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Development of carrier based *bio fertilizer* using endophytic bacteria isolated from okra (*Abelmoschus esculentus L.)*Leaves

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ABSTRACT:

The miscellaneous microorganisms are associated with plants which play a momentous role in plant defense responses. Endophytes spend the whole or at least a part of their life cycle colonizing in the healthy living tissue of the host plant, typically causing no obvious symptoms of disease. The *Abelmoschus esculentus* plant part (leaves) was utilized to isolate bacterial endophytes. 20 bacterial colonies with different colony morphology were selected from different plates for differential staining and biochemical tests (MR-VP, starch, oxidase, catalase, citrate, motility, cetrimide test etc.) Around 3 colonies were selected based on their plant growth promoting Activity (PGPA) which includes phosphate solubilization, Ammonia production, IAA production, Siderophore production, HCN production. From the results obtained through PGPA, a single endophyte was molecular characterized using 16srRNA sequencing. The bacterial endophyte was identified as *Pseudomonas* sp. The selected endophyte was mixed with carrier sugarcane bagasse, applied to plants and the growth parameters were observed high in endophyte applied plants.

KEYWORDS: Endophytes, *Abelmoschus esculentus,* PGPA, 16srRNA, *Pseudomonas*, sugarcane bagasse.

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INTRODUCTION:

Agriculture is the major sector of our country's economy. The research and development systems play a major role in disseminating agricultural biotechnology thereby enhancing the farmer's income. However there is a need to equip the farmers with (basic knowledge of agriculture) appropriate farm management decisions and to follow modern technology. In recent years, the agricultural practices are under pressure in order to provide increased yields to feed the growing global population, which is expected to reach 9.7 billion by 2050[1].

There are many immature fruits, vegetables in Tamilnadu. Among them, brinjal and okra occupy major area in cultivation and major vegetables by consumption. Bhendi(*Abelmoschus esculentus L.)* is an economically important vegetable crop. It is commercially grown in India. India ranks first in the world with 3.5 million tons (70% of the total world population) ofbhendi produced from over 0.35 million ha land [2].

The term endophyte (Gr. Endon-within, phyton-plant) was first coined by De Bary in 1866. There are many bacteria with the capacity of colonizing plants which utilizes the nutrient niche of root surface in the rhizosphere and most of them may even actively switch from the root surface to endophytic lifestyles [3, 4].

Biofertilizers are broadly used to speed up the microbial processes and enlarge availability of nutrients which is easily assimilated by plants. The soil fertility of plants can also be improved by fixing the atmospheric nitrogen and solubilizing insoluble phosphates and by the production of growth promoting substances in soil[5].

MATERIALS AND METHODS:

Collection of sample:

Okra plant was collected from the Agriculture field in Vadapudupatti, Theni district, Tamilnadu. The plants were taken to the laboratory in a sterile manner by placing them in polythene bags with temperature maintained at 4°C.

Isolation of endophytic bacteria:

5cm leaf bits of okra was taken and surface sterilized using 70% of ethanol, 2% of sodium hypochlorite and 0.1 % of mercuric chloride and rinsed three times in sterile distilled water for 2 minutes[6]. After sterilization, okra leaves were macerated and extracted from which 1ml was serially diluted and appropriate dilutions namely 10-4, 10-5 and 10-6 was plated on nutrient agar plates and incubated at 37.C for 48 hours.

Biochemical tests:

20 predominant colonies from nutrient agar plates were chosen and subjected for biochemical tests namely Gram's staining, Indole Production, Methyl Red, Voges Proskauer, Citrate utilization, Oxidase, catalase, motility and spore test[7].

Identification of endophytic bacteria:

The 3 bacterial isolates were chosen namely AEL1, AEL4, AEL6 and plated on Cetrimide, King's B agar medium for further identification of endophytes[8].

Screening for plant growth promoting activities of endophytes:**[PGPA]**

The 3 bacterial isolates AEL1, AEL4, AEL6 were screened for Indole acetic acid [IAA] [9], Phosphate solubilization activity [10, 11, 12]the Siderophore production[13], and HCN production[14].

