. Along with medicinal properties, these plants are serving as a host for microbes during their life cycle. Endophytes are part of almost every plant including medicinal plants Amla, the endosymbiotic mutualistic relationship between endophytes

and plants is in a way beneficial for plants but not well studied in all the prospective of plant growth promotion [2].

Plant growth-promoting bacteria (PGPB) harbours many plant growth-promoting traits because of which they increase plant growth and plant health directly or indirectly (Figure 1). PGPB can directly increase crop yield by solubilizing phosphate, fixing atmospheric nitrogen and/or by siderophore production. Additionally, they may indirectly promote plant growth by producing some bioactive compounds, limiting damage to plant growth by pathogenic agents and producing some phytohormones like indole-3- acetic acid [3].

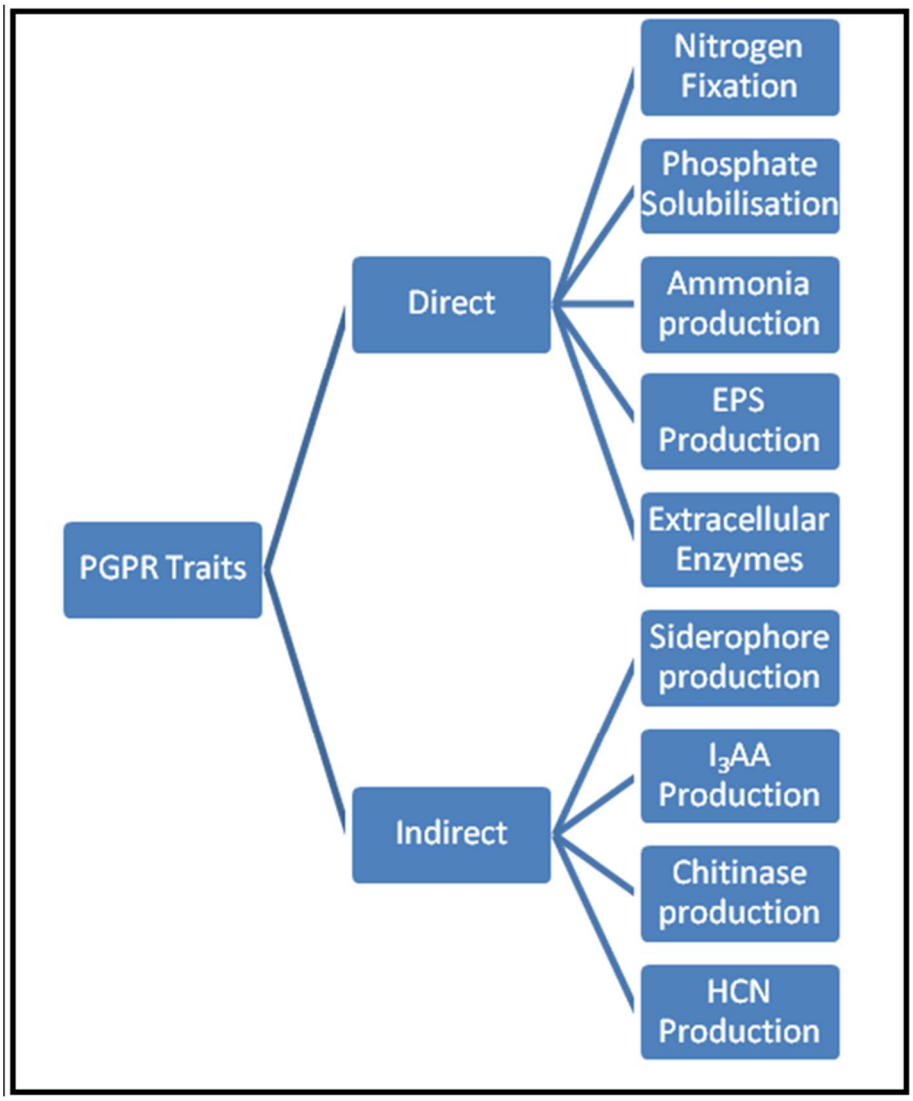

Figure 1. Plant growth promoting traits.

Plant growth-promoting bacteria suppress plant pathogens and promote plant growth by being effective broadly against vivid organisms including insects, nematodes, bacteria, fungi, viruses and parasitic plants. Bacteria like *Azospirillum* sp., *Klebsiella* sp., *Arthrobacter* sp., *Serratia* sp., *Burkholderia* sp., *Bacillus* sp., *Arthrobacter* sp., *Enterobacter* sp., *Pseudomonas* sp. has been reported to show plant grow promotion via the mechanism of plant protection against the pathogen. In addition to nitrogen-fixing bacteria, non-nitrogen fixers like *Bacillus subtilis* isolated from the roots of a banana plant are working against *Fusarium* wilt effectively and are being developed as bio-organic fertilizers [4].

Endophytic isolates from Amla have not been studied in depth enough for their plant growth promotion activity and their application in the field of the agriculture sector. Endophyte serves as an excellent study model for studying plant-microbe interaction as plant growth promotion influenced by plant growth-promoting endophytic bacteria in a variety of plants including rice, soybean, pea, lentil, cucumber is been scientifically reported in several studies. Along with plant growth promotion, bioactive compounds produced by endophytic microbes resembles the metabolites produced by medicinal plants; detailed

investigation and study of endophyte present in medicinal plants can open a door of possibilities for novel bioactive compounds as well [5].

