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Root Endophytic Bacterium *Pseudomonas fluorescens* Strain REB-4 from Berseem (*Trifolium alexandrinum* L.) with Plant Growth Promotion and Antifungal Activities

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ABSTRACT

Background: Berseem is a highly nutritious leguminous fodder crop which is used in rotation with cereal and other fodder crops due to its beneficial effects on soil nutrients. Endophytes isolated from roots of such nutritious plants are known to promote their growth as well as that of crop it is rotated with.

Methods: In the current study, screening of berseem root endophytic bacteria (REB) was carried out. Morphologically distinct colonies were evaluated for their growth promotion traits in terms of phosphate solubilization, hormone production and enzyme synthesis. Their effect on plant growth parameters was also recorded. The *in vitro* antifungal activity was assayed using dual culture technique. The most efficient isolate was identified based on 16s rRNA gene sequence homology and subjected to biochemical analysis.

Result: All the isolates exhibited plant growth promoting traits and secreted high levels of enzymes. They showed *in vitro* antagonistic activities against pathogenic fungi belonging to various genera which could be due to enhanced secretion of chitinase enzyme or other bioactive molecules. The most efficient isolate was found to be Gram negative and molecularly identified as *Pseudomonas fluorescens* which can be used for enhanced production of berseem in future.

Key words: 16S rRNA, Berseem, Biocontrol, PGP activities, *Pseudomonas fluorescens,* Root endophytes.

INTRODUCTION

Endophytes belong to symbiotic group of microorganisms that colonize the internal tissues of plants asymptomatically, being found in nearly every plant worldwide (Santoyo *et al*., 2016). They spend their life cycle as an obligate or facultative microorganism and cause no harm to the host plants. Since few decades, researches into how plant growth can be promoted under biotic and abiotic stresses have mainly concentrated on plant growth promoting rhizobacteria (PGPR). However, more recently attention has been focused on the beneficial aspects of endophytic bacteria which have been isolated from a variety of plants and exhibit an excellent potential as plant growth promoters in agricultural crops under biotic and abiotic stresses (Etesami *et al*., 2013; Khare *et al*., 2018).

Bacterial endophytes promote the plant growth in various ways for example, through enhancing bio-availability of soil nutrients, secretion of growth hormones, supplying biologically fixed nitrogen and providing tolerance to pest and diseases (Compant *et al*., 2005). The seed priming with endophytic bacteria increases seed germination, plant biomass and seed production through production of auxin like compounds (Mahmood *et al*., 2016) and modulates host plant response to abiotic stress (Lastochkina *et al*., 2020). Other beneficial effects of endophyte association with the host plant include modification of root architecture, enhanced uptake of minerals, alteration of nitrogen accumulation and metabolism (Hardoim and Hardoim, 2017). The well-studied and most abundant organisms isolated from plant tissues belong to Actinobacteria, Proteobacteria and Firmicutes and include members of the genera *Streptomyces*, *Pseudomonas*, *Azoarcus*, *Enterobacter*, *Burkholderia*, *Stenotrophomonas* and *Bacillus*, among others (Malfanova *et al*., 2013; Lopez *et al*., 2018).

For this study, it was hypothesized that isolated endophytic bacteria could exhibit growth promotion and biocontrol activities. Hence, the present study was undertaken to (i) evaluate PGP traits of endophytic isolates and ii) test their biocontrol ability against seed and soil borne pathogenic fungi.

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MATERIALS AND METHODS

Collection and isolation of endophytic bacteria

Root samples were collected from healthy berseem (*Trifolium alexandrinum* L.) plants, grown in fields of Central Research Farm, Jhansi, India and washed with distilled water. They were cut into small 1 cm segments, surface

sterilized using 2.0 % NaOCl for 3 min and 70% ethanol for 2 min and rinsed three times with double distilled water. The final rinsing water was spread onto nutrient agar (NA) plates to check the effectiveness of the sterilization process. Plates were incubated at 25±1°C for 10 days and observed daily. Morphologically distinct colonies (REB 1-6) were purified and maintained on NA. For broth cultures, bacteria were grown in 50 ml nutrient broth (NB) medium and incubated for 72 h in dark at 30°C in a shaking incubator at 150 rpm unless otherwise mentioned.

