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Morphological and biochemical characterization of endophytic bacteria from periwinkle [*Catharanthus roseus* (L.) G. Don]

BP Chauhan and NK Singh

Abstract
Catharanthus roseus is an important medicinal plant, belongs to the family Apocynaceae and is a vital source of alkaloids. Fifteen endophytic bacterial isolates selected which has showing highest Total antioxidant capacity were screened and further characterized based on morphological and biochemical characteristic. Nine isolates were tested gram negative and six gram positive. Cultural characteristics of the isolates varied widely on nutrient agar Petriplates and slants. The bacterial colonies appeared creamy white and showed circular form and irregular or entire colony margins on nutrient agar Petriplates. The isolates metabolized various carbohydrate sources. All the isolates were positive for esculin hydrolysis and citrate and malonate utilization. However, none of the isolates could utilize lactose, xylitol, D-Arbinose and sorbitol. Dendrogram based on carbohydrate utilization pattern grouped these fifteen bacterial isolates into two broad groups (Cluster A and culture B) with the Jaccard's similarity co-efficient of 0.46.

Keywords: Endophytic bacteria, periwinkle, gram staining, carbohydrate utilization profile, UPGMA

Introduction
Plants are one of the most important natural sources of medicines. Currently, large numbers of medicines in practice are of plant origin and are derived exclusively from plants. Medicinal plants are the chief source of secondary metabolites that are used as drugs and essential oils of wide therapeutic applications. The important advantages of medicinal plants for therapeutic uses in various ailments are their safety in addition to being inexpensive, effective, and easily accessible. These advantages of medicinal plants forced the traditional medical experts for extensive use in their day to day practice. Plants which are used in traditional medicines are of significant importance and therefore considerable research has been carried out on medicinal plants for bioactive compounds however limited research has been performed on the associated microorganisms and their role in production of bioactive compounds. Endophytes are regarded an important chemical synthesizer inside plants. They play an important role as a selection system for microbes to produce pharmacologically active substances with generally low toxicity toward mammals (Rahman *et al.*, 2017) [21].
Endophytes are generally endosymbiotic microorganisms (commonly bacteria or fungi) that systematically colonize and proliferate within plant tissues without causing any signs of disease or harm (Nair and Padmavathy, 2014) [17]. During colonization of plant tissue, endophytes are also capable of establishing symbiotic relationship with the plant thus making them efficient biocontrol and medicinal agents. Several research reports have demonstrated the activity of bacterial endophytes against various pathogens (Atiphasaworn *et al.*, 2017; Wang, 2019) [4, 32]. As such, there is continued research interest in developing drugs from endophytic compounds which could serve as an alternative to synthetic pharmaceuticals and plant-derived medicines. Endophytes are known to promote plant growth, enhance defense, increase their tolerance to abiotic and biotic stress, and improve nutrient uptake (Shahzad *et al.*, 2018) [25]. Endophytes may actively influence host's biosynthesis pathways and gene expression systems to increase the production of particular secondary metabolite. An important advantage of endophytes is that they can be easily isolated, cultured, are amenable to genetic manipulations, and can be scaled up for bioactive compound production (Xu *et al.*, 2008) [33]. In view of increased importance of bacterial endophytes to both plant and human health, there is an increased focus on developing endophytes into herbal remedies (Photolo *et al.*, 2020) [20].
Endophytic bacteria living in plant tissues are generally deprived of doing substantive

harm or gain benefit other than residency (Kado, 1992) [12]. De Bary (1866) [8] was the first to coin the term endophyte (Gr. *endon*, within; *phyton*, plant). An endophyte is a microorganism that spends either complete or a part of its lifecycle inside the healthy tissues of a living plant, without causing any symptoms of disease (Tan and Zou, 2001) [30]. Endophytic bacteria can be isolated from plant tissues and grown in laboratory on fermentation mediums. In fermentation medium endophytic bacteria can produce similar compounds present in host plants with the help of an enzyme activity. Use of endophytes for production of bioactive compounds has advantage of faster production of uniform quality compounds on a large scale and the possibility of obtaining new bioactive components under different culture conditions (Sarjono *et al.*, 2019) [23]. Endophytes are sometimes responsible for the medicinal properties of the host plants. Endophytes are known to synthesize bioactive compounds that can be used by plants for defense against pathogens and some of these may be a valued drug (Rahman *et al.*, 2017) [21].

Endophytic microorganisms that reside inside living plant tissues are promising and useful but less explored sources of novel natural products for useful in agriculture, medicine, and industry (Strobel and Daisy, 2003) [28]. The importance of endophytes as a source of pharmaceutical bioactive compounds has been demonstrated over a long period, as many of endophytes have been exploited to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs, and many related compounds (Anjum and Chandra, 2015) [3]. Moreover, in view of increasing prevalence of antibiotic-resistant human and plant pathogens, there is an increasing demand for new antimicrobials from natural sources. Bacterial endophytes are believed to have a resistance mechanism against pathogen attack and thus have emerged as a promising source of new antimicrobial compounds.

The application of beneficial endophytic bacteria has opened up new possibilities in the field of biotechnology. In the last decade, role of various endophytic bacteria have been reviewed by several authors (Santoyo *et al.*, 2016) [22]. Endophytic bacteria have been reported to play an important role in growth promotion, nutrient management, disease control, and biotic and abiotic stress tolerance in food and non-food crop plants. Endophytic bacteria are also known to produce several enzymes like, serine-type fibrinolytic enzymes, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, exo-β-agarase and indole-3-acetic acid (IAA). Recent studies showed that L-asparaginase enzyme and a quinoline alkaloid compound (Camptothecin) produced from endophytic bacteria have potential anticancer properties. Therefore, endophytic bacteria represent a potential source for the discovery of new and novel compounds of medicinal importance (Alam *et al.*, 2013) [1].

