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Isolation, identification and characterization of endophytic bacteria- *Azospirillum sp*. and *Pseudomonas sp*. from Brinjal (*Solanum melongena* l.)

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ABSTRACT

The aim of the present study was to isolate, identify and characterize the bacterial endophytes that reside inside the plant tissue of brinjal as native. Totally twenty isolates (*Azospirillum sp*. and *Pseudomonas sp*.) were isolated from brinjal from three different localities namely, Annamalai university, Putthur, Tamilnadu and Karaikal, Pondicherry, India. All the bacterial isolates were evaluated for its biochemical characterization, IAA production and nitrogen fixation. The results showed that, among the isolates of *Azospirillum sp*., isolate number ABRK 10 and in concerned with Pseudomonas sp., isolate number PBRU3 fix high nitrogen, hence they were selected as efficient strains and molecularly characterized by sequencing their 16srDNA. Sequencing results confirmed that their sequence are native to *Azospirillum brasilense* and *Pseudomonas fluorescens* respectively. This study indicates that there is huge number of endophytic microbes occupy a relatively privileged niche within plant and usually contribute to plant health. From this isolation, it was clear that, bacterial population occupy variety of ecological habitat as unique inside their own host plant as species specific to face their own nutrition, competition and contribute plant health and defence.

Keywords: Endophytes, brinjal, *Azospirillum brasilense, Pseudomonas fluorescens*.

INTRODUCTION

Plant tissues are non-sterile; spaces within them are inhabited by different species of fungi and bacteria known as “endophytes”. Most of these microorganisms are not pathogenic to the host plant. Moreover the association between the plants and its endophytes is very often mutualistic. Endophytic bacteria had been isolated from many different plants including (pine, yew), fodders (alfalfa, sorghum, clover), vegetables

(carrot, radish, tomatoes, sweet potatoes, lettuce, soya bean), fruits (banana, pineapple, citrus), cereal grains (maize, rice, wheat) and other crops (marigold, sugarcane, coffee) (Rosen Blueth and Maritine-Romero, 2006). Numerous studies have demonstrated that endophytes synthesize bioactive compounds which can, for example stimulate plant growth and increase resistance to plant pathogens. It is believed that some part of all the metabolites detected in plant tissues originate from colonizing bacteria.

Among the diversity of endophytes studied in all crops, vegetables are poorly studied. So the aim of the present research was to study the diversity and widespread application of endophytes as inoculants in agriculture fields - *Azospirillum brasilense* and *Pseudomonas fluorescens* colonizing interior tissues of brinjal as their native host. Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of sub-tropics and tropics. The name brinjal is popular in Indian subcontinents. The name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except in higher altitudes. It is a versatile crop adapted to different agro - climatic conditions and can be grown throughout the year. It is a perennial but grown commercially as an annual crop. A number of cultivars are being grown in India and consumer preference dependent upon fruit color, size and shape.

Brinjal fruit (unripe) is primarily consumed as cooked vegetable in various ways and dried shoots are used as fuel in rural areas. It is low in calories and fats, contains mostly water, some protein, fiber and carbohydrates. It is a good source of minerals and vitamins and is rich in total water soluble sugars, free reducing sugars, amide proteins among other nutrients. Brinjal is known to have ayurvedic medicinal properties and is good for diabetic patients. It has also been recommended as an excellent remedy for those suffering from liver complaints (Shukla and Naik, 1993)

MATERIALS AND METHODS

Collection of samples

Eggplant variety *Annamalai* was collected from Agriculture field of Annamalainagar, Faculty of Agriculture, Annamalai University, Puthur, Tamilnadu and Karaikal, Pondicherry, India. The plant samples collected from three localities were transported in refrigerated box (4°C) to the laboratory.

Method of isolation

Plant parts such as root, stem and leaves of the samples were initially washed with tap water to remove adhering soil particles and soaked in disinfectant solution of 0.1% mercuric chloride for 2 minutes. Then the samples were thoroughly washed in tap water followed by sterile distilled water for 3-4 times. Then they were toweled with sterile filter paper and weighed 1gm and macerated in mortar and pestle and the extract volume was made to 10 mL by adding with sterile distilled water. From that, 1mL was taken and serially diluted with sterilized distilled water.