MATERIAL AND METHODS

Sample collection

Plant parts of a healthy tree of *Phyllanthus emblica* L. were collected from its natural habitat; sample collection was done from the north region of Gujarat state, India [Latitude: 23.48444 Longitude: 72.398375 (Ganpat University, Kherva, Mehsana)]. All plant parts including leaves, fruits, stems, and roots were collected and placed in sterile zip-lock bags and stored at 4 °C until further processing [6].

Sample preparation and isolation of endophytes

All plant parts of the Amla plant (leaves, stem, roots, and fruits) were washed with tap water to remove the soil debris and adhering epiphytes, and then they were surface sterilized using 70% ethanol and 2% sodium hypochlorite for 30 seconds and 5 minutes eventually. After the surface sterilization process, they were gently washed twice with sterile distilled water. Samples were then processed for isolation of endophytic microbes within 48 h [1].

For further isolation, all surface sterilized parts were cut with a sterile scissor and then cut into 0.5 cm long fragments. One gram of each sample was macerated in surface disinfected mortar and pestle. The homogenate tissue suspension was then centrifuged at 2200×*g* for 5 min, the supernatant was collected, and 0.2 ml of aliquot was inoculated on a nutrient agar media plate in triplicates using the spread plate technique [5].

Characterization of endophytic isolates

All the Amla endophytes isolated from various plant parts of amla were then characterized for their physiological and metabolic characteristics. Preliminary morphological characterization was done using Gram's staining technique (cross-checked by 3% KOH test) followed by cultural and biochemical characterization. For cultural characterization, colony morphological characteristics were observed and all the endophytic isolates were then inoculated into various substrate-containing media like Suger Broth, Glucose phosphate broth, Simmon's citrate agar slant, Urea broth, Starch agar medium, Gelatin agar medium and Tributyrin agar medium to analyze their metabolic activity for various biochemical tests like sugar fermentation test, citrate utilization test, methyl red test, Voges-Proskauer test and various qualitative enzymatic assays [7].

Analysis of amla endophytic isolates for plant growth-promoting traits

Nitrogen fixation

For testing biological nitrogen fixation activity, endophytic isolates were inoculated on two nitrogen-free media - Yeast Extract Mannitol Agar medium (YEMA), g/L – yeast extract 1, mannitol 10, di-potassium phosphate 0.5, magnesium sulphate 0.2, sodium chloride 0.1, calcium carbonate 1, agar 30 and pH 6.8±0.2, which is a specific medium for isolation of symbiotic nitrogen-fixing bacteria and Ashby's Mannitol Agar medium which is a particular medium for non-symbiotic nitrogen-fixing bacteria, having composition g/L – dihydrogen potassium phosphate 0.2, magnesium sulphate 0.2, sodium chloride 0.2, calcium carbonate 5.0, mannitol 10.0, calcium sulphate 0.1, agar 30, pH 7.0±0.2. After inoculation of isolates on these media plates they were kept for incubation at 28±2 °C for 96 to 120 h and plates were observed for colony growth [5].

Phosphate solubilization

Pikovskaya medium (g/L- magnesium sulphate heptahydrate 0.1, potassium chloride 0.2, tri-calcium phosphate 5.0, ammonium sulphate 0.5, ferrous sulphate heptahydrate 0.002, manganese (II) sulphate dehydrate 0.002, yeast extract 0.5 agar 20.0, pH 7.0±0.2) containing 2.4 mg/mL bromo phenol blue was inoculated with the endophytic isolates of Amla and incubated for 48 - 72 h. After completion of the incubation period plates were observed for yellow-coloured tri-calcium phosphate hydrolyzing zones [8]. Then phosphate solubilization index was calculated using Eq. 1 [9].

$$\text{Phosphate solubilization index (PSI)} = \frac{\text{Colony diameter+Clearing zone}}{\text{Colony diameter}} \quad (1)$$

Indole-3-acetic acid (IAA)

Qualitative and quantitative analysis of IAA production was studied. For qualitative analysis, the endophytic isolates were inoculated into 50 ml of nutrient broth supplemented with 0.5% (v/v) of L-tryptophan and incubated for ten days at 28±2 °C in a pre-fumigated incubator. After the period of incubation, the culture was centrifuged for 10 min at 5000×*g* and then the supernatant was analyzed for the presence of IAA. For detection of IAA production, 2 ml of Salkowski reagent was added to 1 ml of supernatant and tubes were incubated for 30 min in the dark. The development of pink colour was

observed as a positive result and compared to the un-inoculated medium which remained colourless and acted as a negative control [10].

Further, for the quantitative analysis, after incubation of 72 h, 1.5 ml of sample was taken from the culture broth and kept for centrifugation at 16,000×*g* for 5 min. Then 1 ml of supernatant was withdrawn carefully into a fresh test tube, and an equal amount of Salkowski reagent was added, vortexed gently and incubated in the dark for 30 min. Production of IAA was confirmed by measuring the intensity of the pink colour developed after 30 min of incubation in the dark. Quantification of IAA was done by taking absorbance at 536 nm spectrophotometrically against an uninoculated medium as blank. The amount of IAA produced was calculated quantitatively using the standard curve [11].