Less than 1% of endophytes are known, which suggest that millions of endophytic microorganisms are yet to be to be studied systematically. Phenols present in plants are one of the largest groups that act as antioxidant compounds, in leaves, flowers, and roots. Until recently, very few reports exist on the antioxidant properties of the diverse and varied endophytic bacteria from different host plants (Gunatilaka, 2006) [10]. Antioxidants are the chemical compounds that are able to eliminate, cleanse and resist the formation of reactive oxygen and free radicals in the body. Free radicals are unstable atoms or molecules due to presence of unpaired electrons in their outer orbitals so it is very reactive to get electron pairs by binding to body cells. If this happens continuously it can cause cell damage and even death (Triandriani *et al.*, 2020) [31]. Although, many methods are available to evaluate the antioxidant activity of bioactive compounds, due to the complexity involved in the *in vivo* mechanisms of action, more than a single *in vitro* chemical method has been suggested to evaluate and compare the antioxidant properties of natural products. Moreover, due to the involvement of multiple reaction characteristics and mechanisms, no single assay is capable of accurately reflecting all antioxidants in a mixed or complex system (Du *et al.*, 2009) [9]. Therefore, it is imperative to use two complementary tests to evaluate the *in vitro* antioxidant properties of different solvent extracts of the endophytic bacteria.

Materials and Methods

Morphological and biochemical characterization from bacterial isolates

The fifteen best performing endophytic bacterial isolates which has highest antioxidant property were obtained from the explants (Root, stem, leaf, petals) of periwinkle plants were subjected to detailed studies (Chauhan *et al.*, 2023) [7].

Morphological examination of the isolates

The Morphological examination of the isolates was done by Gram's staining the pure culture of the isolates and observations for cell shape, cell size and arrangement of cells were recorded (Cappucino and Sherman 1992) [6].

The procedure for Gram staining is described below.

1. Took a clean and dry glass slide and put a drop of sterilized distilled water on one side of the slide.
2. With the help of a sterilized inoculation loop, a colony of actively growing bacterial culture was transferred on the slide at the position of water drop and a thin and uniform smear was prepared.
3. This smear was air-dried and heat-fixed.
4. The bacterial smear was flooded with crystal violet for 1 min.
5. Washed the slide in a gentle and indirect stream of tap water for 2 seconds.
6. The slide was flooded with the mordant (Gram's iodine) and waited for 1 min.
7. Washed the slide in a gentle and indirect stream of tap water for 2 seconds.
8. The slide was flooded with decolorizing agent (95% ethyl alcohol), waited for 15 seconds and washed the slide in a gentle and indirect stream of tap water for 2 seconds.
9. Flooded the slide with counter stain (safranin) and waited for 1 min.
10. Washed the slide in a gentle and indirect stream of tap water until no color appears in the effluent and then blot dried the slide with absorbent paper.
11. The stained slides were observed under oil immersion using a bright-field microscope (E200, Nikon, Japan) and the morphological details (cell shape, cell arrangement in colony and cell length and width) were recorded and analyzed using the NIS-Elements Documentation software (Nikon, Japan).

Cultural characteristics of the isolates

Cultural characteristics of the isolates were studied after growing them on Nutrient agar plates and Nutrient agar slants as per methods described by Cappucino and Sherman (1992) [6] which are represented below.

1. Poured a Petriplate of Nutrient agar and streaked, it with the actively growing endophytic bacterial isolates.
2. Inoculated the actively growing cultures on the surface of Nutrient agar slants with the help of sterilized inoculation loop.
3. Incubated the plates and slants at 37±2 °C temperature for 48 h.
4. The observations regarding cultural characteristics were recorded.

Observations recorded on nutrient agar petriplates

The well isolated colonies of the endophytic bacterial isolates under investigation on nutrient agar petriplates were evaluated as per guideline given in Table.

Table 1: Cultural characteristic of bacteria on Nutrient agar petriplates.

(1)	Size		:	Pinpoint, small, moderate or large
(2)	Pigmentation		:	Colour of colony
(3)	Form		:	The shape of the colony was described as follow.
	(a)	Circular	:	Unbroken, peripheral edge
	(b)	Irregular	:	Indented, peripheral edge
	(c)	Rhizoid	:	Root like, spreading growth
	(d)	Filliform	:	Having the form of or resembling a thread or filament
	(e)	Filamentous	:	Composed of long and thread like structure

Observations recorded on nutrient agar slants

Single straight line of inoculation was done on the surface of slants having nutrient agar medium and were evaluated as per the guideline given in Table.

Table 2: Cultural characteristics of bacteria on Nutrient agar slants.

(a)	Abundance of growth	:	The amount of growth was designated as none, slight, moderate or abundant.
(b)	Pigmentation	:	Presence of the pigments was checked on the organisms and within the medium. Most organisms, however, generally do not produce pigment and appear white to grey.
(c)	Opacity	:	Degrees of opacity was evaluated on the basis of the amount of light transmitted through the growth and were expressed as opaque (No light transmission), translucent (Partial transmission), or transparent (Full transmission).
(d)	Form	:	The appearance of single line streak of growth on the agar surface and were designated as filiform, echinulate, beaded, effuse, arbore scent and rhizoid.

