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Potential of Endophytic Bacteria Isolated from *Vitex negundo* L. to Produce Auxin

Minu M. Ali and Dipak Vora
Dept. of Microbiology, Ramnarain Ruia College, Matunga, Mumbai 400019, INDIA

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Abstract

Endophytic bacteria with plant hormone producing ability were screened. Bacillus endophytes were isolated from the leaves of Vitex negundo L. (nirgundi). The ability of these strains to synthesize auxin (indole-3-acetic acid) was demonstrated. Indole-3-acetic acid (IAA) was extracted from the endophytes. IAA production by these endophytes was established by spectrophotometry (42µg/ml) and rapid in situ assay. IAA production was further confirmed by thin-layer chromatography (TLC) as well as by high-performance liquid chromatography (HPLC). The released IAA increased with the increase of the initial concentration of L-tryptophan in the medium. Effect of auxin producing endophytes on plant growth was demonstrated by conducting pot assay. These endophytic strains showed plant growth promoting characteristics and also exhibited obvious promoting effects on Phaseolus vulgaris. Plant associated endophytes with the ability to produce phytohormone can be valuable biological resources and have vast agricultural potential.

Keywords: Vitex negundo, IAA, endophytes, TLC, HPLC, plant growth, *Phaseolus vulgaris.*

Introduction

Plant hormones are naturally occurring compounds synthesized by plants with extensive applications in agriculture, horticulture, and biotechnology to modify plant growth and development. Some of these plant hormones such as indole-3-acetic acid (IAA) have also been synthesized chemically. These phytohormones commonly act at locations distant from the biological site of production. There are several phytohormone groups including auxins, gibberellins and cytokinins. The most widely studied and used plant hormones are the auxins. IAA is the major auxin involved in many of the physiological processes in plants including cell enlargement, proliferation, tissue differentiation, phototropism and geotropism[1]. In fact, auxins have myriad regulatory functions in plants, including stimulating cell enlargement, root initiation, and lateral root formation, cambium cell division, and differentiation of phloem, xylem[2].

Vitex negundo L. is a plant with myriad medicinal properties and has been used extensively for treatment of a plethora of ailments. The plant has also shown potential as an effective bio-control agent and is also a component of a number of commercially available herbal formulations[3]. Endophytes are microorganisms that grow in living tissues of plants, apparently without inflicting negative effects on the host. Bacterial endophytes have been isolated from surface-sterilized plant tissue or extracted from internal plant tissue[4]. The potential of endophytic bacteria to fix nitrogen and promote plant growth has renewed the interest in such associations. Endophytic bacteria have been isolated from many different plants including trees (pine, yew), fodder plants (alfalfa, sorghum, clover), vegetables (carrot, radish, tomatoes, sweet potatoes, lettuce, soybean), fruits (banana, pineapple), cereals (maize, rice, wheat), and other crops (sugarcane, marigold, coffee)[5,6].

The aim of this study was to examine whether the leaf tissues of *Vitex negundo* L. are colonized by endophytic bacteria and to further isolate and identify them. Additionally they were tested for production of IAA. The effect of these endophytes on plant growth was established.

Material and Methods

The plant material used in this study was the leaves of *Vitex negundo* L. collected from the Aayurmed Biotech farm located at Vikramghad in the state of Maharashtra. The sample herbarium was authenticated from St.Xaviers Blatter herbarium.

Isolation of endophytic bacteria: Seven healthy plants were removed carefully and washed under tap water to remove soil before separating the leaves. These leaves were rinsed in sterile distilled water and then surface-disinfected by soaking in 70% ethanol for 30sec followed by a 5% hypochloride rinse for 3 min. The tissue was then washed 3 times with sterile distilled water[7,8]. A 100µl sample of the water from the third rinse was surface spread on sterile nutrient agar (NA) to verify the efficacy of sterilization [9]. The sterilized leaves were laid, with the exposed inner surface facing downwards, on plates of sterile nutrient agar (NA) [7]. All plates were incubated for several days at 25°C.

