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Isolation, Morphological and Cultural Characterization of Bacterial Endophytes Associated with Different Groundnut Varieties

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ABSTRACT

The present investigation was conducted to determine the morphological and cultural traits of the bacterial endophytes associated with groundnut, which were obtained from different parts like root, stem and leaves of groundnut. Out of the 125 morpho types, 25 were selected based on morphological and physiological characterization and pure cultures obtained by quadrant streak method. Morphological and physiological characterization of isolates was done based on colony shape, size, elevation, surface, margin, colour, pigmentation, motility, gram's reaction and cell shape. The colony characters in agar plate varied from cream white to yellow pigmented, with wrinkled, smooth and glistening surfaces and on the nutrient broth medium varied from surface growth, clouding of the broth and sedimentation. Among 25 isolates obtained, 68 % (18 isolates) were found to be Gram negative (–ve) and 32 % (Seven isolates) were found to be gram positive (+ve). Around 80 % of isolates (20 isolates) were found to have rod shaped cells with varied size ranging from small rods to long rods and 20% (Five isolates) of the isolates found to have cocci shaped cells. About 60 % (15 isolates) of the isolates were found to have motility function while 40 % (10 isolates) of bacterial isolates were found to be non-motile and motility is one of the attributes of being an endophyte.

Key Words: *Broth, Glistening, Gram's reaction, Morphotypes, Motility, Nutrient, Pigmentation, Quadrant streak method, Sedimentation, Wrinkled. .*

The bacterial endophytes are the manufacturers of bioactive compounds which can be widely used in agricultural, medical and industrial application. The word endophyte refers to a meaning of in the plant (endon = within, phyton = plant). Endophytes are most commonly deûned as those organisms whose infections are inconspicuous, the infected host tissues are at least transiently symptomless and the microbial colonization can be demonstrated to be internal.

Endophytes colonize the interior parts of the host plant through horizontal (from the environment) or vertical transmission (via seeds). On the basis of evolutionary relatedness, taxonomy, host plant and ecological niche, endophytes are divided into two major groups: (1) Clavicipitaceous or class I endophytes which inhabit some grasses and (ii) Non clavicipitaceous or class II endophytes which are associated in the asymptomatic tissues of non-vascular plants such as ferns, allies, conifers and angiosperms (Harman, 2000). Two general types of soil bacteria have been shown to have the capacity to act as PGPB (Plant Growth Promoting Bacteria); rhizospheric bacteria, that are typically found around the roots of plants; and endophytic bacteria that are found within the tissues of the plant itself do not withstanding the fact that endophytic bacteria may also be found free-living in the soil (Santoyo *et al.,* 2016).

In accordance with their life strategies, bacterial endophytes can be classified as obligate or 'facultative' (Hardoim *et al*., 2008). Obligate endophytes are strictly dependent on the host plant for their growth and survival and transmission to other plants occurs vertically or via vectors. Facultative endophytes have a stage in their life cycle in which they exist outside host plants.

Endophytic bacterial diversity can be considered a subset of the rhizosphere and/or root-associated bacterial population (Marquez-Santacruz *et al.,* 2010). Groundnut also hosts a diverse group of endophytic bacteria which has proved to be advantage for the growth and development of the crop. Endophytic bacteria seem to be distributed in most of the plant species and have been isolated from plant parts, namely, stems, leaves, roots, flowers, fruits and seeds (Lodowyckx *et al*., 2002).

Microbes profit from plants because of the enhanced availability of nutrients, whereas plants can receive benefits from bacterial associates by growth

enhancement or stress reduction (Hardoim *et al.,* 2008). These endophytes do not cause damage to the host organism but they promote plant growth by one or more of these three factors; the production and secretion of plant growth regulators (Verma *et al.,* 2001).

The endophytic occurrence of particular bacteria is the result of chance factors, determined by the chances of developing roots coming into contact with effective levels of bacteria that can become endophytic, and deterministic factors, determined by the presence of dedicated genetic systems that enable bacterial plant crosstalk and an active endophytic colonization process. Basumatary *et al*. (2021) isolated and evaluated endophytic bacteria from leaves and stems of tomato and characterized by morphological, cultural, biochemical and molecular approaches.

The most common way of entry is through the roots, through primary and lateral root hair cells, root cracks and wounds, as well as hydrolysis of root cells. Other ways sites include stomata, particularly on leaves and young stems; lenticels and germinating radicles (Hardoim *et al.,* 2008). Endophytes are found in plants of most ecosystems and are of agricultural importance since they help to improve crops yields, by stimulating plants growth and immune response excluding plant pathogens by niche competition, as well as actively participating in phenylpropanoid metabolisms and antioxidant activities (Pandey *et al.,* 2018). The endophytic colonization and movement of microorganisms may be greatly influenced by the micro-environment, as well as developmental and environmental factors.