Biochemical characterization

Gram's reaction of the bacterial isolates was recorded as described (Section 3.3.1). Further, biochemical characterization of the isolate was done by carbohydrate utilization pattern; carbohydrate utilization profile is regarded as one of the most important criteria for phenotypic characterization of the bacterial isolates. The carbon utilization profiles was generated using Hicarbohydrate™ kit (KB009, Himedia Laboratories, Mumbai, India) following manufacturer's protocol. Single colony was inoculated into 5ml Brain Heart Infusion Broth and incubated at 35-37 °C for 4-6 h until the inoculum turbidity become 0.5 O.D. at 620 nm. Each well of the Hicarbohydrate™ kit was inoculated with an aliquot of 50 µl of bacterial suspension and the plates were incubated at 35±2 °C for 18-24 h and the results was recorded on the basis of colour change of the medium in wells of the kit as per manufacturer's protocol.

The ability of an isolate to utilize a carbohydrate was used to generate binary matrix for all the isolates. The similarity matrix was constructed following SIMQUAL program and the data were analyzed using a numerical taxonomy and multivariate analysis (NTSYSpe 2.02i) software package (Rohlf, 2000) [35]. The dendrogram was based on the proximity matrix obtained from the Jaccard coefficient and Sequential Agglomerative Hierarchiel Non-overlapping (SAHN) method and clustering was done using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) Method (Sneath and Sokal, 1973) [27].

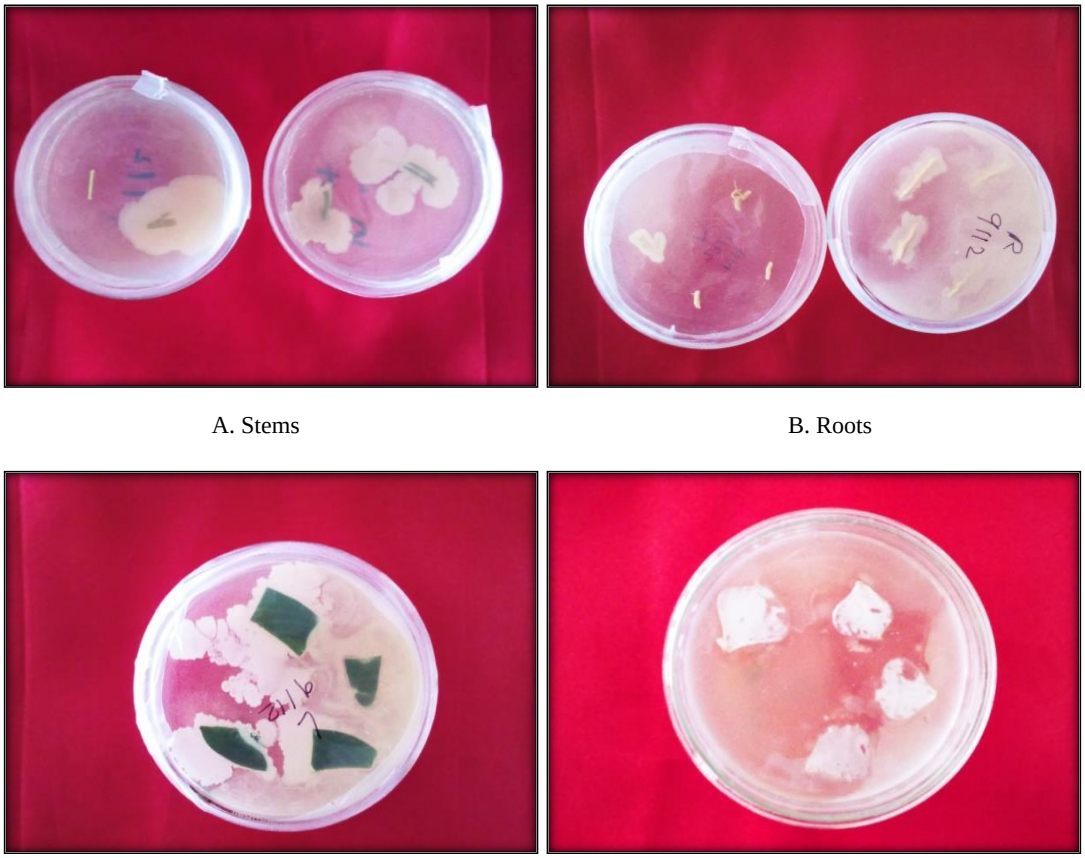
Results and Discussion

Morphological and biochemical characterization from bacterial isolates

The fifteen best performing endophytic bacterial isolates which has highest antioxidant property were obtained from the explants (Root, stem, leaf, petals) of periwinkle plants were subjected to detailed studies (Plate 1), including their phenotypic characterization through microscopic examination (Cell shape, cell length and width and arrangement of cells in colony).