Siderophore production

Production of siderophore was checked qualitatively using a CAS (chrome azurol S) agar plate. For preparing CAS agar medium 10 ml of MM9 salt solution was added to 75 ml of double distilled water, then 3.22 g of PIPES [piperazine-N, N'-bis (2-ethanesulfonic acid)] was added until pH reached 6.8, after this bacteriological agar powder was added as per 3% concentration. One millilitre of 20% glucose solution and 3 ml of sterile Casamino acid solution was then added into MM9 solution in the next step. This medium was then autoclaved and allowed to cool till 50 °C. Further, a 10 ml solution of blue dye was gently added from a side wall of the medium-containing flask and gently agitated without disturbing autoclaved medium. Endophytic isolates were inoculated into the medium after pouring and allowing it to solidify. After incubating it for 7 days plates were checked for a holo orange zone [12].

For quantitative determination, 10 µl of freshly grown culture (activated 16 to 18 h before inoculation) was added in 1.5 ml sterile centrifuge tubes containing LB broth and incubated at 28±2 °C for 48 h. After incubation tubes were centrifuged for 10 minutes at 16,000×*g*, the supernatant was collected and 0.5 ml of supernatant was mixed with 0.5 ml of CAS reagent. The absorbance of this solution system was measured after 20 min of incubation period at 630 nm using a spectrophotometer [13]. Siderophore production in per cent siderophore unit (PSU) was then calculated using Eq. 2.

$$\text{Siderophore production PSU} = \frac{(Ar - As) \times 100}{Ar} \qquad (2)$$

Here, Ar = absorbance of reference (CAS solution and uninoculated broth); As = absorbance of a sample (CAS solution and supernatant).

Chitinase production

Active cultures of all Amla endophytes were inoculated with a semi-minimal medium containing colloidal chitin. After inoculation, all inoculated plates were incubated at 28±2 °C for 5 to 7 days. To observe the hydrolytic zone on the medium plate, at the time of result observation 1% Congo red dye solution was added, allowed to react for 10 min and then washed with 1 M NaCl solution [14].

Ammonia production

Endophytic isolates of Amla were tested for the production of ammonia in peptone water. For qualitative detection, each tube of 10 ml peptone water was inoculated with freshly grown cultures. To check the qualitative analysis of ammonia production the red litmus paper strip was inserted and the broth tube was incubated for 48-72 h at 28±2 °C [15].

For quantitative analysis, endophytic isolates were tested for the production of ammonia in a medium supplemented with peptone and NaCl. Actively growing cultures were inoculated in 10 ml peptone water and incubated at 28±2 °C for 72 h. After incubation, all the tubes were observed for the development of a yellow to brown colour with the addition of 0.5 ml Nessler's reagent [16]. Absorbance was noted at 450 nm using a spectrophotometer and the concentration of ammonia produced by the bacterial endophytes was calculated using an ammonium sulphate standard curve [17].

HCN production

Endophytic isolates were inoculated on an HCN stimulation medium (supplemented with 4.4 g glycine per litre of nutrient agar). After inoculation a disc of Whatman filter paper no. 1 (5.5 cm diameter) dipped into HCN revealing solution i.e., 0.5% picric acid and 2% Na_2CO_3 was placed in lids of all the Petri plates, then all the plates were sealed with parafilm tape and kept at 28±2 °C temperature for 4 days. After incubation, plates were observed for the orange-brown colour of filter paper [18].

EPS production

For the production of exopolysaccharide (EPS) all the endophytes were inoculated into a sterile modified medium developed by Pereira Duta et al. (2006) with the composition in g/L mannitol 10, dipotassium hydrogen phosphate 0.1, potassium dihydrogen phosphate 0.4, magnesium sulphate 0.2, yeast extract 0.4, sodium chloride 0.1, manganese chloride 0.15 and pH 7.0; after inoculation, all flasks were incubated at 28±2 °C for 7 days [19].

Extraction and quantification of EPS

The inoculated medium (5 ml) after the incubation of 7 days was collected and diluted with Milli Q water (1:1 v/v). This diluted culture was centrifuged at 16,000×*g* for 15 min at 4 °C and supernatant was collected and added with 75% Acetone in a 1:4 ratio. This mixture was again centrifuged at 5,000×*g* for 15 min at 4 °C to precipitate EPS. Pellets were re-suspended again and kept at 4 °C overnight. After refrigeration, the centrifugation step was repeated and the pellet was measured for its wet weight. For dry weight, pellet was dried overnight at 55 °C. Dry weight and wet weight were measured for further comparative study [20].

RESULTS AND DISCUSSION

Morphological, cultural and biochemical characterization

Inoculation of macerated tissue suspension of different parts of the Amla plant on different culture media like nutrient agar medium, actinomycetes isolation agar and nitrogen-free medium has resulted in the isolation of 25 endophytic isolates including fungi. From 21 amla endophytes, 8 isolates comprising of seven bacterial isolates (designated as RN1, RN2, RN3, YR1, YR2, NR1, NR3) and one actinomycete (designated as RAC) belongs to roots; 8 isolates including six bacterial isolates (namely SN1, SN2, SN3, SN4, SY1, SY12) and two actinomycetes (namely SAC, AIA1) were isolated from stem sample, while 2 isolates (designated as LN1 and LN2) and 3 isolates (namely FN1, FN2 and FN3) belong to homogenate tissue suspension of Amla leaves and amla fruit sample respectively. Morphological analysis of all the isolates was done based on Gram's staining which was confirmed by the 3% KOH test. All the amla endophytes were analyzed for their metabolic activity by performing various biochemical tests and have shown diverse metabolic activity as shown in Table 1. Amla plant has not been well studied concerning their endophytic variety and availability. Exploration of different plant parts of *Phyllanthus emblica* L. has resulted in the isolation of 21 different isolates including bacteria and actinomycetes. Out of 21 isolates, 14 isolates were obtained on nutrient agar medium; 3 were on actinomycetes isolation agar and 4 isolates grew on nitrogen-free medium. Endophytes from various other traditional medicinal plants showing plant growth-promoting activity have been reported previously, which includes endophytes of Artemisia *annua*, *Gynura procumbens*, *Tridax procumbens* and some Chinese herbs [21]. Gohain et al., 2015 [22] have reported an antimicrobial biosynthetic potential of endophytic actinomycetes isolated from *Emblica officinalis* G. (Amla plant).