Isolation of bacterial colony

About 0.1mL each from the dilutions of 10^{-4}, 10^{-5} and 10^{-6} was transferred to petri-plates individually containing nitrogen free malate medium (Dobereiner,1992) and King's B (Schaad, 1980) medium for the isolation of *Azospirillum sp.* and *Pseudomonas sp.* respectively. The petri plates were incubated at 28ºC for 48hrs. Meanwhile 0.5mL of the extracts was transferred to a 5mL sterile semi solid NFB medium and incubated to observe the sub-surface pellicle formation of *Azospirillum sp*. The sub-surface pellicle with change of colour of the medium into blue was further streaked on NFB solid medium to get individual colony of *Azospirillum sp*. The individual colonies from NFB and King's B medium exhibited yellow fluorescent colour and were transferred to slants containing the respective medium. This was incubated at 28ºC for 48 hrs, after that maintained at 4ºC in refrigerator for further study.

Purification of *Azospirillum sp.* and *Pseudomonas sp.*

The isolates of *Azospirillum sp*. were further confirmed by streaking on to potato infusion agar (BMS) plates and incubated at 32ºC for 7days. Individual colonies showing scarlet with typical pink colour often wrinkled colonies were transferred to nutrient agar slants for further study. The petriplates containing King's B medium for *Pseudomonas fluorescens* were examined after incubation under ultraviolet light at 360µm for the confirmation of colonies exhibiting fluorescence.

Characterization of *Azospirillum sp.* and *pseudomonas sp.*

Microscopic observation of the wet mounts of the 72hrs old cultures was carried out for their shape and motility.

Further the isolates were subjected to various biochemical tests for their characterization. The representative isolates of *Azospirillum sp*. and *Pseudomonas sp*. were identified by preliminary identification tests (John Holt *et al.,* 1994) like the Gram Stain, Catalse test, Oxidase test, Spore formation, Carbohydrate fermentation test, Motility test (Hanging drop method) Biotin requirement (Allen, 1953) Nitrate and Nitrite reductase (Yordi and Ruoff, 1981).

Nitrogen Fixation and IAA production

The nitrogen fixation of the isolates was determined by Microkjeldahl Assay described by Humphris, 1956 and IAA production (Gorden and Paleg, 1994).

Molecular characterization and Phylogenetic analysis of endophytic bacterial isolates by 16srRNA sequencing

Isolation of DNA

Total genomic DNA was extracted by standard method. All the isolates were grown at 30°C for 5 days in shake flasks containing 100 mL of NFB and King's B liquid medium for isolates of AORU5 and PBRU3 respectively. Pellet was obtained by centrifugation and washed twice with distilled water. Approximately 200 mg of pellet was used for the genomic DNA extraction. The pellet was suspended in 500 µL of the lysis solution [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 250 mM NaCl and 2% SDS]. Lysozyme was added to obtain a final concentration of 1 mg/mL and then incubated at 37°C for 60 min. After the addition of 10 µl of proteinase K (10 mg/mL), the mixture was incubated at 65°C for 30 min. The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The organic extraction was repeated, and the supernatant was taken and 4M Ammonium acetate and two volumes of isopropanol were added. Total genomic DNA was precipitated by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in Tris EDTA buffer (pH 8.0) and stored at -20°C.

16SrRNA Sequence

Amplification of 16SrRNA gene sequence was achieved by using 27F (forward primer) and 1492R (reverse primer). PCR setup was prepared to 25µl containing 100ng of template DNA, 2 mM $MgCl_2$, 5µm of primers, 2.5 µl of 10X assay buffer which includes (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% Gelatin), 10 mM each of dNTPs and 5units/µl of Taq DNA Polymerase. Initial denaturation at 94ºC for 5 min, 35 cycles of 1 min at 94ºC, 1 min at 45ºC and 1 min at 72ºC, followed by a final 10 min extension at 72ºC.

The amplified products of approximately 1,461 bp of AORU5 and 1.341 bp of PBRU3 were sequenced by pyro sequencing method. Sequence was run in the blast search through National Centre for Biotechnology Information (NCBI) database using the BLAST Programme. Closely related sequences were downloaded and aligned using CLUSTAL X. A phylogenetic tree was constructed using the Neighbour joining method. A phylogenetic and molecular evolutionary analysis was performed using MEGA (Molecular Evolutionary Genetics analysis) version 4.0. Based on the homology 100% similarities were observed with *Azospirillum brasilense* and *Pseudomonas fluorescens* of the isolated AORU5 and PBRU3 respectively.

RESULTS

A total of twenty eight isolates were isolated from brinjal. Of the 28 isolated, 15 were *Azospirillum sp.* and 13 were *Pseudomonas sp.* Eight *Azospirillum sp.* were isolated from the plant sample collected from Annamalainagar, which includes five from root, two from stem and one from leaf. From plant sample of Karaikal, two from root, two from stem and one isolate from leaf were isolated. From the plants collected from Puthur, two were isolated from root.