A flagellate, rod-shaped novel endophytic bacterium named as M15^T was isolated from the roots of rice. Later this bacterium was subjected to various morphological and biochemical tests such as cell shape, motility, Gram staining, catalase and oxidase activities. Upon molecular identification by 16S rRNA sequencing, this was identified as *Rhizobium oryzihabitans* sp., nov., (Zhao *et al*., 2020). A total of 32 bacterial endophytes were isolated from four plant tissues (root, stem, leaf and grain) of six rice varieties cultivated in central-eastern and northeastern states of India. Out of 32 isolates, (19) 59.3 % were positive for Gram's reaction (17) 53.1% were able to produce indole acetic acid. (Kumar et al., 2020).

An experiment was carried out by Alsulton *et al*. (2019) to isolate and characterize the bacterial endophytes from cocoa plants. A total of 103 bacterial endophytes were isolated from healthy ties of com plant such as leaves, branches and fruits. Later these endophytes were checked for their antagonistic properties against *Phytopthora palmivora* causing black pod of cocoa in Malaysia. Mintoo *et al.* (2019) isolated six different bacterial endophytes from different parts of Indian long pepper (*Piper longum*) plants viz, roots, nodes, internodes, petioles, leaves and spikes. Later, they were biochemically characterized and identified by 16S rRNA sequencing. Majority of the bacteria were the members of the phyla Proteobacteria and Firmicutes which normally help in plant growth promotion.

MATERIAL AND METHODS

Site of Sample Collection and crops used for sampling.

The sample collection site was collected AICRP, RARS, Tirupati, Andhra Pradesh, India, where groundnut research plots are being carried out. Healthy leaves, stems and root samples were collected during *rabi* season of 2022-23. Twenty three varieties of groundnut were selected. Ten Plants were selected randomly in their seg staged and uprooted manually along with the root system, shoot seg & root segref for collection.

Sample preparation

The groundnut samples were analysed in Department of Agricultural Microbiology for isolation of bacterial endophytes. All samples were washed to isolate to remove adherent soil particles. Followed by surface stemilage of samplesSurface sterilization of groundnut samples with 70 % ethyl alcohol for 3 minutes followed by washing with sterile distilled water to remove alcohol. later plant samples were surface sterilized with 1.2 % (w/v) of Sodium Hypochlorite solution (NaOCl) for 20 min at 110 rpm and followed by washed with sterile distilled water for 5-6 times. To check the sterility of samples, 0.1 ml aliquot from final wash was spread on nutrient agar plates. (Gyaneshwar *et al*., 2001). If any growth was detected in the sterility check, samples were discarded. The surface sterilized samples were then used for isolation of endophytic bacteria by culture dependent based technique.

Isolation of bacterial endophytes

The bacterial endophytes were isolated according to the procedure by Bacon *et al*. (2002). Root, shoot and leaf segments of 2 cm length were excised using flame sterilized scalpel by cutting little bit portion on either side of the leaf, stem and root section. All the individual samples were blotted dry with filter paper and then weighed to have final sample of 0.5 gm. The surface sterilization of the shoot, leaf and root pieces was done with above mentioned sterilization steps. Efficiency of surface disinfestations depend on selection of disinfectant, its strength, duration of immersion in disinfectant. The procedure for surface disinfestation and isolation conditions were standardised prior to experimentation. The cut ends of plant sample sections were removed with flame sterilized scalpel and were placed properly with the cut surface touching the agar media. The plates were incubated for three to five days at 28 $\pm 1^0$C. Single colonies from the plates were picked up and purified by repeated streaking on fuel form medium and stored under refrigerated conditions for further studies. This procedure was repeated several times till pure colony was achieved.

Morphological tests

The following morphological tests *viz.,* cell shape, Gram reaction, and motility were carried out to characterize the endophytes.

(a) Cell shape (Aneja, 2006)

The purified bacterial endophytic cultures were taken at log phase and microscopically observed for the cell morphological characteristics.

(b) Colony morphology: Morphological characters of each isolate were examined on NA medium. characters like shape, elevation, surface, margin, colour etc., were observed.

(c) Gram staining (Hucker and Conn, 1923)

Gram staining for bacterial endophytes was carried out as per modified Hucker's method. A drop of sterile distilled water was placed in the centre of a glass slide. A loopful of inoculum from the actively growing culture was taken, mixed with water drop and spread out at the centre of the slide using the tip of an inoculation loop to make a thin suspension. The smear was air dried and fixed by mild heating by passing the slide (other/back side of the smear) three to four times over the flame. The smear was then flooded with Crystal violet solution for 60 seconds and washed gently with distilled water for a few seconds. Then the slide was flooded with Gram's iodine solution for 60 seconds and washed gently with 95% alcohol. This was followed by washing with running water for 15 to 30 seconds and blot dried carefully. This smear was counter stained with safranin solution for 60 seconds. Finally, the slide was washed gently with tap water and air dried. The slides were observed under the light microscope and under oil-immersion(100x) and the isolates that appeared as violet is classified as gram-positive bacteria and the bacteria that appeared as pink is classified as gram-negative bacteria.