Microscopic examination of the isolates

Microscopic examination of the isolates was done by Gram's staining using the methodologies of Cappuccino and Sherman (1992) [6]. All the endophytic bacterial isolates were rod shaped; however they varied in their reaction to Gram's staining; few were Gram negative and others were Gram positive. The cells of all the isolates appeared singly and rod shaped on the slide under microscope. Thus, all the isolates were monobacillus in nature. The length of the isolates ranged from 2.35 µm (P1) to 5.33 µm (S4) whereas cell width ranged from 1.89 µm (R1) to 4.27 µm (P2) (Table 4.4). The Gram's staining of the endophytic bacterial isolates showed that nine isolates (R1, R4, R5, R6, S1, S3, S6, P1 and P2) were Gram positive whereas six isolates (R2, R3, S2, S4, S5 and L1) were Gram negative in nature (Plate 2). Thus, in our observation, 60% of the isolates were Gram positive whereas 40% of the isolates were Gram negative.



A. Stems B. Roots

C. Leaves D. Petals

Plate 1: Isolation of endophytic bacteria from periwinkle plants [*Catharanthus roseus* (L.) G. DON]

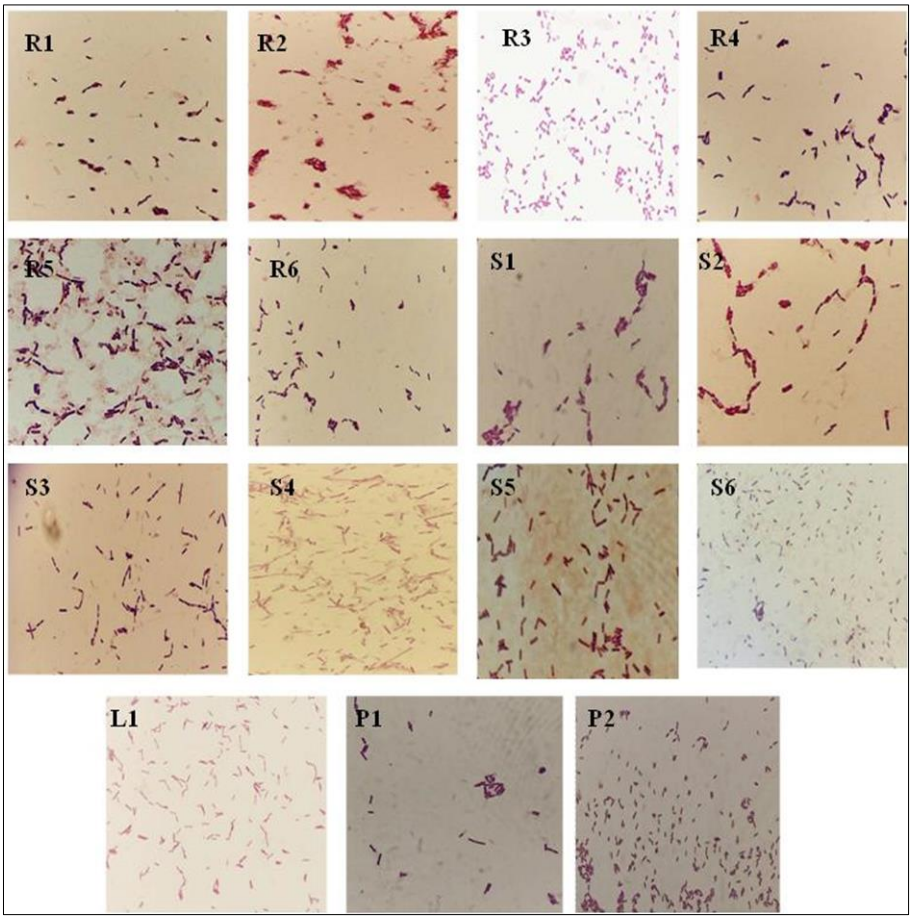


Plate 2: Gram's staining of the endophytic bacterial isolates from periwinkle plants [*Catharanthus roseus* (L.) G. DON]

Table 3: Morphological description of the endophytic bacterial isolates

Sr. No.	Isolates	Cell Shape	Length (µm)	Width (µm)	Cell arrangement	Gram's Staining
1	R1	Rod	2.46	1.89	Mono	+Ve
2	R2	Rod	3.63	2.48	Mono	−Ve
3	R3	Rod	3.79	2.73	Mono	−Ve
4	R4	Rod	3.43	2.71	Mono	+Ve
5	R5	Rod	3.78	2.83	Mono	+Ve
6	R6	Rod	2.95	2.92	Mono	+Ve
7	S1	Rod	3.36	3.09	Mono	+Ve
8	S2	Rod	4.82	3.93	Mono	−Ve
9	S3	Rod	4.01	2.90	Mono	+Ve
10	S4	Rod	5.33	4.07	Mono	−Ve
11	S5	Rod	4.01	3.94	Mono	−Ve
12	S6	Rod	4.30	3.65	Mono	+Ve
13	L1	Rod	2.70	1.91	Mono	−Ve
14	P1	Rod	2.35	2.00	Mono	+Ve
15	P2	Rod	4.72	4.27	Mono	+Ve
Note: The values for cell length and width indicate mean of twenty randomly selected bacterial cells for each isolate						

The Gram's staining is based upon the biochemical characteristics of the cell wall because this reaction depends upon the presence of relative amount of peptidoglycan and lipids in the cell wall and on the presence of outer membrane (Present only in Gram negative bacteria). This staining technique is also visualized to see bacteria under microscope and to determine their morphological characteristics.

Moreover, the bacteria that produce spore are more resistant to extreme environmental conditions because of their reduced cell metabolism or dormancy, if present in poor environment. *Bacillus* which forms spores results in resistance to extreme environmental stresses such as heat, UV light and γ-radiation, mechanical disturbances, enzymatic reactions and toxic chemicals. In addition, spores play a role in bacterial resistance to environmental stresses; spores can survive for very long periods in more stable environmental conditions (Amrullah *et al.,* 2018) [2].

