Bacterial Endophytes: Isolation and plant growth promoting traits from *Rhododendron* Species of Darjeeling Himalaya

SHREYASI BISWAS AND MAHUYA MUKHOPADHYAY

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Department of Botany,
University of Calcutta,
Kolkata 700 019, India

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Bacterial Endophytes: Isolation and plant growth promoting traits from *Rhododendron* Species of Darjeeling Himalaya

SHREYASI BISWAS[1] , UPAKAR RAI[2] AND MAHUYA MUKHOPADHYAY[3]*
[1&3]Department of Microbiology, Lady Brabourne College,P-1/2, Suhrawardy Avenue, Kolkata-700017
[2]Department of Botany, St. Joshep's College, North Point, Darjelling 734104, West Bengal

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The study was undertaken to isolate the bacterial endophytes from one *Rhododendron* species- *Rhododendron grande* Wight, and one subspecies of *Rhododendron arboretum* Smith- *R. arboreum* Smith sub sp. *cinnamomeum* (Wall. ex G.Don) Tagg. A total of 52 endophytic bacteria were isolated from leaves and stems. Their phytohormone production, nitrogen fixation, phosphate solubilisation, ammonia production abilities were explored. In this report, only 13 endophytic isolates were documented based on their gibberellins production, nitrogen fixation along with the other potentials mentioned earlier. Isolate RGRADS03 from the stem samples of *R. grande* (RGRAD) was reported with the highest gibberellin (292.7±3.01µg/ml) production among the 13 isolates. The isolates from *R. arboreum* subsp. *cinnamomeum* (RACD) also had quite impressive gibberellins production(range: 102.6-201.5µg/ml). 4 isolates from leaf samples of *R.grande* and a total of 6 isolates from leaf and stems of *R.arboreum*subsp. *cinnamomeum* were reported to fix nitrogen.Apart from this, all the 13 isolates from both the rhododendron species had ammoniaand auxin (indole acetic acid) production ability. In case of phosphate solubilisation isolatedstrains of *R. arboreum* subsp. *cinnamomeum* were more potent than the strains of *R. grande,* as the 3 isolates RACDL14,RACDL17 and RACDS04 have Solubilizing Index of 2.08±0.15,2.43±0.1,2.16±0.04 respectively.

Keywords: Endophytic bacteria, gibberellins, IAA, nitrogen-fixation, phosphate solubilization

INTRODUCTION

Endophytes can be defined as ensymbiotic microorganisms that thrive inside the plant tissues without causing harm to their host plants. They are different from phytopathogens as they always improve the growth and development of their host plants either direct or indirect way. A single plant is a reservoir of not single but many beneficial microbes (Webster *et al.,* 2020; West *et al.,* 2010). Bacterial and fungal endophytes have been isolated from several plant species like crops,angiosperms, economically important plants as well (Faria *et al.,* 2013; Nath *et al.,* 2015).They exhibit complex interactions with their host plants including mutualism, antagonism and sometimes parasitism (Nair & Padmavathy, 2014). The endophytes may colonize in the roots, stems, leaves, petioles, fruit, buds, seeds. Their interaction with plants depend on some factors like plant species, development stage of the host plants, environmental conditions (Geisen *et al*.2021; Shimanovich and Faeth, 2019).

Endophytes whether bacteria or fungi have significant impact on plants. The bacterial endophytes can synthesize important phytohormones like auxins, gibberellins, cytokinins, adscisic acid (Mukhopadhyay and Chakraborty, 2019; Shahzad *et al*. 2016; Dudeja*et al*. 2012; Shahzad *et al*. 2017). Hence these endophytes are beneficial for plants. The endophytes are also capable ofproducing siderophores, solubilize phosphate, fix atmospheric nitrogen (Joe *et al*. 2016; Loaces *et al*. 2011). Endophytic fungi are potent producers of antimicrobial components which may antagonize the harmful effects of phytopathogens (Kumar & Kaushik, 2012; Zheng et al., 2017).Rhododendron is a unique horticulture plant with immense ethnomedicinal properties. This genus belongs to the family Ericaceae and founded by Carl Linnaeus, 1975. These plants are widely spread