Molecular characterization of endophytic bacteria*:*

Based on the results obtained from Plant growth promoting activities of endophytic bacteria, AEL6 was considered as the effective endophytic bacteria to promote plant growth and hence AEL6 culture was molecularly characterized for identification on Genus and species. Bacterial isolate AEL6 was grown in nutrient broth medium for 24 h at 28°C and genomic DNA was extracted using phenol/chloroform procedure[15]. Amplification of the 16S rRNA gene was performed with the universal primer pairs of fD2 (5′-AGA GTT TGA TCA TGG CTC AG-3′,) and rP1 [5′-ACG GTT ACC TTG TTA CGA CTT-3′,][16]. The PCR products were sequenced and 16S rRNA gene sequences obtained was further aligned to other closely related bacterial species deposited in NCBI database using the BlastN program.

Preparation of carrier based bio fertilizer:

The carrier chosen for the present study is sugarcane bagasse which possesses high water holding capacity, high storage validity and increase crop yield and quality. The carrier was sterilized at 121° C for 60 minutes. The Pikovskaya's broth culture of *Pseudomonasaeruginosa* and carrier material was mixed in ratio 2ml broth : 1kg carrier. Okra seeds were sterilized with 70% ethanol for 2 min and in 2% sodium hypochlorite for 2 min, followed by washing ten times in sterile distilled water. For this experiment, pure cultures were grown in Pikovskaya's broth at 28°C.The following treatment was investigated with three replicates of the experiment.(1) Control and (2) *Pseudomonasaeruginosa*. Pots were sterilized with 20% sodium hypochlorite solution and filled with sterile loam soil. The okra seeds (10 seeds in each pot) were sown in pots filled with sterile soil. On days 4 and 5 after sowing, the okra seeds were thinned to one plant per hole. The pots were watered to 50% water-holding capacity and were maintained at this moisture content by watering every day. The pot culture studies were carried out for okra plant using the carrier based *bio fertilizer* for a period of 45 days. The plant growth and yield parameters such as seed germination, leaf length, whole plant length, flowering and yield at a regular interval of five days were recorded.

RESULT AND DISCUSSION:

A total of 20 endophytic bacteria were isolated from okra plant leaves. These isolates were evaluated for their biochemical characteristics (Table-1).

TABLE 1: Biochemical test:

Sample	Gram staining	MR	VP	Citrate	Oxidase	Catalase	Indole	Motility	Spore test
AEL1	-	+	-	+	+	+	-	-	-
AEL2	-	+	-	+	+	-	-	-	-
AEL3	-	+	-	+	+	-	-	-	-
AEL4	-	+	-	+	+	+	-	-	-
AEL5	-	+	-	+	+	-	-	-	-
AEL6	-	+	-	+	+	+	-	-	-
AEL7	+	-	-	+	+	-	+	-	-
AEL8	-	+	-	+	-	+	-	-	-
AEL9	+	+	-	+	-	-	+	-	+
AEL10	+	+	-	+	-	-	+	-	+
AEL11	-	+	-	+	+	-	-	-	-
AEL12	+	+	-	+	-	-	+	-	-
AEL13	+	-	+	+	+	-	+	-	-
AEL14	+	-	+	+	+	-	+	-	-
AEL15	-	+	-	+	+	-	-	-	-
AEL16	+	-	-	+	-	-	-	-	-
AEL17	+	-	-	+	+	-	-	-	-
AEL18	+	-	-	+	+	-	-	-	-
AEL19	+	-	-	+	+	-	-	-	-
AEL20	-	+	-	+	+	-	-	-	-

AEL – *Abelmoschus esculentus* leaf

In the present study, the organism revealed Gram positive (10), Methyl Red positive (13), Voges Proskauer positive (2), Citrate utilization positive (20), Oxidase positive (15), Catalase positive (4), Indole production positive (6), motility negative (20) and spore formation positive (2).If the organisms show negative result for Gram stain, Voges Proskauer, Indole production, Motility and spore tests but positive result for Methyl red, Citrate utilization, oxidase and catalase tests, the possibility of *Pseudomonas* sp. is high[17].

Identification of endophytic bacteria:

The selective media such as King's B & Cetrimide showed positive growth for AEL1, AEL4, AEL6and hence the bacterial isolates may be *Pseudomonas* sp. The similar results have been observed by Sivagamasundari*et al*., 2014[8].