Out of 35 endophytic bacteria isolated from four medicinal plants by Anjuman and Chandra (2015) [3], 18 isolates were found Gram-positive cocci, 11 were Gram-negative bacilli, 6 were found to be Gram-positive bacilli, 18 isolates showed positive results for endospore staining, 24 gave positive results for catalase test, 13 gave positive results for oxidase test, and 15 isolates were found to be motile. However, out of sixteen morphologically different endophytic bacterial isolates collected from the fresh and healthy leaves of seven different medicinal plants: *Codiaeum variegatum* pictum (Croton), *Adhathoda vasica Nees* (Adulsa), *Neolamarckia cadamba* (Kadamba), *Azadirachta indica* (Neem), *Curcuma longa* (Turmeric), *Hibiscus rosa-sinensis* (Hibiscus), and S*araca asoca* (Ashoka), five isolates were Gram positive cocci, *i.e*., N1, N2, N5, T_1 and T_2, five isolates were Gram positive bacilli, *i.e*., N3, H1, H3, AD1 and C2, one isolate was gram negative bacilli, *i.e*., K2, three isolates were Gram positive coccobacilli, *i.e*., N4, T_3 and H2 and two isolates were Gram negative coccobacilli, *i.e*., A1 and A2 (Jain *et al.,* 2017) [11].

However, Lopez *et al*. (2011) [15] reported that all the bacterial root endophytes from cactus were Gram negative except one which was contrary to the findings of dominance of Gram negative bacterial endophytes (Sgroy *et al*., 2019) [24]. Sgroy *et al*. (2019) [24] reported 68.9% Gram positive bacteria and 31.1% Gram negative in the root of *Prosopis strombulifera*. While, Panchal and Ingle (2011) [19] found 91.6% root endophytes to be Gram positive. However, Zinniel *et al.* (2002) [34] reported that among the endophytic bacteria Gram-negative bacteria outnumber the gram positive bacteria in most of the agronomic crops. Moreover, Bind *et al*. (2019) [5] isolated endophytic bacteria from pigeon pea and noted that out of 40 endophytic bacterial isolates 25 of the isolates were Gram negative while 15 were Gram positive.

Cultural characterization of bacterial isolates on nutrient agar petriplates

The isolates exhibited wide morphological variation when grown on petriplates containing Nutrient agar. The colonies of all the isolates were small to medium in size and all produced creamy white pigmentation (Table 4.5). The shape of the colony growing on nutrient agar medium in petriplates indicates that all the isolates produced circular colonies.

Appearance of the outer edge of the colony *i.e*. margin of the colony growing on nutrient agar medium in petriplates revealed irregular margins in the isolates R1, R2, R4, S2, S4, L1, P1, and P2 whereas entire margin in the isolates R3, R5, R6, S1, S3, S5 and S6.

Table 4: Cultural characteristics of endophytic bacterial isolates on nutrient agar petriplates

Sr. No.	Isolates	Pigmentation	Form	Margin	Elevation
1	R1	Creamy white	Circular	Irregular	Convex
2	R2	Creamy white	Circular	Irregular	Convex
3	R3	Creamy white	Circular	Entire	Convex
4	R4	Creamy white	Circular	Irregular	Convex
5	R5	Creamy white	Circular	Entire	Raised
6	R6	Creamy white	Circular	Entire	Raised
7	S1	Creamy white	Circular	Entire	Flat
8	S2	Creamy white	Circular	Irregular	Convex
9	S3	Creamy white	Circular	Entire	Flat
10	S4	Creamy white	Circular	Irregular	Convex
11	S5	Creamy white	Circular	Entire	Raised
12	S6	Creamy white	Circular	Entire	Convex
13	L1	Creamy white	Circular	Irregular	Flat
14	P1	Creamy white	Circular	Irregular	Flat
15	P2	Creamy white	Circular	Irregular	Flat

The morphological and cultural characteristics of plant associated bacteria are important in their identification and classification. It indicates phenotype of the microorganism and its diversity is influenced by the genetic make-up of the organism as well the environment. The phenotypic variation

indicates the ability of an organism to survive, adapt and acclimatize in diverse climatic conditions.

Elevation of the colonies implies the degree to which colony growth is raised on the agar surface. Elevation was recorded for all the isolates. The isolates R5, R6, and S5 showed raised type of elevation whereas R1, R2, R3, R4, S2, S4, S6 showed convex type of elevations. The isolates S1, S3, L1, P1 and P2 showed flat type of elevation on the agar surface of petriplate containing nutrient agar medium.

The morphological and biochemical characterization of root endophyte associated with brown sarson (*Brassica rapa* L.) was estimated by Padder *et al.* (2017) [18]. They selected a total of 81 morphologically dissimilar isolates and characterized them on the basis of Gram's staining, cell and colony morphology. It was observed that Gram negative bacteria formed the dominant group. The colony characterization revealed that circular forms dominated, likewise the colonies with entire margins and convex elevation dominated among all the isolates.

EC3 bacterial endophyte had morphological characteristic like white color, round shaped colonies, convex elevation on Nutrient agar medium and, cell shape was monobacillus (Sarjono *et al.*, 2019) [23]. These characteristics were of similar to that of our observations. However, Amrullah *et al.* (2018) [2] isolated endophytic bacteria from red betel root. The bacterial endophytes gave creamy wet pigmentation colour, round form, flat edge, flat elevation and Gram positive in nature.