*Correspondence :mahuya.mukhopadhyay@ladybrabourne.com

in the temperate, subalpine and alpine climates in the mountains of northern hemisphere.The spread of rhododendrons in Indian Himalayan region showed the presence of 87 species, 12 subspecies and 8 varieties according to a report in the year of 2010 (Sekar & Srivastava, 2010).From Darjeeling Himalaya, 21 taxa have been reported (Rai *et al.*, 2013). There is new species of rhododendrons reported in Singalila National Park (Rai *et al.*, 2014). Rhododendrons have important phytochemicals and these are very important mankind (Kumar *et al.*, 2019). These plants have been used to treat blood dysentery, asthma, nasal ulcer, blurry vision, gout, diabetes, heart problems, coughs, piles, liver disorders and also have anticancer properties (Madhvi *et al.,* 2019). The local tribes of mountains use these plants to prepare jams, jellies, alcoholic beverages. Due to the human interference the population of rhododendrons aredecreasing. Major threats faced by them are due to deforestation, extraction for firewoodand incense by local people. If the proper conservation is not taken, then the threatened species of *Rhdodoendrons* will be wiped out in near future.

MATERIALS AND METHODS

Sample Collection

The leaf and stem samples were obtained from two rhododendron species – *Rhododendron grande* Wight and *R. arboreum* Smith sub sp. *cinnamomeum*-(Wall. ex G.Don) Tagg.The samples were obtained from St. Joseph College campus, Darjeeling in the month of November, 2020 and taken to laboratory in aseptic condition.

Isolation of Bacterial Endophytes

The surface sterilization is one of the most important steps in case of endophyte isolation.The sterilization process as follows – at first the leaf and stem samples were washed under tap water for 3-4 times, then dipped the samples in 70% ethanol for 1 minute, immersed in 2.5% sodium hypochlorite solution for 5 minutes, followed by 70% ethanol wash for another 1-2 minutes and then final rinsing in sterile distilledwater for 8-9 times. The whole process was done under Laminar flow cabinet.The samples were macerated and dissolved in sterile saline water aseptically.100µl of inoculum from stock as well as diluted solutions of samples was inoculatedon Nutrient agar, King'B agar, Yeast Mannitol agar and Tryptic Soy agar and incubated for 24-72 h and Nystatin (50 µg/ml) was used as antifungal agent (Borah *et al*., 2019 with modification).The sterilized plant samples along with the final step rinsed water were inoculatedon Nutrient agar for control. If growth occurs then whole surface sterilization needs repetition to confirm endophytes' growth. Total 52 bacterial endophytes were isolated and kept them in 4°C for further use.

Gibberrellin Production Assay

To detect the gibberellic acid production ability, twenty-four h fresh cultures of isolatedbacterial endophytes were inoculated in 25 ml Nutrient broth and incubated for 4 days at 28±2°C. At day 4, the cultures were harvested at 10000g for 15 minutes. 15 ml of supernatant was mixed with 2ml of zinc acetate and kept for 2 mins. Then 2ml of potassium ferrocyanide was added into this. Centrifuged at 2500 g for 15 mins. to geta clear supernatant. Finally, 5ml clear supernatant was taken out and mixed with 5ml of30% hydrochloric acid, incubated for 75 minutes at 20°C. The absorbance was measured at 254 nm in UV-Vis spectrophotometer (Sharma *et al*. 2014). The media without any culture was act as control.The standard curve was prepared(Holbrook *et al*.1961) using commercial gibberellins (100-1000 µg/ml). So, the concentration of gibberellins of endophytes was calculated from the standard curve.

Indole Acetic Acid Production Assay

For Indole acetic acid (IAA) determination, 5µl of twenty-four h fresh cultures were incubated in 25ml Nutrient broth in shaking condition (120 rpm) for 48 h at 28±2°C.The media was supplemented with 0.2% L-tryptophan and without any bacterial culturethe media was regarded as control. The endophytic isolates were harvested at 10000g for20 mins. The pellet was discarded and supernatant was used for the detection. One part of supernatant was mixed with 2 parts of Salkowski reagent, incubated in dark for 30 minutes.The appearance of pink or light red color of the samples indicates the positive result.The absorbance was measured at 530nm in UV-Vis spectrophotometer and concentrationsof the samples were calculated from the standard curve of IAA ranging 10-100µg/ml (Gordon and Weber,1951).

Detection of Nitrogen Fixation Ability

The isolated strains were inoculated on Glucose Nitrogen-Free Mineral media slants and incubated for 7 days at 30°C. Bromothymol blue was applied as indicator in this mediathe change in color of media from light green to dark green or blue indicates the ability of nitrogen fixation by these endophytes (Latt *et al*. 2018).