Morphological and physiological characterization: Staining: Gram staining, capsule staining and endospore staining were carried out using standard staining protocols.

Biochemical characterization of the test isolates: All the isolated endophytes were characterized using selective/ differential media like MacConkeys agar, Cetrimide agar and Salmonella-Shigella agar (36±1°C for 24 h). Single well-isolated colonies were purified & identified biochemically. Further they were tested using IMViC, oxidase, urease, catalase, pigment production and starch hydrolysis and motility as per the established methods[10,11].

IAA production test: In this study using Gordon and Weber spectrophotometric method, log phase culture (5µl) was inoculated in LB (Luria Bertani) medium (5ml) amended with L-tryptophan (0, 1, 2, and 5mg/ml). The test tubes were incubated on a rotary shaker in the dark for 24h and then centrifuged at 10,000 rpm for 15 minutes. Add o-phosphoric acid (2 – 3 drops) to supernatant (2 ml). The aliquots were shaken, 4ml of Salkowski reagent added and votexed thoroughly. The samples were incubated for 25 minutes at room temperature and their absorbance was read at 530 nm. Auxin quantification value was recorded by extrapolating calibration curve made by using IAA as standard (10 - 100µg/ml)[12,13,14.]

Rapid in-situ assay for IAA production: Agar plates were inoculated with toothpicks into a grid pattern from agar cultures. Grid plates consisted of replicated rows of isolates per plate. Each inoculated plate was overlaid with nitrocellulose disk (82-mm-diameter). Grid plates were overlaid immediately after inoculation. All the plates were further incubated until colonies size diameter reached to 0.5 to 2 mm. The membrane was removed and treated with Salkowski reagent till adequate colour development is achieved. Isolates producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony 1[5].

A dilution series with concentrations of IAA from 0.05 to 8 nmol per 0.5ul aliquot was applied directly onto nitrocellulose disks in a grid pattern of two replications of each dilution. Stock solutions of IAA were freshly prepared as ethanol solutions, and dilutions were made in distilled water (IAA). The resulting spots formed on the nitrocellulose membranes were approximately 2 mm in diameter, approximately the size of bacterial colonies when assayed.

Extraction and purification of IAA: Single bacterial colony was inoculated in 200 ml of nutrient broth amended with 5 mg/ml of tryptophan and incubated on a shaker incubator (48h). Centrifugation was done at 10,000 rpm for 30 min to separate bacterial cells from the supernatant.

This was then acidified to pH 2.5 to 3.0 with 1N HCL and extracted twice with double volume of ethyl acetate. Ethyl acetate fraction extracted was evaporated to dryness in a rotatory evaporator at 40ºC and dissolved in 300 ml of methanol. The extract was stored at -20 ºC till estimation[16,17].

Thin layer chromatography: Thin-layer chromatography (TLC) of IAA was performed according to Lwin *et al,*[18]. The IAA extracts dissolved in methanol were applied to and developed on silica gel TLC plates (Silica gel G f 254, thickness 0.25 mm) and developed in ethyl acetate: chloroform: formic acid (55:35:10). Spots were identified by spraying the plates with Salkowski reagent or Ehmann's reagent. Rf values of observed spots were compared with Rf value of authentic IAA spots[12,19].

High pressure liquid chromatography: The ethyl acetate extract of the isolates were analyzed by HPLC. HPLC chromatograms were produced by injecting 20 µl of the filtered extract onto a Cosmosil C-18 (150 mm x 4.6 mm, internal diameter) in a chromatograph equipped with an ultraviolet detector absorbing at 233 nm. The solvent system used to separate IAA was 0.1 M Potassium dihydrogen orthophosphate Buffer: methanol 65:35 (v/v), flow rate was 1 ml/min [17]. The sample temperature was maintained at 4ºC.