Table 1. Results of biochemical test of endophytic isolates of amla plant

Biochemical tests												
Endophytic Isolate	Sugar fermentation test					Citrate Utilization test	MR Test	VP Test	Nitrate reduction test	Ammonia production	H_2S production		Catalase test
	1	2	3	4	5					Red litmus paper test	Lead acetate paper test	TSI slant test	
RN1	-	+	A	-	-	-	-	-	-	+	-	-	++
RN2	A	+	+	-	A	-	-	+	-	+	-	-	+
RN3	-	+	+	-	-	-	+	-	-	+	-	-	+
SN1	+	+	+	A	A	-	+	-	-	+++	-	+	++
SN2	+	+	A	+	A	-	-	+	-	-	-	-	+
SN3	-	+	+	-	-	-	-	-	-	++	-	-	++
SN4	-	+	-	-	-	+	-	+	-	+	-	-	+
FN1	A	+	+	-	A	-	-	-	+	++	-	-	++
FN2	-	+	+	-	-	-	-	-	-	++	-	-	++
FN3	-	-	+	-	A	-	-	+	-	+	-	-	+
YR1	A	+	+	A	A	+	-	-	-	+	+	-	++
YR2	-	-	+	-	-	+	-	+	-	+	+	+	++
SY1	A	+	A	-	A	-	+	-	-	+	-	-	++
SY12	-	-	-	-	-	-	+	-	-	+	+	-	+
RAC	-	+	-	-	-	-	-	+	-	+	-	-	+
SAC	A	+	A	-	A	-	-	-	-	++	-	+	++
LN1	A	-	-	A	A	-	-	-	-	+	-	-	+
LN2	-	+	+	-	-	-	-	-	-	++	-	-	++
NR1	A	A	+	-	A	-	-	-	-	++	-	-	+
NR3	-	+	-	-	-	-	-	+	-	+	-	-	++
AIA1	-	+	-	-	A	-	-	-	-	++	-	-	+

Key: '+' – Positive; '++': Present in high amount; '-'– Negative; 'A'- Only acid; 1,2,3,4,5- Glucose, Xylose, Galactose, Lactose and Maltose respectively

Cultural characteristics (size, shape, margin, texture, consistency, elevation, opacity and pigmentation) of all the isolates were studied; 10 isolates of nutrient agar media (out of 14) with moist consistency and smooth texture, 4 endophytic isolates gave pigmented colonies and 1 endophytic isolate formed colony with filamentous margin. The majority of endophytic isolates recovered from the actinomycetes isolation agar gave colonies with dry consistency and dry texture; isolate RAC and AIA1 formed choky white colonies while SAC grew by forming dry colonies with light brown colour.

From all 21 isolates, 47.62% (10 out of 21) isolates are Gram-positive rods, 14.28% are Gram-positive filamentous-shaped microorganisms, 9.52% are Gram-positive spherical-shaped bacteria and 26.57% are Gram-negative rod-shaped bacteria.

Enzymatic assay

Secretion of lytic enzymes like amylase, lipase and proteases by microorganisms associated with plants can promote plant growth by increasing nutrient availability and inhibiting plant pathogens. As explained by Wang et al., 2021 [23] proteases produced by PGPB can degrade cell wall components of various pathogens and can offer plant protection. As the cell wall of pathogenic fungi and oomycetes are composed of chitin, glucan, cellulose and other biopolymers, they can be degraded by enzymes like lipase, cellulase and chitinase produced by microorganisms [24]. In the current study, all the isolates were studied for the production of lytic enzymes like lipase, protease and amylase (Table 2). From all 21 endophytic isolates of Amla, 57.14% isolates (12 out of 21) showed amylase activity by forming a zone of starch hydrolysis on a starch agar plate; 38.09% endophytic isolates (8 out of 21) gave proteolytic activity on casein agar medium and 19.04% (4 out of 21) endophytic isolates displayed lipase activity positive. Amongst all, isolates SN1 and FN3 showed significant amylase activity while isolate RN1 gave a good zone of protein hydrolysis on the gelatin agar plate. Hawar, 2022 [25] has reported that the production of extracellular enzymes by endophytic isolates of the medicinal plant *Phyllanthus emblica* L. can improve plant growth by making nutrients available to the host plant.

Plant growth-promoting traits

Nitrogen fixation

Plant growth-promoting bacteria can add some beneficial effects on plant growth and crop production through some direct plant growth-promoting mechanisms like nitrogen fixation, nutrient solubilization and the production of growth regulators [26]. As shown in Table 2, out of all 21 endophytic isolates of the Amla plant four bacterial isolates YR1, YR2, SY1 and SY12 showed nitrogen fixation ability as they grew on a specific nitrogen-free medium (YEMA). The shortage of nitrogen has increased the demand for chemical fertilizers supplementing nitrogen in the soil. Ultimately, these chemicals are creating huge problems in the environment at the ecosystem level. Several endophytic microorganisms have been recognized that restore nitrogen symbiotically [27]. The nitrogen fixation ability of endophytes helps in the promotion of plant growth.