Detection of Ammonia Production

To screen the ammonia production ability, the bacterial endophytes were incubated in10ml of Peptone water broth for 24 to 72 h at 30°C. After incubation 0.5 ml of Nessler's reagent was added in the test organisms. Formation of yellow to brown color indicates the ammonia production (Cappuccino and Sherman,1992).

Detection of Phosphate Solubilisation

The phopspahte solubilization assay was performed using Pikovskaya's media (Pikovskaya, 1948).Isolated strains were inoculated on this media and incubated for 5 days at 28±2°C. Observation of clear zones around the bacterial colonies were regarded as phosphate solubilization capability of these endophytes. This result was interpreted in terms of Solubilization Index(SI) = (Colony diameter + Halo zone diameter) **/** Colony diameter (Fankem *et al*. 2006).

Statistical Analysis

The data obtained from this study were statistically analyzed. The three growth parameters IAA, gibberellins and phosphate solubilization abilities of the isolated bacterial endophytes were analyzed by two ways ANOVA using Microsoft Excel 2007 at $p<0.05$ significance level.

RESULTS AND DISCUSSION

A total of 52 bacterial endophytes were isolated from the leaf and stem samples of two *Rhododendron* species. Out of these, 13 endophytic strains were selected based on their gibberellin production and nitrogen fixation. These strains were also capable of producing IAA (indole acetic acid), ammonia and solubilize inorganic phosphate. The other isolates also had these traits, but these 13 isolates were more potent in terms of these specific growth traits mentioned above.The isolates were diverse in terms of their colony morphology as enlisted in (Table1).

Gibberellin is an important phytohormone that helps in plant growth (Colebrook *et al*., 2014).In this study bacterial endophytic isolates from leaf and stem samples of *Rhododendron grande* Wight and *R. arboreum* Smith subsp. *cinnamomeum*-(Wall. ex G.Don) Tagg had produced quite high amount of gibberellins (Table 2 and Fig.1). Isolate RGRADS03 produced highest amount of gibberellins- 292.7±3.01 µg/ml followed by RGRADS11, RGRADL08, RGRADL09, RACDL14 and RGRADL07. The isolates from *R. grande* showed significant amount of gibberellins production ranging 118.4-292.7 µg/ml and in case of *R. cinnamomeum* the concentration of gibberellins produced by endophytes ranged from102.6-201.5 µg/ml. Auxin is another important hormone that helps in root development, apical dominance, phototropism in plants. The indole acetic acid (IAA) is the most important auxin for plant growth and development reported in many studies (Kumar *et al*. 2017;Dhungana and Itoh, 2019). Endophytic isolates RACDS04 from *R.cinnamomeum* had highest concentration of IAA production 57.5±0.21 µg/ml.In case of *R. cinnamomeum* (RACD) strains the IAA production was ranged from 24.5 - 57.5 µg/ml (Table2 and Fig. 2). The bacterial endophytes from *R. grande* (RGRAD) had equivalent ability as the strain of *R. cinnamomeum*in case of IAA production. The highest IAA producer in case of *R. grande* was RGRADL09, an isolate from leaves (43±0.23 µg/ml). Nitrogen is the vital component of chlorophyll, proteins, nucleic acids, enzymes of plants (Han *et al*. 2015). So, it is very important for the growth of plants. Endophytes are able to fix atmospheric nitrogen and make it available to plants (Hongrittipun *et al*. 2014). Out of 52 isolates, only10 isolates were able to fix nitrogen (Table 2). The isolates RGRADL07, RGRADL08, RGRADL09 and RGRADL10 from *R.grande* leaves were only capable to fix nitrogen. But the isolated endophytes from stem samples of the same *Rhdododendron* species were unable in this. But in case of *R.cinnamomeum* 4 endophytic isolates from leaves RACDL02, RACDL14, RACDL16, RACDL17 and 2 isolates from stems RACDS04, RACDS12 had shown nitrogen fixation. Ammonia is also important for plant growth in an indirect way by suppressing the growth of phytopathogens or directly by