Endophytic colonies from sweet potato roots were reported by Khan *et al.* (2009) [36]. These bacteria were of similar morphology, round shaped, and color white and pale to bright yellow. Among the endophytes, Gram negative bacteria predominated; 51 out of 81 isolates (62.96%) were Gram negative. The endophytes having circular forms (58.02%), entire margins (60.49%), convex elevation (38.27%) and rod shape (67.90%) predominated among all the isolates.

Cultural characterization of bacterial isolates on nutrient agar slants

The fifteen isolates were cultured on Nutrient agar slants as per the guidelines of Cappuccino and Sherman (1992) [6]. The cultural characteristics of the isolates for a single straight line of inoculation on the surface of Nutrient agar slants were evaluated by observing abundance of growth, pigmentation, opacity and form (Table 5). The isolates varied widely in terms of amount of growth, pigmentation and their form on Nutrient agar slants.

The amount of growth among the isolates varied widely, few endophytic bacterial isolates showed abundant growth while others showed slight and moderate growth. Isolates, S1, S2, S3, S4, S5 and S6 (Stem bacterial endophytes) showed slight growth on Nutrient agar slants. Isolates, P1, P2 (petals bacterial endophytes) and L1 (leaf bacterial endophytes) showed moderate growth whereas six bacterial isolates R1, R2, R3, R4, R5 and R6 showed abundant growth on Nutrient agar slants. All the fifteen isolates under investigation produced creamy white pigments. All the fifteen isolates allowed partial transmission of light, which indicated that these isolates were translucent in opacity.

Table 5: Cultural characteristics of endophytic bacterial isolates on nutrient agar slants

Sr. No.	Isolates	Amount of growth	Pigmentation	Opacity	Form
1	R1	Abundant	Creamy white	Translucent	Effuse
2	R2	Abundant	Creamy white	Translucent	Effuse
3	R3	Abundant	Creamy white	Translucent	Beaded
4	R4	Abundant	Creamy white	Translucent	Effuse
5	R5	Abundant	Creamy white	Translucent	Effuse
6	R6	Abundant	Creamy white	Translucent	Effuse
7	S1	Slight	Creamy white	Translucent	Arborescent
8	S2	Slight	Creamy white	Translucent	Effuse
9	S3	Slight	Creamy white	Translucent	Arborescent
10	S4	Slight	Creamy white	Translucent	Effuse
11	S5	Slight	Creamy white	Translucent	Effuse
12	S6	Slight	Creamy white	Translucent	Beaded
13	L1	Moderate	Creamy white	Translucent	Effuse
14	P1	Moderate	Creamy white	Translucent	Effuse
15	P2	Moderate	Creamy white	Translucent	Effuse

The single line of streak on the agar surface showed effuse form of growth in the isolates R1, R2, R4, R5, R6, S2, S4, S5, L1, P1, P2; arborescent form of growth in S1 and S3; and beaded form of growth in the isolates R3 and S6 (Table 5).

Biochemical characterization

Carbohydrate utilization profile

The phenotypic diversity of these endophytic bacterial isolates was determined based on carbohydrate utilization pattern consisting of 35 various carbohydrate sources (Table 6). The isolate showing positive reaction for utilization of carbohydrate are indicated by '+', the isolate which does not utilize a particular carbohydrate are indicated by '-'and the isolate showing intermediate reaction are indicated by '±'. All the isolates were positive for utilization of esculin hydrolysis and citrate and malonate utilization. However, none of the isolates were able to utilize lactose, sodium- gluconate, methyl-D-mannoside, xylitol, D- Arbinose and sorbitol. Moreover, the isolates under investigation showed a varying degree of utilization of a large number of carbohydrate sources like, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, glycerol, salicin, dulcitol, inositol, sorbitol, mannitol, adonitol, arabitol, erythritol, α-methyl-D-glucoside, rhamnose, cellobiose, melezitose and ONPG.

Moreover, among the isolates, R4 and R5 (root bacterial endophytes) were most versatile and could metabolize 23 carbon sources whereas isolate S5 (stem bacterial endophytes) was least efficient and could metabolize only 6 carbon sources and L1 (Leaf bacterial endophytes) followed by the isolate from leaf which could metabolize only 7 carbon sources.