Table 2. Qualitative direct and indirect plant growth promoting traits and enzymatic assay of Amla endophytes

Isolate	Nitrogen fixation	EPS Production	Ammonia Production (Nessler's test)	Phosphate solubilization	Detection of enzymatic activity				HCN Production
					Amylase	Protease	Lipase	Chitinase	
RN1	-	+	++	+	+	++	-	-	-
RN2	-	+	++	+	+	-	-	-	-
RN3	-	+	+	+	+	-	+	-	-
SN1	-	+	++	-	++	-	-	-	-
SN2	-	-	+	+	-	+	+	+++	-
SN3	-	+	+	++	-	-	-	+	-
SN4	-	++	+	+	+	-	-	++	-
FN1	-	-	++	-	-	-	+	-	-
FN2	-	+	++	+	-	-	-	++	-
FN3	-	+	+	+	++	+	+	-	-
YR1	+	+	+	+	-	+	-	+	-
YR2	+	++	++	-	-	-	-	-	+
SY1	+	+	+	-	-	-	-	+	-
SY12	+	+	+	+	+	+	-	-	-
RAC	-	-	+	++	+	-	-	++	-
SAC	-	-	+	-	+	+	-	+	-

LN1	-	+	++	-	+	+	-	+	-
LN2	-	+	+	+	+	+	-	++	-
NR1	-	-	+	++	+	-	-	+	-
NR3	-	-	++	+	-	-	-	-	-
AIA1	-	+	+	++	-	-	-	-	-

Key: '+' – Positive; '++'; Present in high amount; '-'– Negative

Phosphate solubilization

Hassan et al., 2021 [28] explained that solubilization of inorganic phosphate by endophytes isolated from medicinal plants can promote plant growth and have reported that endophytic isolate has shown inorganic phosphate solubilization by forming maximum solubilization zone up to 13 mm. Khan et al., 2019 [29] reported maximum phosphate solubilization by bacterial isolates with a phosphate solubilizing index of 2.82. As shown in Figure 2, amongst 15 Amla endophytic bacterial isolates, SN3 showed the highest phosphate solubilization ability with a phosphate solubilizing index of 3.0 and 14 mm solubilizing zone on the Pikovskaya agar medium plate. Endophytic isolates YR1, SY12, and NR3 gave good phosphate solubilization activity with a phosphate solubilizing index of 2.75, 2.66 and 2.5 respectively. Phosphorous solubilizer *Piriformospora indica*, *Trichoderma harzianum* and *Phomopsis liquidambari* have shown an increase in growth, quality and yield of their respective host plants *Brassica campestris*, maize and rice respectively[30]. Phosphorus is an important nutrient for the development of plant; solubilization of insoluble phosphate from soil can help plant for better growth. The phosphate solubilization index value was calculated for all 15 amla endophytes showing positive results and represented in **Figure 2**, which suggests isolates SN3 and AIA1 are having highest phosphate solubilization ability.