Concerned with *Pseudomonas sp*. six were isolated from plants collected in Annamalainagar. Of which three were isolated from root, two from stem and only one from leaf. From the plants collected at Karaikal, two were isolated from root, each one from stem and one from leaf. From Puthur plant sample, two from root and one from stem were isolated.

The maximum percentage (3.05) of nitrogen fixation recorded with the ABRK10 and minimum of 1.14% recorded with ABRU2 of isolates of *Azospirillum*. The IAA production ranged from 0.18 to 1.60 µg. In concerned with isolates of *Pseudomonas,* the nitrogen fixation was observed with only two isolates (0.81% and 1.02%). But all the isolates produced IAA ranged from 0.05 to 1.80 µg.

Table: 1 List of bacterial endophytes isolated from different tissues and locality of Brinjal.

S. NO.	BACTERIAL ISOLATES	BACTERIAL NAME	ISOLATED FROM	LOCALITY
1.	ABRU1	*Azospirillum sp.*	Root	Annamalainagar
2.	ABRU2	*Azospirillum sp.*	Root	Annamalainagar
3.	ABRU3	*Azospirillum sp.*	Root	Annamalainagar
4.	ABRU4	*Azospirillum sp.*	Root	Annamalainagar
5.	ABRU5	*Azospirillum sp.*	Root	Annamalainagar
6.	ABSU6	*Azospirillum sp.*	Stem	Annamalainagar
7.	ABSU7	*Azospirillum sp.*	Stem	Annamalainagar
8.	ABLU8	*Azospirillum sp.*	Leaf	Annamalainagar
9	ABRK9	*Azospirillum sp.*	Root	Karaikal
10.	ABRK10	*Azospirillum sp.*	Root	Karaikal
11.	ABRK11	*Azospirillum sp.*	Stem	Karaikal
12.	ABSK12	*Azospirillum sp.*	Stem	Karaikal
13.	ABLK13	*Azospirillum sp.*	Leaf	Karaikal
14.	ABRP14	*Azospirillum sp.*	Root	Putthur
15.	ABSP15	*Azospirillum sp.*	Root	Putthur
16.	PBRU1	*Pseudomonas sp.*	Root	Annamalainagar
17.	PBRU2	*Pseudomonas sp.*	Root	Annamalainagar
18.	PBRU3	*Pseudomonas sp.*	Root	Annamalainagar
19.	PBSU4	*Pseudomonas sp.*	Stem	Annamalainagar
20.	PBSU5	*Pseudomonas sp.*	Stem	Annamalainagar
21.	PBLU6	*Pseudomonas sp.*	Leaf	Annamalainagar
22.	PBRK7	*Pseudomonas sp.*	Root	Karaikal
23.	PBRK8	*Pseudomonas sp.*	Root	Karaikal
24.	PBSK9	*Pseudomonas sp.*	Stem	Karaikal
25.	PBLK10	*Pseudomonas sp.*	leaf	Karaikal
26.	PBRP11	*Pseudomonas sp.*	Root	Putthur
27.	PBRP12	*Pseudomonas sp.*	Root	Putthur
28.	PBSP13	*Pseudomonas sp.*	Stem	Putthur

Table 2: Nitrogen fixation and IAA production of obtained isolates of *Azospirillum sp.* from brinjal.

ISOLATE NUMBER	N FIXATION %	IAA PRODUCTION ($\mu g/ML^{-1}$)
ABRU1	2.21±0.055	1.20±0.025
ABRU2	1.14±0.041	1.60±0.035
ABRU3	2.05±0.026	0.45±0.035
ABRU4	1.52±0.040	1.20±0.612
ABRU5	2.52±0.068	1.16±0.049
ABSU6	1.96 ±0.040	0.33±0.035
ABSU7	2.44±0.045	0.82±0.041
ABLU8	1.52±0.040	0.18±0.007
ABRK9	2.05±0.035	1.09±0.021
ABRK10	3.05±0.056	1.10±0.028
ABRK11	2.02±0.047	1.72±0.049
ABSK12	1.82±0.045	0.19±0.035
ABLK13	1.78±0.045	0.57±0.070
ABRP14	2.18±0.030	1.19±0.014
ABSP15	2.05±0.045	0.65±0.025

Values are ± SD of three samples

Table 3: Nitrogen fixation and IAA production of obtained isolates of *Pseudomonas sp.* from brinjal.