Table1. Colony Morphology of Isolated Bacterial endophytes

Bacterial Endophytes	Colony Morphology					
	Size	Shape	Edge	Opacity	Elevation	Color
RGRADL07	Very small	Dots	Entire	Translucent	Slight raised	White
RGRADL08	Medium	Round	Entire	Opaque	Raised	White
RGRADL09	Medium	Round	Entire	Opaque	Convex	Milky white
RGRADL10	Medium	Irregular	Undulate	Translucent	Raised	Yellow
RGRADS03	Small	Irregular	Undulate	Opaque	Flat	Cream
RGRADS10	Medium	Round	Entire	Translucent	Raised	Light white
RACDL02	Small	Irreglar	Undulate	Translucent	Convex	White
RACDL14	Medium	Irregular	Undulate	Opaque	Slight raised	White
RACDL16	Small	Round	Entire	Opaque	Raised	Yellow
RACDL17	Large	Round	Entire	Opaque	Raised (waxy)	Light white
RACDS04	Small	Round	Entire	Opaque	Flat	White
RACDS12	Medium	Irregular	Undulate	Opaque	Flat	Yellow

Note: RGRADL means bacterial endophytes isolated from leaves of *Rhododendron grande*. RGRADS= Isolated strains from stem samples. RACDL= Strains from leaf sample from *R.cinnamomeum* and RACDS= stem sample endophytes.

Table 2. Isolated bacterial endophytes and their plant growth promoting factors

Isolated Endophytes	Gibberellin±SD (µg/ml)	IAA±SD (µg/ml)	Nitrogen fixation ability	Ammonia		Phosphate	
				Ability	Color	Ability	SI±SD
RGRADL07	211.9±2.9	21.5±0.21	+	++	Dark yellow	+	1.98±0.13
RGRADL08	234.6±2.1	29.2±0.52	+	++	Dark yellow	++	2.86±0.09
RGARDL09	232.6±3.10	43±0.23	+	++	Dark yellow	+	1.86±0.15
RGRADL10	118.4±2.11	33.5±0.24	+	++	Dark yellow	++	2.13±0.18
RGRADS03	292.7±3.01	20.2±0.41	-	+++	Brown	+	1.8±0.09
RGRADS10	194.5±3.02	23±0.05	-	++	Dark yellow	-	0
RGRADS11	242±3.4	22.24±0.11	-	++	Dark yellow	-	0
RACDL02	143.3±2.14	27.1±0.21	+	+	Yellow	-	0
RACDL14	201.5±4.02	32.8±0.36	+	+	Yellow	++	2.08±0.15
RACDL16	102.6±4.06	24.5±0.23	+	+	Yellow	-	0
RACDL17	173±3.01	38.6±0.31	+	++	Dark yellow	++	2.43±0.1
RACDS04	199±2.16	57.5±0.21	+	++	Dark yellow	++	2.16±0.04
RACDS12	161.2±4.10	36.8±0.12	+	+	Yellow	-	0

Note: Concentrationof Gibberellin and IAA ; ± Standard deviation , SI = Solubilisation Index
+ (<2) = Mild Phosphate solubilizer,++ (>2<3) = Moderate solubilizer, +++ (>3) = Strong solubilizer