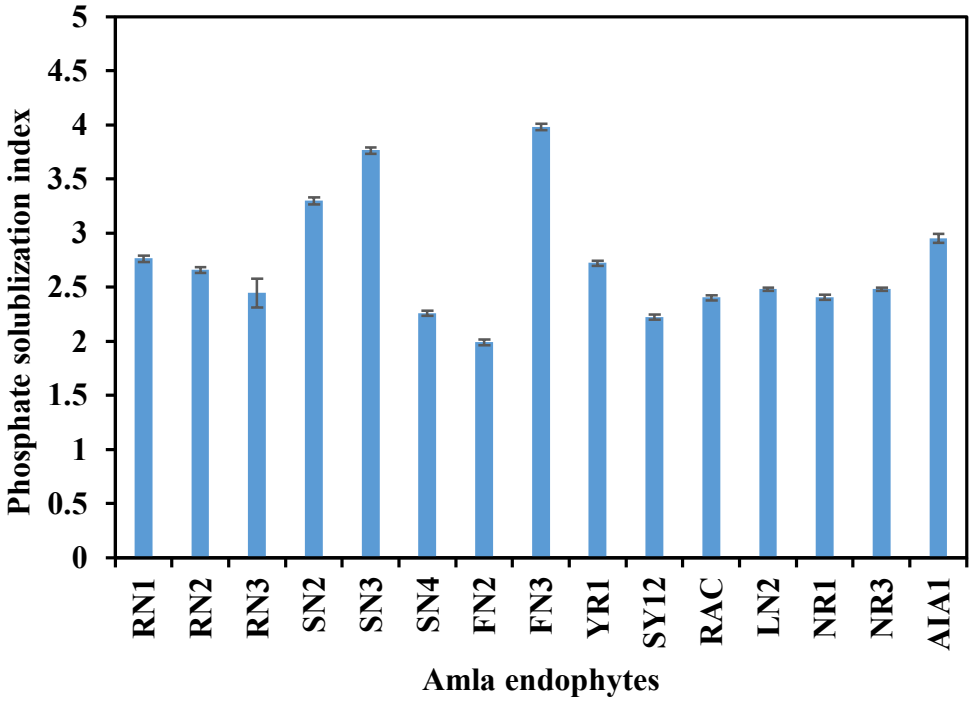

Figure 2. Phosphate solubilization index of endophytic isolates of Amla plant

Chitinase production

Chitinase is the enzyme that can hydrolyze chitin. This enzyme has been explored for its inhibitory effect on plant-harming fungi and insects [31], as it breaks downs the fungal cell wall and exoskeleton of many vertebrates made up of biopolymer chitin. Chitinase production can be detected by detecting N-acetyl-D glucosamine a monomer of chitin [32]. Amongst all the endophytic isolates of the Amla plant sample, 52.38% of isolates (11 out of 21) showed chitinase activity (Table 2); endophytic isolate SN2 gave the highest chitinase activity with a substantial zone of decolourization followed by isolates SN4, FN2, RAC and LN2. Taechowisan et al., 2003 [33] have reported that purified chitinase enzyme from *Streptomyces aureofaciens* CMUAc130 showed some antifungal activity against plant pathogen *Fusarium oxysporium* by inhibiting their hyphal growth.

Indole -3 acetic acid production (IAA)

Plant hormones play a vital role in the development and growth of plant cells, Jahn et al., 2021 [34] have explained that IAA levels highly influence the processing the root initiation, cell growth, tropism and senescence. Microbial IAA acts as an intermediary between microorganisms and plants and plays an important role in signalling between both symbionts. Out of 21 amla endophytes, 9 isolates exhibited production of IAA (ranging from 1.46 ± 0.10 µg/ml to 14.5 ± 0.10 µg/ml); amongst which isolates RN2, YR1, and SN4 gave good IAA production. Taghinasab & Jabaji, 2020 [35] have reported some of the bacterial and fungal endophytes including *Bacillus* sp., *Pantoea vagans* MOSEL-t13, *Serratia marcesence, Bipolaris* sp. CS-1 and *P. geniculata* which are producing IAA and helping plant growth. IAA is a plant growth-promoting hormone belonging to class auxin which plays a very important role in cell elongation and cell division. From all 21 microbial isolates as shown in Figure 3, 9 isolates namely, RN2, SN1, SN4, FN1, FN3, YR1, YR2, SY1, and SY12 showed a positive result.

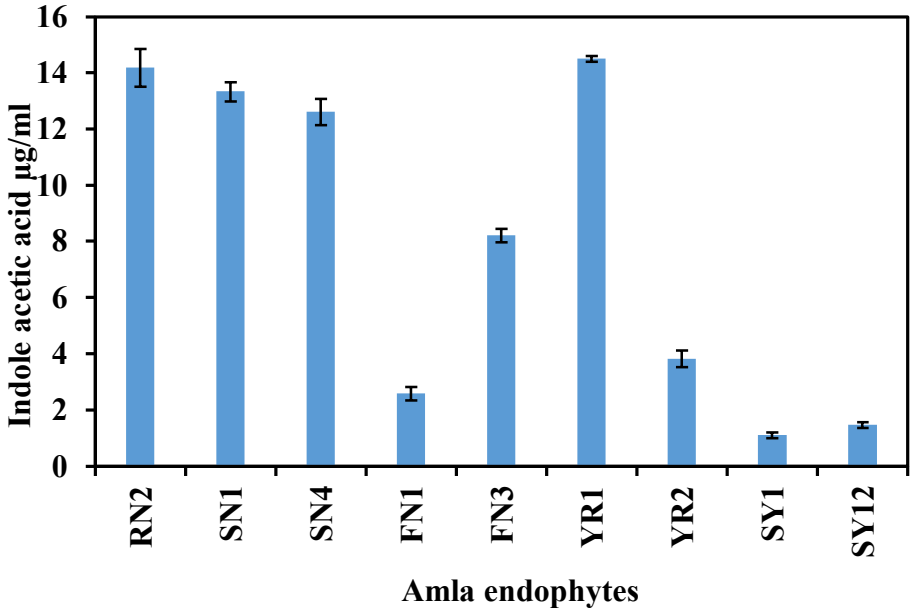

Figure 3: IAA production by endophytic isolates of Amla plants

Siderophore production

Endophytic bacteria *Pantoea agglomerans* and *Bacillus safensis* have been reported with 66% and 50% siderophore activity respectively [36]. Amla endophyte SY12 has shown 97.46±0.45% SU followed by 95.4 ± 0.2 and 95.3 ± 0.2 by NR1 and NR3 respectively, which is the highest amongst all following the isolates showed activity zone on CAS agar plate. Jain et al., 2021 [37] have reported siderophore production by endophytes of leaves and roots ranging from 15.57 to 83.38% SU. The results of the quantitative analysis of siderophore production are described in **Figure 4**. Though some of the isolates have not shown positive results in qualitative analysis, they have shown good per cent decolourization of CAS dye. The percentage activity of all the endophytic isolates ranged from 68.18±0.09% SU to 97.46±0.45% SU. Amongst all, isolates SY12, NR1, NR3, YR2 and RN2 showed considerable siderophore production.