Table 6: Carbohydrate utilization profile of endophytic bacterial isolates

Sr. No.	Test	R1	R2	R3	R4	R5	R6	S1	S2	S3	S4	S5	S6	P1	P2	L1
1	Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Xylose	±	+	+	+	+	±	-	-	-	-	-	-	-	-	-
3	Maltose	+	+	+	+	+	+	+	+	±	+	±	+	±	+	+
4	Fructose	+	+	+	+	+	+	+	+	+	+	±	+	±	±	+
5	Dextrose	+	+	+	+	+	+	+	±	±	+	±	+	+	+	+
6	Galactose	+	±	+	+	+	±	±	±	±	±	±	±	-	-	-
7	Raffinose	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
8	Trehalose	+	+	+	+	+	+	+	+	+	±	±	+	±	+	-
9	Melibiose	+	+	+	+	+	+	+	±	+	+	±	±	-	-	-
10	Sucrose	±	+	+	+	+	+	-	±	-	-	±	-	+	+	-
11	L-Arabinose	±	+	+	+	+	+	+	±	±	+	±	+	-	-	-
12	Mannose	+	+	+	+	+	±	-	-	-	-	-	-	±	±	-
13	Inulin	±	±	+	±	+	+	-	-	-	-	-	-	-	-	-
14	Sodium- gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	Glycerol	+	+	+	±	+	±	-	-	-	-	-	-	-	-	+
16	Salicin	-	-	-	-	-	-	+	+	+	±	±	±	±	+	-
17	Dulcitol	-	-	-	-	-	-	±	+	+	+	±	±	+	±	-
18	Inositol	±	±	±	+	-	-	-	-	-	±	±	±	-	-	±
19	Sorbitol	-	-	-	±	-	-	±	+	+	±	+	±	±	-	-
20	Mannitol	+	±	+	±	+	+	-	±	-	-	-	-	-	±	-
21	Adonitol	+	±	+	+	-	-	-	-	±	±	±	±	-	±	-
22	Arabitol	+	+	+	+	+	+	-	-	-	±	±	±	±	±	-
23	Erythritol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
24	α-Methyl- D-glucoside	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
25	Rhamnose	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-
26	Cellobiose	+	±	±	+	+	+	±	+	±	±	+	+	-	-	-
27	Melezitose	±	±	±	±	+	±	-	-	-	-	-	-	-	-	-
28	Methyl-D- Mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	ONPG	+	+	-	+	+	+	+	+	±	+	+	+	-	-	-
31	Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	D- Arbinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	Malonate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
35	Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The numerical analysis of phenotypic characteristics based on ability of the isolates to metabolize various carbon sources revealed a high degree of metabolic polymorphism. The dendrogram based on proximity matrix obtained from the Jaccard similarity coefficient and Sequential Agglomerative Hierarchical Non-overlapping (SAHN) algorithm and clustering using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) grouped these fifteen isolates of bacteria into two broad groups (Cluster A and Culture B) at the Jaccard's similarity co-efficient of 0.46 (Figure 1).

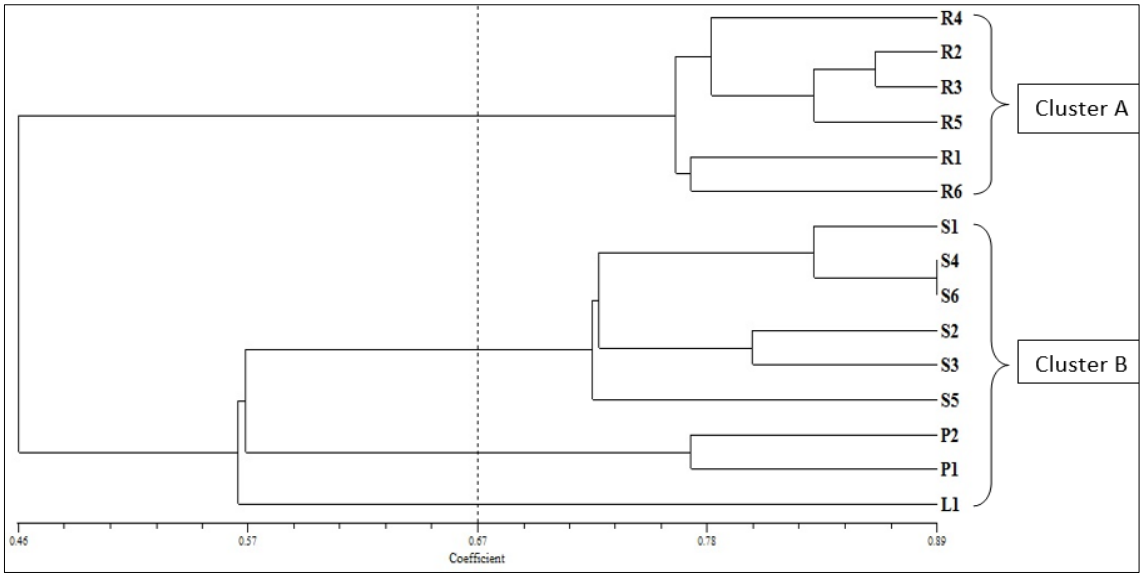


Fig 1: Dendrogram showing diversity of endophytic bacterial isolates based on Carbohydrate utilization profile using Jaccard's co-efficient and Unweighted Pair Group Method with Arithmetic Average (UPGMA)

All the bacterial isolates from roots (R1, R2, R3, R4, R5, and R6) were closely related to each other on the basis of carbohydrate utilization profile which make cluster A. The cluster A comprised of the six isolates, which was further divided into two subgroups. One sub-group of this cluster comprised of four isolate R4, R2, R3, and R5. However, the second subgroup of cluster A contained only two isolate R1 and R6. The cluster B comprised of the nine isolates, which was further sub-divided into two subgroups; one subgroup contain only one isolate which was represented by leaf bacterial isolate L1 whereas the second subgroup was comprised of the stem bacterial isolates S1, S2, S3, S4, S5, S6 as well as the isolates from petals (P1 and P2).

Carbohydrates provide carbon to the organism, which is one of the most important building blocks of the biological system. It is one of the most abundant elements in the cell of an organism. The ability of an organism to utilize a large number of carbons gives an important insight about metabolic/nutritional adaptability of an organism. A bacterium having ability to utilize large number of carbon can adapt and acclimatize in diverse type of soil and environment having different minerals and nutrient capability. Differential utilization of carbon sources by isolates of endophytic bacteria obtained from the explants (Root, stem, leaf, petals) of periwinkle plants, as determined by Hi-carbohydrate^{TM} test kit, may play an important role in adaptation and acclimatization of the isolates to a variety of habitats and agro-climatic environments, crop plants and soil types. The importance of carbohydrate utilization by bacteria in their characterization increases many fold because carbohydrates serve as primary substrate for the synthesis of many important metabolites and commercial products by microorganisms (Naik *et al.,* 2008) [16].