Retention times for peaks were compared to those of authentic standards (IAA) added to the medium and extracted by the same procedures. Quantitation was done by comparison of peak heights.

Pot Experiment: Soil samples were air dried sieved (2mm/10 mesh) and autoclaved. The seeds of beans (*Phaseolus vulgaris*) were emersed in 95% ethanol and 2%$HgCl_2$ for 3mins for surface sterilization. The seeds are then washed with sterile distilled water thrice. Seeds were dipped in overnight suspension of 1ml (0.5$Absorbance_{540nm}$) of endophytic isolate for 10mins. Seeds were further dried and applied to sterile soil to a depth of 4mm. Experiment was performed in duplicate with 10 seeds for each isolate. Sterile seeds without treatment with inoculated media were used as control. The pots were kept in sunlight and uprooted on the 16th day to measure root length and shoot height[20,21].

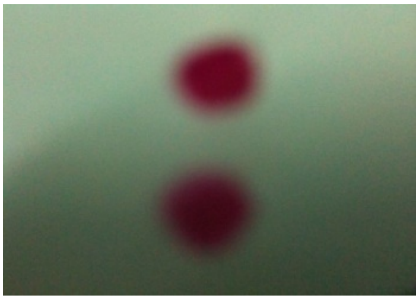
Results and Discussion

The endophytic isolates of *Bacillus sp*. were isolated from leaf tissue of *Vitex negundo* L. These isolates were identified as *Bacillus sp*. on the basis of results of biochemical tests and sugar fermentation behaviour. These isolated *Bacillus sp.* were further screened for their ability to produce plant growth regulator, IAA. Varying levels of IAA production were recorded with different concentrations of tryptophan, i.e. 0, 1, 2 and 5 mg/ml (table 1). A significant increase in the production of IAA (ranging from 6µg/ml to 42µg/ml) was recorded with increase in tryptophan (table 1).

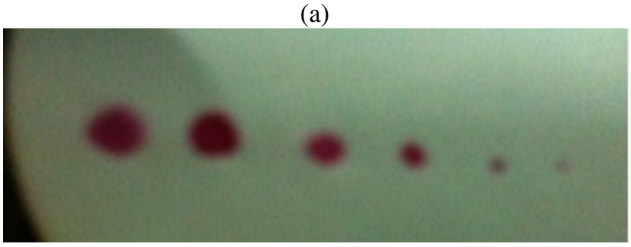
All the colonies of endophytic bacillus immobilized on nitrocellulose membrane on treatment with Salkowski reagent produced a deep red ring in the membrane surrounding the colonies (figure 1). The colour of the reaction is similar to that produced with IAA in solution assays with this reagent.

Table-1
IAA production by endophytic *Bacillus* isolates after 48h of incubation

Tryptophan mg/ml	IAA production (µg/ml) by endophyte	
	Isolate 1	Isolate 2
0	6 µg/ml	9 µg/ml
1	14 µg/ml	15 µg/ml
2	29 µg/ml	30 µg/ml
5	40 µg/ml	42 µg/ml



(a)



(b)

Figure-1
(a) Colonies of isolates after reaction with the salkowski reagent. (b) Concentrations of IAA (from the left) 8, 4, 2, 1, 0.5 and 0.4 nmol. The high concentrations of IAA produce a deep red colour and lower concentrations yield a pink colour on exposure to salkowski reagent.

IAA production by endophytes was further confirmed by TLC. The spots of standard IAA and ethyl acetate extracts of the respective culture were tested in solvent system of ethyl acetate: chloroform: formic acid (55:35:10). Rf value of culture spots and standard IAA spot were observed to be same. Chromatograms of culture spots thus confirmed IAA production.

The HPLC of the ethyl acetate extracts of the endophyte culture broth confirmed that IAA is produced by all the bacterial isolates. Chromatographs obtained of the ethyl acetate extract of both the isolates were compared with the standard IAA chromatograph obtained. Endophytic isolates show IAA production (figure 2 and figure 3).