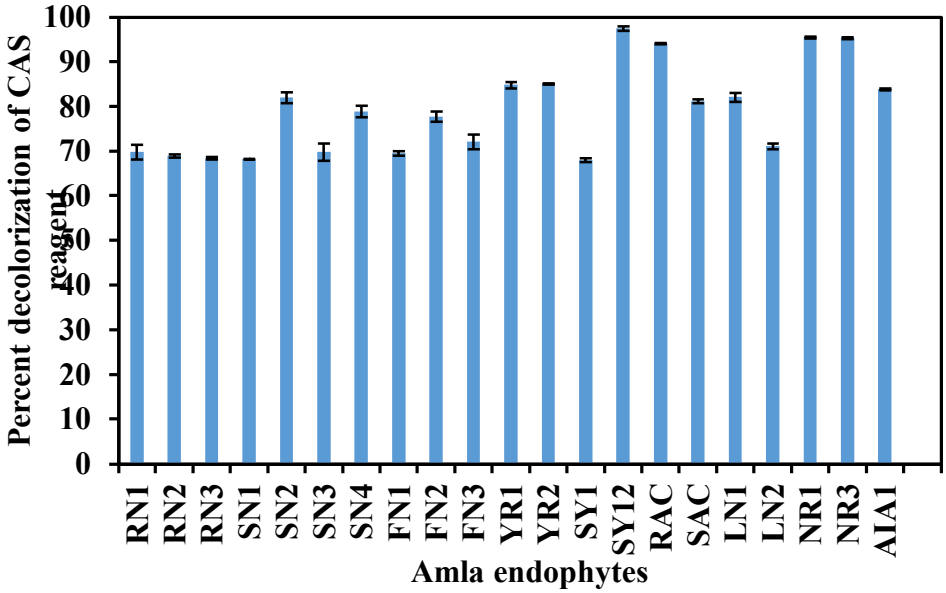

Figure 4. Siderophore production in terms of per cent decolourization of CAS reagent by endophytic isolates of Amla plant

Ammonia production

Endophytic microbes can promote plant growth by the production of ammonia, as it is involved in synthesizing various nitrogen-containing plant biomolecules. It has been reported by [38] that ammonia production by endophytes notably improves root and shoot growth. In the present study, all endophytic isolates are showing ammonia production ranging from 1.10±0.10 to 14.50±0.10 µg/ml; amongst which isolate LN2 showed the highest activity (10 µg/ml). Chaudhary et al., 2021 [39]have reported that ammonia production by *Rhizobium pusense* MB-17a creates alkaline conditions and works as a limiting factor for fungal growth. In the qualitative test (as shown in Table 2) out of 21 endophytic isolates except for isolate SN2, all 20 isolates gave positive results by turning red litmus paper blue because of ammonia production. While in quantitative analysis, all the isolates were found to be positive for ammonia production. Amongst all isolates, as shown in Figure 5 LN2, NR3 and AIA1 have shown the highest ammonia production and SN2, SN1and RN1 have shown the lowest production.

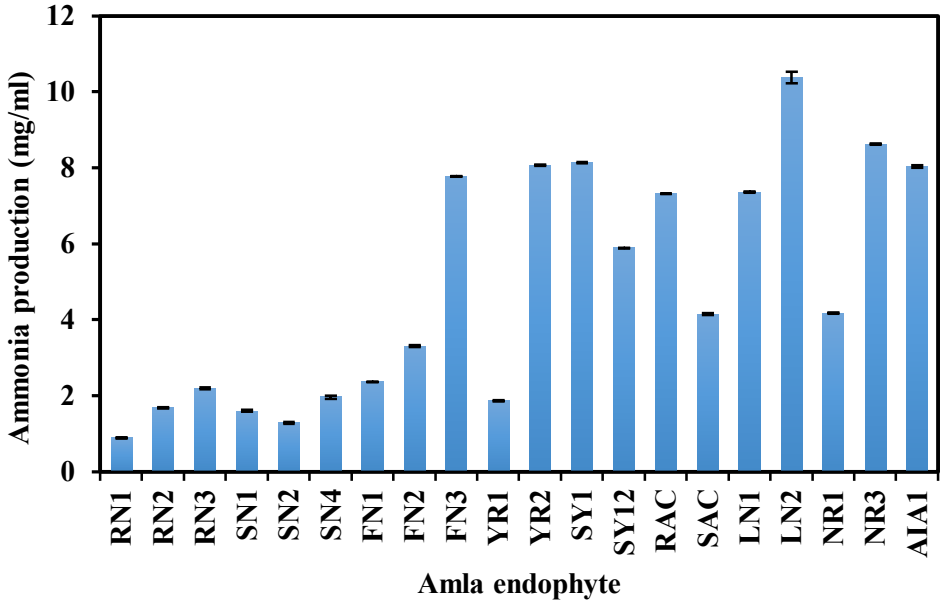

Figure 5. Ammonia production by Amla endophytes

HCN production

Abdel-Hamid [38] have explained that the volatile compounds produced by plant-associated endophytes can injure plant pathogenic microbes. Hydrogen cyanide (HCN) is a secondary volatile compound produced by endophytes. It has been reported that HCN produced by endophytic microbes can suppress infection of plant pathogenic microbes as well as nematodes. The endophytic isolate YR2 of the Amla plant showed the production of HCN by changing the yellowish filter paper (containing HCN-revealing solution) into oranges brown. HCN produced by plant growth-promoting bacteria has been reported to promote plant growth by inactivating pathogenic organisms and increasing phosphate solubilization [40]. Amongst all the isolates isolated from homogenate tissues sample of different parts of the Amla plant, only endophytic isolate YR2 showed positive results (Table 2) for HCN production by giving colour change of picric acid and sodium carbonate suspended filter paper from yellow to orange.