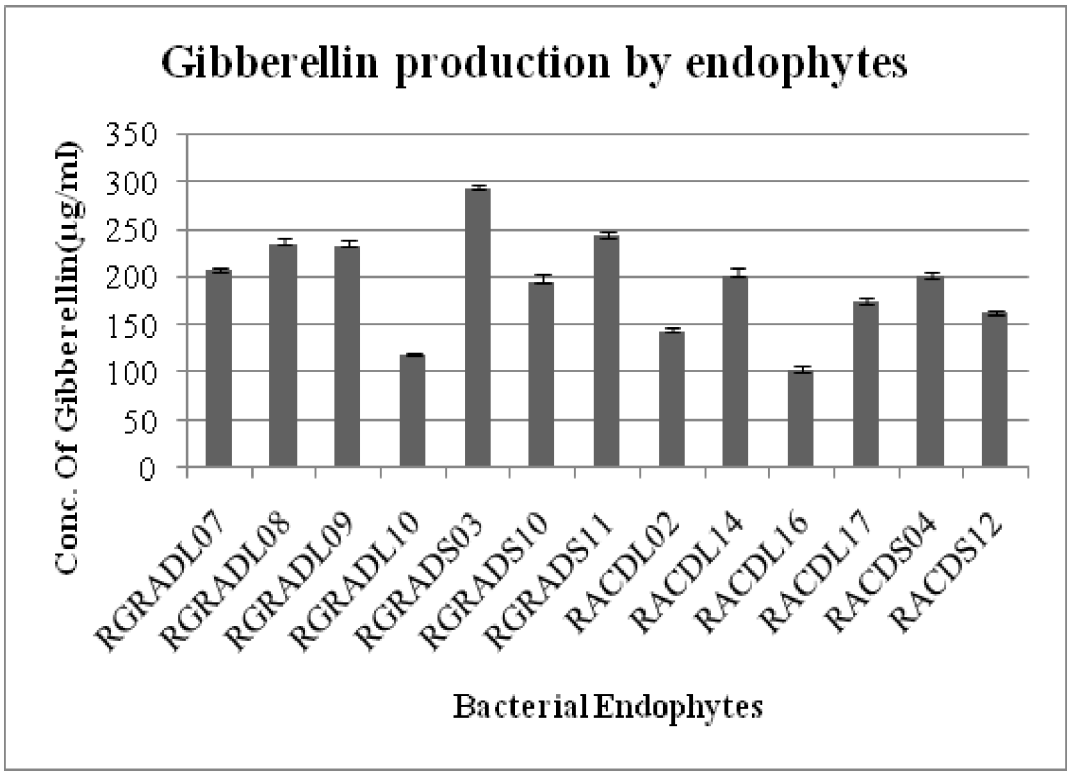

Fig.1. Gibberellin production abilities of isolated bacterial endophytes

Note: Each bar represents the Mean ± Standard deviation of three replicates of gibberellins production amount by the isolated bacterial endophytes. The data were significantly different ($p<0.05$). The isolates had abilities to produce different concentration of gibberellins.

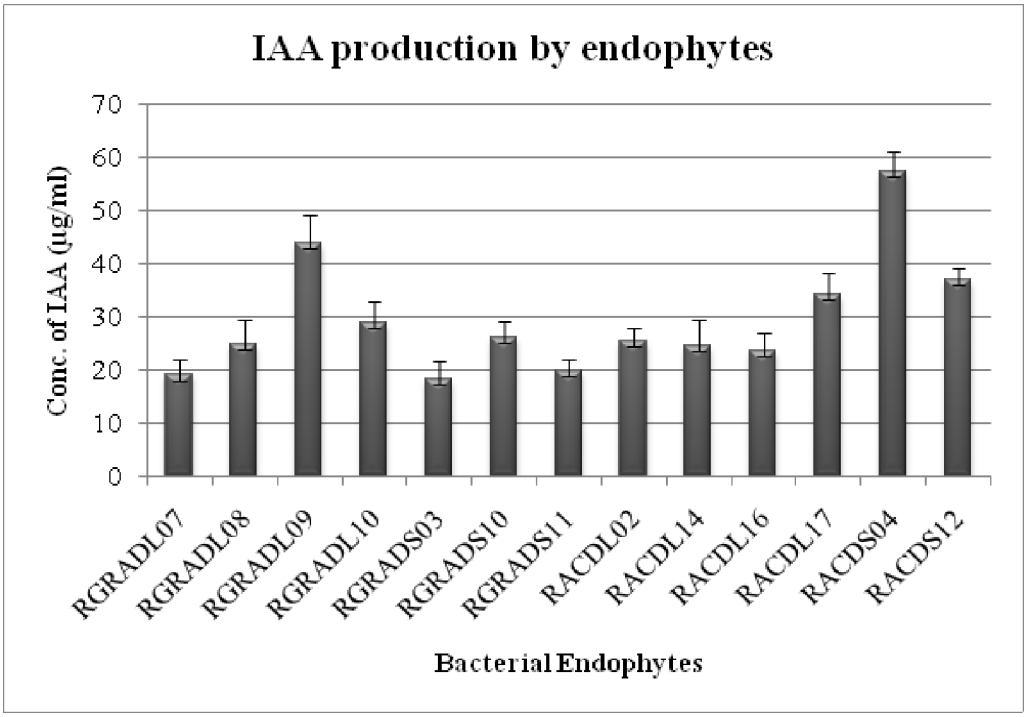

Fig. 2. IAA production abilities of isolated bacterial endophytes

Note: The conc. of Indole acetic acid production by the isolated endophytic strains were represented as Mean ± Standard deviation of three replicates. The data were significant at $p<0.05$ level.