Pot assay was carried to establish effect of isolated endophytes in plant growth. Both the isolates show significant increase in the plant root length and shoot height. Seeds of *Phaseolus vulgaris* show increase in root length (0.79cm) and shoot height (7.3cm) after treatment with the isolated endophytes (table 2 and figure 4).

Table-2
Root length and shoot height of 10 bean plants treated with IAA producing endophytic *Bacillus* isolates

Test	Mean in cm		Difference from control	
	Root length	Shoot height	Root length	Shoot height
Control	8.465±SD 0.23	12.45±SD 0.38	--	--
Bacillus isolate 1	9.25±SD 0.07	18.25±SD 0.2	0.79	5.8
Bacillus isolate 2	9.41±SD 0.23	19.75±SD 0.31	0.095	7.3

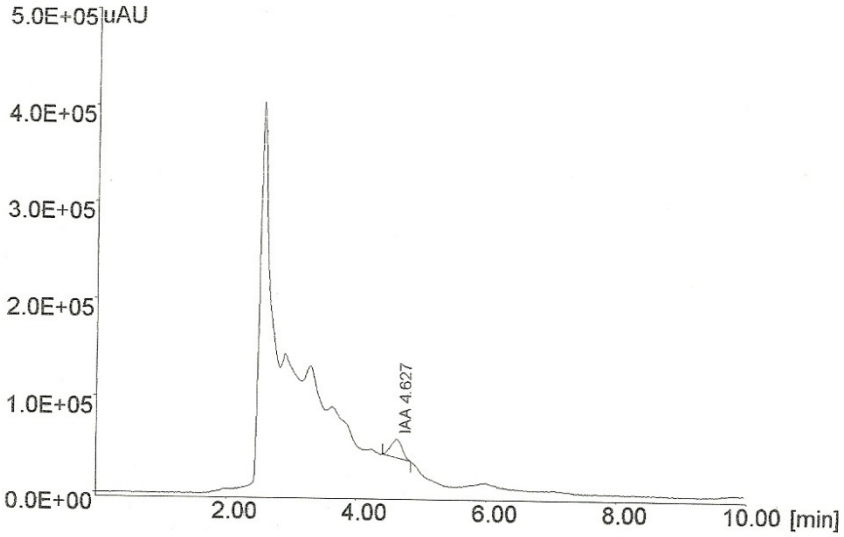


Figure-2
HPLC chromatograph of ethyl acetate extracts of the culture broth inoculated with *Baciilus* isolate 1

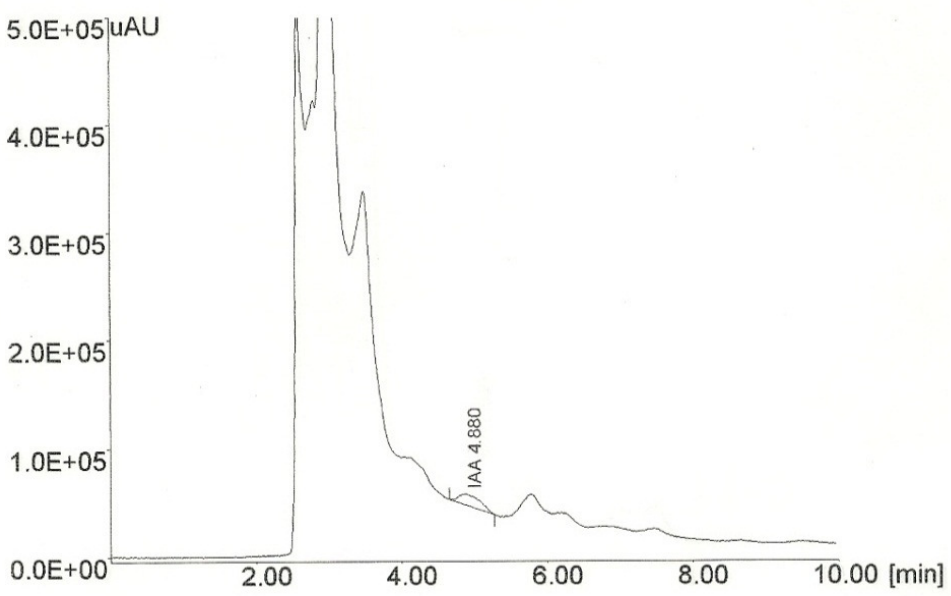


Figure-3
HPLC chromatograph of ethyl acetate extracts of the culture broth inoculated with *Bacillus* isolate 2