EPS production

The results for the presence or absence of EPS production are shown in Table 2. From a total of 21 isolates, 15 endophytic isolates showed an ability to produce exopolysaccharides. The dry weight and wet weight of the same are described in Figure 6.

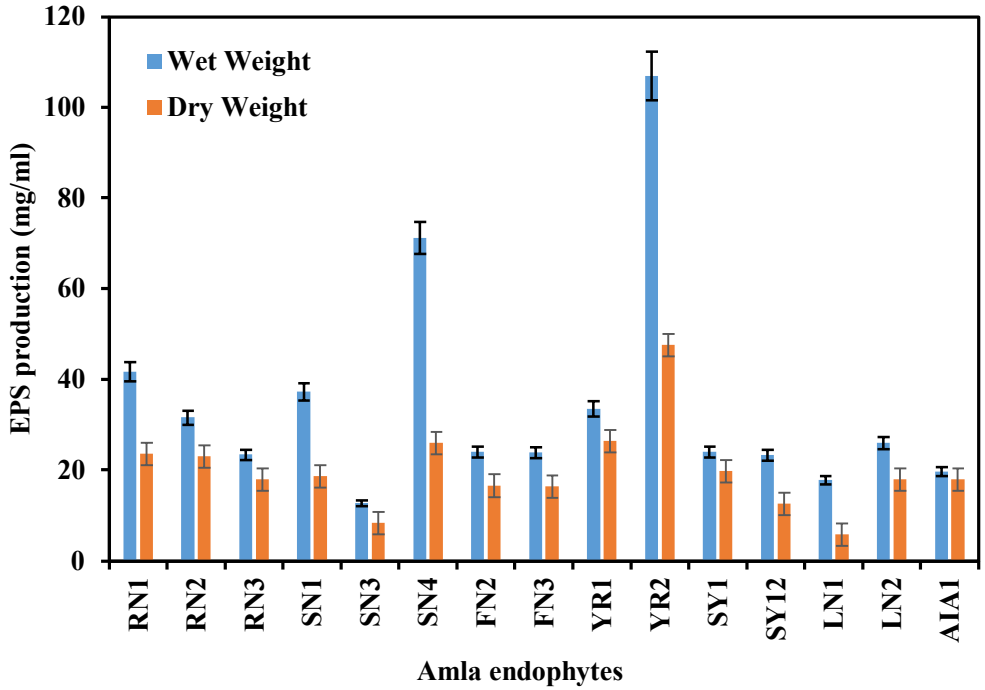

Figure 6. Exopolysaccharide production by Amla endophytes

Exopolysaccharide produced by endophytes has very good potential to improve plant growth as they are involved in the formation of biofilms and protect plants against abiotic stress like drought. Along with improving plant health they also play important role in human health improvement as they possess some antioxidant, antitumor, and anti-inflammation activities [41]. In the current study, 71.42% (15 out of 21) of endophytes isolated from various parts of Amla plants have produced exopolysaccharide substances. Amongst all isolates, YR2 has produced EPS in the highest amount. *Burkholderia vietnamiensis, Enteobacter asburiae, Pseudomonas* sp., *Sphingomonas yanoikuyae, Rhodotorula graminis* are some of the reported examples of endophytic microorganisms producing exopolysaccharides and helping their host plant against biotic stress like drought and salinity [42].

CONCLUSION

In the current study, *Phyllanthus emblica* L. (Indian gooseberry, Amla) has been explored for the presence of endophytes. In this hunt, a total of 25 endophytes were found from samples of Amla plant parts (leaves, root, fruit, and stem) including 4 fungal endophytes, 18 bacterial endophytes and 3 actinomycetes. From 21 endophytic isolates (bacteria and actinomycetes), 15 isolates represented noteworthy enzymatic activity in qualitative enzyme assay. Endophytic isolate FN3 revealed the production of all three lytic enzymes (amylase, protease and lipase) which can help plant in defence against plant pathogen and the

degradation of complex nutrients. Endophytic isolate YR2 produced HCN along with siderophore which can be proven good symbiont by protecting the plant against the infection of nematode and pathogenic fungi. Amla endophytes YR1, YR2, SY1 and SY12 has the capacity for nitrogen fixation; amongst them, YR1 and SY1 are also capable of solubilizing phosphate. Along with nitrogen fixation and phosphate solubilization, Amla endophyte YR1 showed the ability to produce IAA, EPS, siderophore and chitinase. Inoculum development in form of a bacterial consortium or seed treatment of these amla endophytes can help in improving plant growth and crop yield as they are showing various direct and indirect plant growth-promoting traits. The impact of these Amla endophytes on plant growth promotion can be further studied in a greenhouse and field study with various crops. As endophytes SY1, SY12, YR1, YR2, SN4, and FN3 are showing multiple beneficial plant growth-promoting traits like phosphate solubilization, IAA production, siderophore production and nitrogen fixation, they can be furtherly developed as bioinoculants and these endophytic isolates of Amla can be applied on plants using various seed treatments solely or in consortia.

ACKNOWLEDGEMENT

The authors are thankful to the Indian Institute of Advance Research (IIAR), Gandhinagar for providing facilities to conduct PGPR characterization. The authors are personally thankful to Dr. Dhara Patel (Faculty, Microbiology Department, IIAR) and Ms. Anushree Kamath (Research student, IIAR) for their help and support.

CONFLICT OF INTEREST STATEMENT AND COMPETING

The authors declare that they have no conflict of interest in the publication.

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