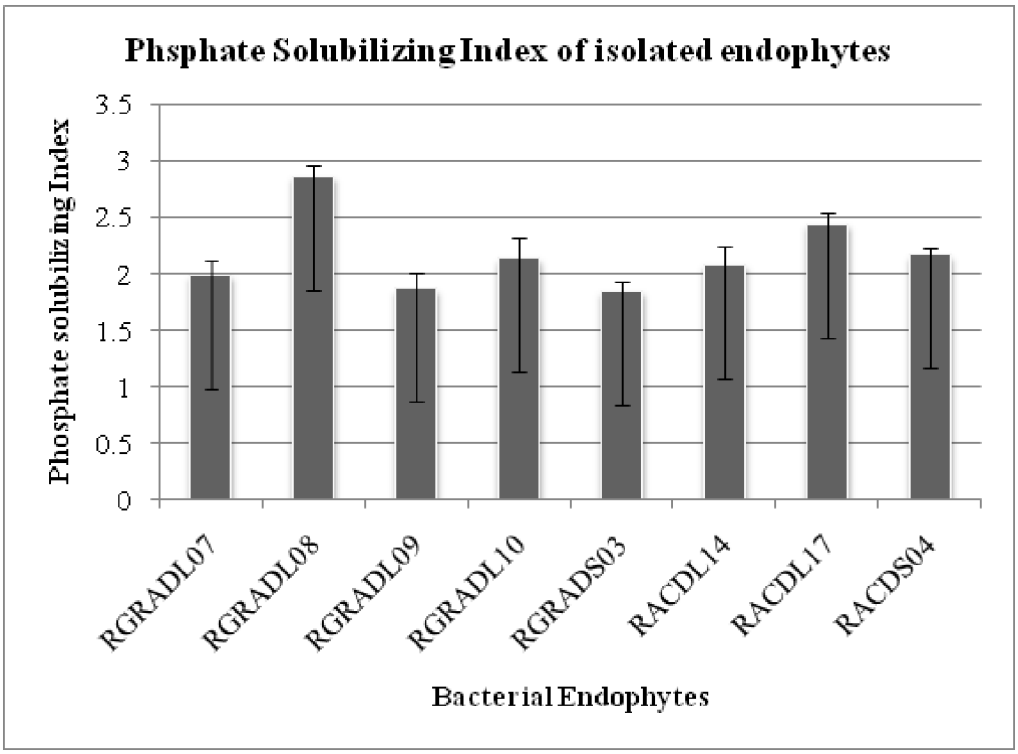

Fig. 3. Phosphate Solubilization Index by the endophytes
Note: Each bar represents the Mean± Standard deviation of Phosphate Solubilization Index of the endophytic isolates and they were significantly different ($p<0.05$) to solubilize inorganic phosphate provided as tricalcium phosphate in the media.

satisfying the nitrogen demand of the host plant (Minaxi *et al*. 2012; Rodrigues *et al*. 2016). In this present study 13 bacterial endophytes were able to produce ammonia (Table 2).The qualitative assay reported that RGRADS03 from *R. grande* was most potent ammonia producer based on the intensity of the color (brown). Rest of the other isolates were mild to moderate ammonia producers (yellow to dark yellow color).Phosphate solubilizing soil bacteria always act to enhance the phosphate uptake by the plants and help in root and shoot development (Zhang *et al*. 2017). Endophytic bacteria usually release organic acid in soils which solubilize phosphate complexes and make available to the plants (Otieno *et al*. 2015).The qualitative assay of phosphate solubilisation was based on the ability of endophytic isolates to solubilize tricalcium phosphate in Pikovskaya's media.Well their ability was described here in terms of phosphate Solubilisation Index (SI).The isolated strains from leaf sample of *R. grande* were capable of solubilizing inorganic phosphate. Endophytic isolate RGRADL08 was moderate solubilizer and among the others having a highest solubilizing index of 2.86±0.09, whereas another endophytefrom this plant species RGRADL10 had solubilizing index of 2.13±0.18 (Table2). It was observed 2 endophytic isolates from leaf sample of *R. cinnamomeum* RACDL14(SI: 2.08±0.15) and RACDL17 (SI:2.43±0.1) were moderate phosphate solubilizers (Fig.3). The statistical analysis showed that all the isolates enlisted in this study have significant differences at $p<0.05$ level, which can be interpreted as the endophytes had the different abilities to produce the phytohormones and for phosphate solubilization under same culture conditions.

CONCLUSION

In our study endophytic bacteria were isolated from an important horticultural plant *Rhdodoedndron* species. The potentials of plant growth promoting factors had been explored and showed a significant phytohormone production and nitrogen fixation abilityalong with phodphate solubilisation and ammonia production. As the species of rhododendrons are decreasing day by day, due to urbanization and environmental pollutionstheir conservation is needed. As the bacterial endophytes produced important components, application of them may be beneficial for their

improved growth. Though there are several methods to grow and preserve plants under greenhouse condition, a natural resource would be an additional way to improve the growth and developments of plants.

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