Plant growth promoting activities:

Phosphate solubilization:

The clear zone was produced by all three strains and are identified as the best potential phosphate solubilizer based on their capacity to solubilize tri calcium phosphate [Ca3 (PO4)2] by the production of clear halo zone on the medium. The zone ranges from 1cm to 1.5cm and maximum phosphate solubilization was expressed by isolate *AEL6* on Pikovskaya's agar plate in the range of 1.5 cm diameter of transparent zone.

Most commonly, phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plant species. The ability of bacteria to solubilize mineral phosphate has gained the development of agricultural microbiologists as it can induce the availability of phosphorus and iron for the plant growth. Plant growth promoting bacteria have been shown to solubilize precipitated phosphorus and improve phosphate availability to plant the represent a possible mechanism of plant growth promotion under field conditions. Alive [free living] phosphate solubilizing bacteria release phosphate from additional soluble inorganic and organic phosphate compounds in soil and so contributes to increase available phosphate from the plants[18].

Siderophore production:

Pseudomonas isolates were screened for siderophore formation. The bacteria grown on CAS agar produced siderophore. The*Pseudomonas* has changed the color of the Chrome Azurol's agar plate's sky blue into orange zone formation. The color changes in CAS agar plates were recommended for the production of siderophore by the microorganisms isolated and color intensity based on the effect of siderophore concentration. The microorganisms producing siderophore hold back some soil borne fungal pathogen through direct role of siderophore – mediated iron competition in the bio control ability[19].

HCN production:

The HCN has been suggested as one of the important antifungal agent to inhibit the pathogen which is produced by *Pseudomonas sp.* The HCN formation by *Pseudomonas*isolates on nutrient agar was expressed in the color change of picric acid contain Whatman filter paper strips from deep yellow (-) to orange or brown (+) at 28°C for 4 days by *Pseudomonas* sp[20].

Indole Acetic Acid production:

The *Pseudomonas*spwhich produces IAA induces the plant growth. Auxin is one of the most considered hormones among plant growth promoters. The best characterized and physiologically most active auxin in plant is IAA. IAA production ranged from 0.09 to 0.14µg/mL. The maximum IAA production is observed in AEL6 *Pseudomonas* sp. The bacterial IAA plays a major role in the development of the host plant root system[21].

TABLE 2: Plant Growth Promoting Activities (PGPA):

Sample	Phosphate solubility	IAA Production	HCN production	Siderophore

	(zone formation)	(µg/mL)		production
AEL 1	1 cm	0.09	-	+
AEL4	0.8 cm	0.08	+	+
AEL6	1.5 cm	0.14	+	+

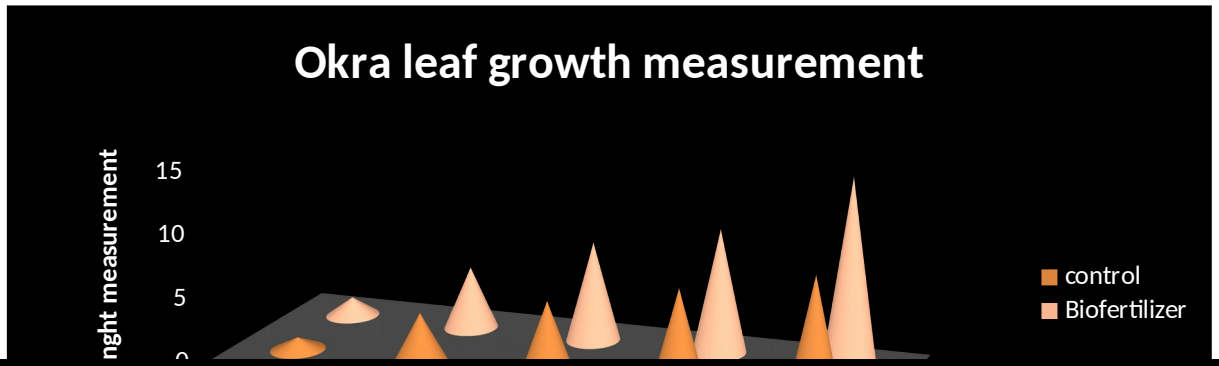
Molecular characterization of endophytic bacteria:

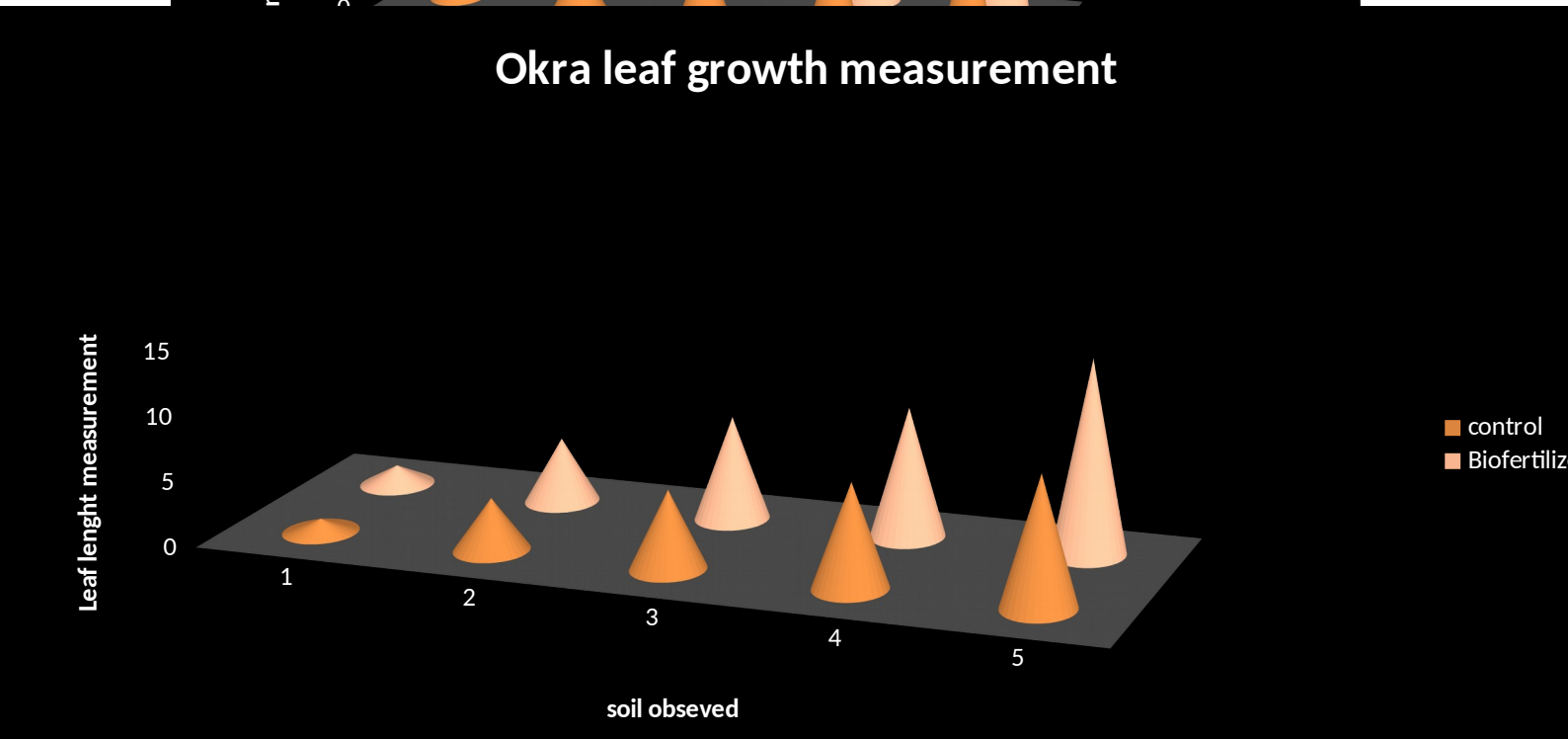
One bacterial strain AEL6 showing potential plant growth promoting activities was selected for molecular characterization. The partial 16S rRNA gene sequences of AEL6 showed similarity with *Pseudomonas* sp. Sequences available in public domain databases[22].