Metabolite utilization profile is also important because changes in metabolite composition in rhizosphere may affect the role and composition of rhizobacterial populations which are dependent upon rhizospheric nutrients for their survival and growth. The ability of the isolates to utilize a wide array of carbohydrate decides their numerical and functional superiority in the agro-ecosystem. Moreover, a larger spectrum of carbon source utilization by bacterial isolates may help in developing media that should stimulate the microbial growth and multiplication and may be used for development of bioinoculants.

The endophytic bacteria grow on a wide variety of carbohydrates depending upon the metabolic pathways they follow and accordingly the expression of these genes leads to the catabolism of a particular or a series of carbohydrates via, tricarboxylic acid cycle, the Entner-Doudoroff, the Embden-Meyerhof-Parnas and the pentose-phosphate pathways (Taghavi *et al.,* 2009) [29]. This could be the possible reason for selective metabolism of different carbohydrates by the isolated endophytic bacteria. The biochemical and physiological tests of endophytic bacteria were carried out by Anjum and Chandra (2015) [3]. In their study, 24 isolates gave positive results for catalase and 13 showed positive results for oxidase test. The results indicated that they can produce catalase and oxidase enzyme which are crucial for the survival and competitiveness of these isolates in the agroecosystems.

The metabolic properties of isolated bacterial root endophytes from brown sarson (*Brassica rapa* L.) was revealed by Padder *et al.* (2017) [18]. All the isolates could metabolize glucose and galactose but a total of 16 and 21 isolates among all the isolated 81 bacterial root endophytes did not metabolize maltose and sucrose, respectively. In the same way there were 47 isolates among all the isolated bacterial root endophytes which were capable of metabolizing all the tested carbohydrates *viz*. glucose, galactose, maltose and sucrose.

The bacterial endophytes associated with *Curcuma longa* L. were characterized by Kumar *et al.* (2016) [14] who observed a huge diversity among the isolates for metabolizing the various carbon sources. For instance, all the isolates metabolized glucose, 50% isolates metabolized maltose and 66.6% metabolized sucrose. Similar findings were also observed by other workers while investigating the diversity in metabolization of various carbohydrates by bacterial root endophytes (Singh *et al*., 2013) [26].

Summary and Conclusion

The 15 screened bacterial isolates were subjected to morphological and biochemical characterization. Microscopic examination of the isolates was done after Gram's staining. Out of fifteen, nine isolates (R1, R4, R5, R6, S1, S3, S6, P1 and P2) were Gram positive whereas six isolates (R2, R3, S2, S4, S5 and L1) were Gram negative in nature. Therefore, we can say that 60% of the isolates were Gram positive whereas 40% of the isolates were Gram negative. The cells of all the isolates appeared singly and rod shaped on the slide under microscope.

Cultural characteristics of the isolates varied widely on Nutrient agar slants and petriplates. Colonies of most of the endophytic bacteria appeared creamy white in colour on Nutrient agar. Single line of streak on the agar slant surface showed effuse form of growth in the isolates R1, R2, R4, R5, R6, S2, S4, S5, L1, P1and P2; arborescent form of growth in S1 and S3; and beaded form of growth in the isolates R3 and S6. All the isolates appeared circular form on Nutrient agar petriplates and the colony margin of the isolates appeared irregular or entire. R1, R2, R4, S2, S4, L1, P1 and P2 bacterial isolate showed irregular margin whereas R3, R5, R6, S1, S3, S5 and S6 possessed entire margin. The isolates R5, R6, and S5 showed raised type of elevation whereas R1, R2, R3, R4, S2, S4, S6 showed convex type of elevations. The isolates S1, S3, L1, P1 and P2 showed flat type of elevation on the agar surface of petriplate containing Nutrient agar medium.

The phenotypic diversity of these endophytic bacterial isolates was determined based on carbohydrate utilization pattern consisting of 35 various sources. All the isolates were positive for esculin hydrolysis and citrate and malonate utilization. However, none of the isolates were able to utilize lactose, sodium- gluconate, methyl-D-mannoside, xylitol, D-Arbinose and sorbitol. Moreover, the isolates under investigation showed a varying degree of utilization of rest of the sources of carbohydrates tested in this study. Among the isolates, R4 and R5 (Root bacterial endophytes) were most versatile and could metabolize 23 carbon sources each whereas isolate S5 (Stem bacterial endophytes) was least efficient and could metabolize only 6 carbon sources.

The numerical analysis based on ability of the isolates to metabolize various carbohydrate sources was done and a dendrogram was prepared based on proximity matrix obtained from the Jaccard similarity coefficient and Sequential Agglomerative Hierarchical Non-overlapping (SAHN) algorithm and clustering using the Unweighted Pair Group

Method with Arithmetic Average (UPGMA) which grouped these fifteen isolates of bacteria into two broad groups (Cluster A and culture B) with the Jaccard's similarity co-efficient of 0.46. The cluster A comprised of the six Isolates (Isolates from roots) whereas cluster B comprised of the nine isolates (Isolates from stems, petals and leaves).

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