(d) Motility in liquid media (Aneja, 2006)

Bacterial endophytes grown for 72 hours were used to observe for the presence of flagella using cavity slide for bacterial motility.(Aneja, 2006)

RESULTS AND DISCUSSION

Isolation and characterization of bacterial endophytes in different groundnut varieties (a) Diversity and composition study of endophytic bacteria from groundnut

A total of 125 culturable endophytic bacterial morphotypes were isolated and pure cultures were obtained by quadrant streak method. Morphological and physiological characterization of isolates was carried out based on colony shape, size, elevation, surface, margin, color, pigmentation, motility, gram's reaction and cell shape.

(b) Naming of the isolates

From the above studies, those bacterial colonies showing distinct colony morphology and growth in broth were selected for further studies. Twenty-five isolates from 27 different varieties of groundnut (Narayani, Rohini, Nitya Haritha, Dharani (TCGS1043), Dheeraj, Vijetha, JL24, TAG 24, Abhaya, Bheema, VisishtaG1694, G2117, Greeshma, Prasuna, Pragathi, Kadiri 2, Kadiri6, Kadiri7, Kadiri8, Kadiri9, Kadiri Harithandhra, Kadiri Lepakshi, Kadiri Amaravati (K 1535), Tirupati 1(TCG 1704), Tirupati2, Tirupati3, Tirupati4) were obtained and purified by streak plate technique. They were named accordingly with different codes. Codes were given to the isolated bacterial endophytes in such a way that first and second letter indicating the variety followed by third letter representing the part of the

plant from which the isolate has been isolated and are presented in Table 1.

Culture based characterization of the bacterial endophytes from groundnut plants.

(a) Morphological characteristics of the endophytic bacterial isolates The cultural characteristics of the bacterial isolates on the nutrient agar medium and nutrient broth medium studied were presented in Table 2. The colony characters in agar plate varied from cream white to yellow pigmented, with wrinkled, smooth and glistening surfaces. The cultural characteristics on the nutrient broth medium varied from surface growth, clouding of the broth and sedimentation. Colony morphology gave an indication of the variation among the bacterial endophytes.

(b) Cultural characteristics of the endophytic bacterial isolates

All the isolates were stained and tested for Gram reaction and the results were presented in Table 3. Among 25 isolates obtained, 68 % (18 isolates) were found to be Gram negative (–ve) and 32 % (Seven isolates) were found to be Gram positive (+ve). Around 80 % of isolates (20 isolates) were rod shaped cells with varied size ranging from small rods to long rods and 20% (Five isolates) of the isolates found to have cocci shaped cells. About 60 % (15 isolates) of the isolates were found to were motile while 40 % (10 isolates) of bacterial isolates were found to be non-motile and motility is one of the attributes of being an endophyte. Colonies of varied sizes like small, medium and large were observed among the obtained endophytic isolates with round to irregular colony shape and varied pigmentation showing white, cream, yellow & greenish-blue tinted colonies. Flagella have been shown to affect endophytic colonization and bacterial mobility within host plants.

In the studies of Zinniel *et al.* (2002), preliminary characterization of endophytic bacteria showed that approximately equal percentages of gram-positive (41 %) and gram-negative (42 %) bacteria were recovered from the agronomic crop plants, whereas gram-negative bacteria (50 %) were isolated more frequently than gram-positive bacteria (21 %) from prairie grass. And more of gram-negative bacteria were found to be motile than gram-positive bacteria.

Further, Duhan et al. (2020) isolated 39 bacterial endophytes from *Tinospora cordifolia* and these bacteria exhibited diversity in colony morphology, white to yellowish white with circular or irregular shapes with wavy margins. Only three isolates are Gram negative and remaining were Gram positive.