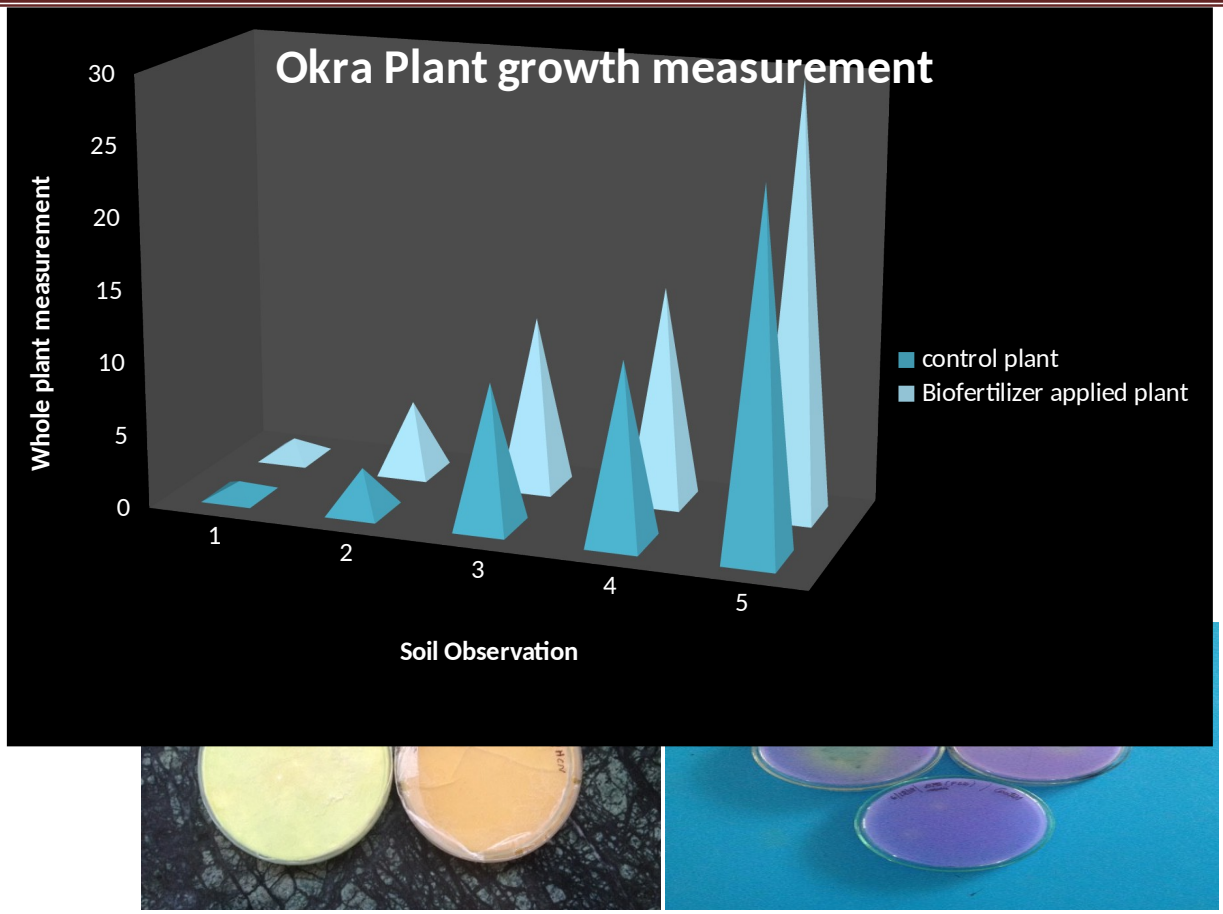
Preparation of carrier based bio fertilizer:

The endophytic bacteria isolated from okra were tested for their influence on growth parameters and showed considerable influence on crops. The okra plants inoculated with AEL6 isolate showed fastseed germination, increased leaf length(fig:1), whole plant length (fig:2), and increased flowering. A corresponding significant increase in the root and shoot biomass was also observed in the endophyte applied plants.

Fig:1 Comparative Analysis of leaf in Abelmoschus Esculentus plant control and bio fertilizer applied plant







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CONFLICT OF INTEREST:

The authors of this paper have no conflict of interest in publishing this paper.

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