Measurement of plant growth promoting (PGP) traits

Phosphate solubilization (PS)

The quantitative phosphate solubilization was carried out in liquid Pikovskaya's medium in 100 ml flasks. The concentration of the soluble phosphate in the supernatant was estimated after 10 days by stannous chloride ($SnCl_2.2H_2O$) method (Gaur, 1990). A simultaneous change in the pH was also recorded in the supernatant on Systronics digital pH meter.

Indole acetic acid (IAA) and gibberellic acid (GA) production

Endophytic bacterial isolates were grown in NB supplemented with 50 µg/ml of L-Tryptophan. The production of IAA was estimated as per Rahman *et al*. (2010) and absorbance was measured at 530 nm using UV-visible spectrophotometer (LABINDIA ® Analytical UV 3200). Similarly, GA production was estimated using the method of Holbrook *et al*. (1961). The absorbance of the sample was measured at 254 nm. The concentration of IAA and GA in sample was determined from standard graph of commercial IAA (10 to 200 µg/ml) and GA (10 to 100 µg/ml) respectively.

Measurement of enzymatic activities

The cell wall degrading enzymes cellulase, glucanase (β-1, 3 glucanase) and chitinase were assayed in culture filtrates of bacterial endophytes after 72 h of growth. The quantitative estimation of cellulose activity was carried out by inoculating 1 ml of 48 h bacterial culture in to 50 ml of minimal medium supplemented with 1% CMC. The cellulase activity was measured by the dinitrosalicylic acid (DNS) method (Miller *et al*., 1959). For quantitative glucanase production, 50 ml of minimal medium supplemented with 0.5 % laminarin azure was inoculated with 1 ml of 48 h old bacterial culture. It was assayed by incubating 0.5 ml of 0.5% (w/v) laminarin (Sigma) in 50 mM acetate buffer (pH 4.8) and 0.5 ml of enzyme solution at 45°C for 30 min and the reducing sugar produced was determined using DNS method. The amount of reducing sugars released was calculated from standard curve recorded for glucose and enzyme activities (cellulase, glucanase) were expressed in U/ml.

The quantitative estimation of chitinase was carried out by inoculating 1 ml of 48 h old bacterial culture in to 50 ml of minimal medium supplemented with 1% colloidal chitin. It was assayed by incubating 0.5 ml of 1% (w/v) colloidal chitin (Sigma) in 50 mM acetate buffer (pH 4.8) and 0.5 ml of enzyme solution at 45°C for 30 min. The activity of enzyme was measured by DNS method. One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 µmol of *N*-acetyl glucosamine per min under the above condition.

Measurement of antifungal activity

All endophytic bacterial isolates were screened for antagonistic effect against seed and soil borne pathogens using dual plate assay. Briefly, mycelial plug (5 mm) of actively growing pathogen was placed in the center of the plate containing Potato Dextrose Agar (PDA) medium. A loopful of 48 h old bacterial culture grown in NA medium was streaked 2.5 cm away from the edge of each plate and perpendicular to the fungi. Plates with only pathogenic fungal culture were used as control. All the plates were incubated at 25°C for 7 days. The antifungal activity was examined by calculating percent inhibition of radial growth of fungus using following formula:

$$PI = \frac{C - T}{C} \times 100$$

Where,

PI = Per cent inhibition.

C = Radial growth of pathogenic fungus in control plate.

T = Radial growth of the pathogenic fungal colony interacting with antagonistic endophytic bacterial isolate.