ISOLATE NUMBER	N FIXATION %	IAA PRODUCTION (μGML^{-1})
PBRU1	0.81±0.050	1.28±0.040
PBRU2	-	1.18±0.041
PBRU3	1.02 ±0.052	1.80±0.050
PBSU4	-	0.40±0.042
PBSU5	-	1.24±0.014
PBLU6	-	0.05±0.035
PBRK7	-	1.16 ±0.030
PBRK9	-	0.52 ±0.047
PBSK10	-	0.52 ±0.047
PBLK11	-	0.17±0.02
PBRP12	-	1.18±0.037
PBRP13	-	1.17±0.051
PBSP14	-	1.05 ±5.557

Values are ± SD of three sample

Table 4: Biochemical characteristic of obtained isolates of *Azospirillum sp.* and *Pseudomonas sp.* from brinjal
+ Positive

ISOLATE NUMBER	APG	UTILIZATION OF DIFFERENT CARBON SOURCE				BN	NRA	NIRA	SF	GS	CA	OA	MO
		MAL	SUC										
ABRU1	+	-	+	+	-	+	+	+	-	-	-	+	+
ABRU2	-	+	+	+	-	+	+	+	-	-	+	+	+
ABRU3	-	+	+	+	-	-	+	+	-	+	+	+	+
ABRU4	+	+	-	+	+	+	+	+	-	+	-	+	+
ABRU5	-	+	+	+	+	-	+	+	-	-	-	+	+
ABSU6	+	+	-	+	+	+	+	+	-	-	-	+	+
ABSU7	+	-	+	-	+	-	+	+	-	-	+	-	+
ABLU8	+	-	-	-	+	+	+	+	-	+	-	+	+
ABRK9	-	-	+	-	-	+	+	+	-	-	+	+	+
ABRK10	+	+	+	-	-	-	+	+	-	-	+	+	+
ABRK11	+	+	+	+	+	+	+	+	-	-	-	+	+
ABSK12	+	-	+	+	-	-	+	+	-	-	+	+	+
ABLK13	-	+	-	+	-	+	+	+	-	+	-	+	+
ABRP14	+	+	+	+	+	-	+	+	-	-	+	+	+
ABSP15	-	+	-	+	+	+	+	+	-	+	-	+	+
PBRU1	-	+	-	-	-	+	+	-	-	-	-	+	+
PBRU2	-	-	-	-	-	+	-	-	-	-	+	-	+
PBRU3	-	-	-	+	+	-	+	-	-	-	+	+	+
PBSU4	+	-	-	+	+	-	-	-	-	-	-	+	+
PBSU5	+	+	+	+	+	-	-	-	-	-	+	-	+
PBLU6	+	+	-	-	+	-	-	-	-	-	+	+	+
PBRK7	+	+	-	-	-	-	-	-	-	-	-	+	+
PBRK8	+	-	-	-	+	-	-	-	-	-	-	-	+
PBSK9	-	+	-	-	-	-	-	-	-	-	+	+	+
PBLK10	+	-	+	+	-	-	-	-	-	-	-	+	+
PBRP11	-	+	+	+	+	-	-	-	-	-	+	+	+
PBRP12	+	+	+	+	+	-	-	-	-	-	-	-	+

APG: ACID PRODUCATION FORM GLUCOSE; **MAL:** MALATE; **SUC:** SUCCINATE; **MAN:** MANNITOAL; **FRU:** FRUCTOSE; **BN**: BIOTIN NEEDS; **NRA:** NITRATE REDUCTASE ACTIVITY; **NIRA:** NITRITE REDUCTASE ACTIVITY; **SF:** SPORE FORMATION, **GS:** GRAM'S STAIN; **CA:** CATALASE ACTIVITY; **OA:** OXIDASE ACTIVITY; **MO:** MOTILITY

On the basis of the results obtained, it could be concluded that brinjal harboring diverse group of bacterial endophytes. However, the benefits of these bacterial endophytes are not yet clearly understood. We hypothesize that isolated bacterial endophytes might be useful to its respective host species. Nonetheless, our research findings could be useful, as a foundation for further research on both in agriculture, particularly vegetable production, as well as its endophytic bacteria.

CONCLUSION

A considerable research effort is required to inoculate endophytic bacteria. In order to guarantee reproducibility, reliable methods of inoculum delivery should be developed. This is especially for the inoculation of trees. It should be noted that the development of successful application technologies would fully depend on improving our understanding of how bacterial endophytes enter and colonize plants. This remark could be applied to all aspects of the ecology of bacterial endophytes and only under those circumstances can the potential use of bacterial endophytes for plant beneficial purposes be fully evaluated.

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