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Table 1: Details of the bacterial endophytes isolated from different groundnut varieties

Sl. No	Variety	Parts	Isolate
1	Narayani	Shoot	NRS3
2	Kadiri-8	Shoot	K8S5
3	Kadiri-9	Root	K9R2
4	Prasuna	Root	PSR4
5	Kadiri Lepakshi	Shoot	KLS7
6	Nityaharitha	Shoot	NHS5
7	Kadiri-7	Root	K7R3
8	Kadiri-7	Leaf	K7L3
9	Kadiri-9	Shoot	K9S3
10	Pragati	Leaf	PGL4
11	Pragati	Shoot	PGS2
12	Rohini	Shoot	RHS5
13	Abhaya	Root	ABR2
14	Kadiri Amaravati	Leaf	KAL4
15	Kadiri Amaravati	Leaf	KAL1
16	Kadiri Harithandhra	Shoot	KHS3
17	Dharani	Shoot	DHS3
18	Tirupati 1	Shoot	T1S2
19	Tirupati 2	Shoot	T2S4
20	Tirupati 3	Shoot	T3S4
21	Greeshma	Shoot	GRS2
22	Bheema	Root	BHR5
23	Dharani	Shoot	DHS5
24	Kadiri 8	Root	K8R2
25	Visishta (G1694)	Leaf	VSL1

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Table 2: Morphological characteristics of the endophytic bacterial isolates of groundnut

S. No.	Isolate	Culture in broth			Colony characteristics in agar
		Surface Growth	Clouding	Sediment	
1	NRS3	Ring	Slight	Moderate	Small, Circular, Smooth, Glistening nearly opaque, Pale Yellow
2	K8S5	Slight	Moderate	Abundant	Small Circular, cream colour, glistening, Smooth margin
3	K9R2	Ring	Slight	Scanty	Medium Circular, Light Green, Transparent, Glistening
4	PSR4	Ring	Abundant	Scanty	Small Round, Yellow, Raised, Smooth
5	KLS7	Ring	None	Scanty	Small, Pale yellow, Glistening, transparent
6	NHS5	Ring	Moderate	Moderate	Small, Round, Pale Yellow, Glistening
7	K7R3	Ring	Slight	Scanty	Circular, yellow pigmented, glistening
8	K7L3	Ring	Slight	Scanty	Small, Cream coloured, Rough surface, Opaque
9	K9S3	None	Moderate	Scanty	Small, Yellow, Transparent, Smooth
10	PGL4	Ring	Slight	Scanty	Small, Pale Yellow, Smooth, Glistening
11	PGS2	Ring	Moderate	Flaky	Circular, red pigmented, glistening
12	RHS5	Ring	Slight	Flaky	Small, Pale white with blue tint, Smooth, Glistening, Transparent
13	ABR2	Ring	Moderate	Scanty	Medium, Yellow, Rough, glistening
14	KAL4	Ring	None	Flaky	Small Circular, Yellow, Smooth, Glistening, Opaque
15	KAL1	Ring	Adequate	Scanty	Large, Round, Yellow, glistening, Opaque
16	KHS3	Ring	Slight	Scanty	Medium, Yellow, Smooth, Opaque, glistening
17	DHS3	Ring	Slight	Scanty	Small, Circular, Pale yellow pigmented, glistening
18	T1S2	None	Slight	Scanty	Small, Circular, Pale yellow, Rough, Transparent
19	T2S4	None	Slight	Scanty	Small, Circular, Yellow colour, glistening, Transparent
20	T3S4	Ring	Heavy	Flaky	Small, Yellow, Smooth, glistening, opaque
21	GRS2	None	Slight	Scanty	Medium, Cream colour, Smooth, Glistening, Opaque
22	BHR5	Ring	Moderate	Slight	Medium, Round, Yellow, Rough, Opaque
23	DHS5	Ring	None	Scanty	Medium, Circular, Pale white, Smooth, glistening, Opaque
24	K8R2	Ring	Slight	Scanty	Large Round, yellow pigmented, Smooth, glistening, Opaque
25	VSL1	Ring	Slight	Flaky	Medium, Cream white, Smooth, glistening, opaque

Table 3: Cultural characteristics of the bacterial endophytes isolated from different groundnut varieties

S. No	Isolate	Gram reaction	Cell shape	Motility
1	NRS3	-	Rod	+
2	K8S5	-	Rod	+
3	K9R2	-	Rod	+
4	PSR4	-	Rod	+
5	KLS7	-	Rod	+
6	NHS5	+	Cocci	-
7	K7R3	-	Short Rods	+
8	K7L3	-	Cocci	-
9	K9S3	-	Long Rods	-
10	PGL4	-	Rods	+
11	PGS2	+	Cocci	-
12	RHS5	-	Rods	+
13	ABR2	+	Rods	-
14	KAL4	+	Short Rods	+
15	KAL1	-	Cocci	-
16	KHS3	-	Rods	-
17	DHS3	-	Rods	+
18	T1S2	+	Rods	-
29	T2S4	+	Rods	+
20	T3S4	-	Cocci	-
21	GRS2	-	Rods	-
22	BHR5	-	Long Rods	+
23	DHS5	+	Rods	+
24	K8R2	+	Rods	+
25	VSL1	-	Rods	+

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