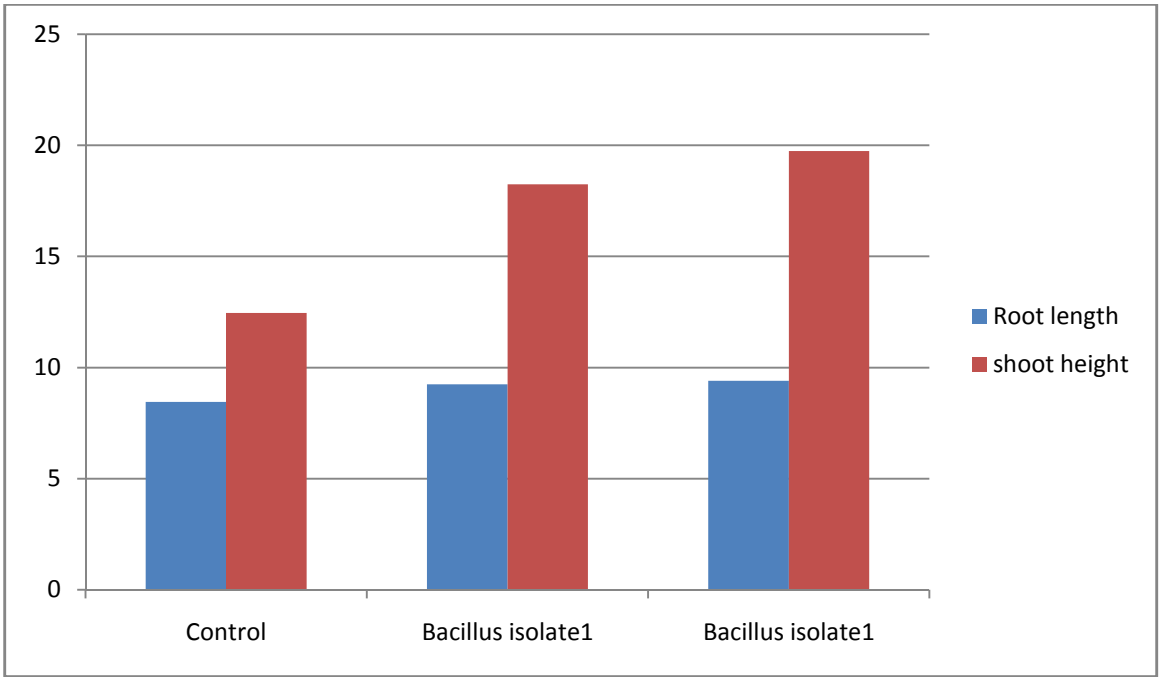


Figure-4
Effect on root length and shoot height of bean plants on treatment with the isolates

Conclusion

Thus the present investigation findings highlight that IAA producing endophytic bacteria from leaves of medicinal plant *vitex negundo* can be easily isolated and may be further exploited after strain improvement for local use. This study demonstrates the occurrence of culturable bacterial endophytes in the medicinally important plant of *vitex negundo* L. Culture filtrates of these isolated endophytes were found to have the ability to produce a significant amount of IAA (auxin), thus were efficient in promoting seed germination and root elongation of the plant. The plant growth promoting properties of these endophytic bacillus strains and their considerable effect on plant growth suggest that these are promising strains with the potential for agricultural, and/or industrial exploitation.

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