Measurement of physiological parameters

All endophytic bacterial cultures, grown overnight, were inoculated in 50 ml NB at 30°C for 48 h with 150 rpm shaking. Subsequently, the accumulated bacterial biomass was harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cell pellets obtained were washed twice with sterile distilled water and finally suspended in phosphate-buffered saline (PBS) of pH 7.0 [approximately 1×10^7 Colony Forming Units ml^{-1}]. Berseem seeds were surface sterilized by immersion in 3% NaOCl for 5 min, followed by 70% ethanol for 2 min. The seeds were then rinsed thrice with sterilized distilled water. The surface-sterilized seeds were soaked in PBS bacterial cells for 4 h. The seeds treated only with PBS were used as controls. Four replicates comprising of 25 seeds for each treatment were placed in petri dishes containing filter paper moistened with sterilized water and kept at 20°C in the dark. Germination was recorded as a radicle protrusion through the seed coat and the germination percentage, fresh weight (FW) and dry weight (DW) were determined. Seedling vigor index was calculated as a product of germination percentage and seedling dry weight in grams (Perry, 1978).

Identification and biochemical characterization of efficient endophytic bacterial isolate

Best performing bacterial isolate was grown in NB medium for 24 h at 25±1°C. Genomic DNA was extracted using phenol/chloroform protocol described by Kheirandish and

Harighi (2015) and PCR was performed using 16S rRNA universal primers The partially amplified gene was sequenced, edited and aligned, using *ClustalW* using MEGA 7.0 (Kumar *et al*., 2018). Aligned sequence of 16S rRNA gene was used for homology search by the Basic Local Alignment Search Tool-nucleotide (BLASTn) (http://www.ncbi.nlm.nih.gov) algorithm at National Center for Biotechnology Information (NCBI). Furtherthis isolate was characterized biochemically using biochemical test kit (KB002 Hiassorted™).

Statistical analysis

All the statistical analysis was performed using SAS version 9.3 software with three replicates of each experiment and were interpreted through one-way ANOVA followed by Duncan's multiple range test (DMRT) at the $p \leq 0.05$ significance level. In this study, the experiments were performed in triplicates and were repeated twice.

RESULTS AND DISCUSSION

Bacterial endophytes isolated from Berseem roots exhibited plant growth promoting activities

Berseem (*Trifolium alexandrinum* L.) is more popularly known as King of fodders due to its high nutritious value and increasing economic importance in India and worldwide (Vijay *et al*., 2017). It is also used in rotation with rice crop in some parts of the world, such as Egypt. Rice seedlings were found to benefit from the growth promoting activities of endophytes isolated from berseem roots (Etesami *et al*., 2013). In the current study, berseem roots were used for isolation of endophytic bacteria and six morphologically different colonies were observed. These isolates were named as REB 1-6 (Root Endophytic Bacteria 1-6) and evaluated for their capability to solubilize phosphate, produce IAA and GA *in vitro* as a measure of their plant growth promoting activities.

All six isolates showed the significant zone of phosphate solubilization (PS) on medium. Clear zones were formed around the colonies after 5 days of incubation on Pikovskaya Agar medium amended with insoluble TCP, an indication of phosphate solubilization ability of the bacterial endophytes. In quantitative estimation (Supplementary Table 1), the strain REB-4 showed the highest phosphate solubilization with a value of 75.23 µg/ml while REB-2 showed the lowest value (57.63 µg/ml). Similarly, all six bacterial endophytic isolates were able to produce IAA and GA ranging from 33.24 to 47.70 µg/ml and 9.40 to 16.53 µg/ml respectively. The production of IAA and GA were highest in isolate REB-4 and lowest in isolate REB-2. Several researchers have reported the ability of root-endophytes isolated from different plant systems to solubilize phosphate *in vitro* and produce IAA and GA (Fadiji and Babalola, 2020). The bacteria that inhabit plant roots secrete an enzyme phytase that can solubilize phosphate and make it available to the host plants. These bacteria also produce growth regulators such as IAA and GA that induce root proliferation of host (Patten and Glick, 2002) which provide an opportunity to the endophytes to colonize the host tissue successfully. The cumulative effect of these properties of endophytes is increase in the overall growth of host plants.

To check the effect of these isolates on plant growth, physiological growth parameters of plants were recorded when their seeds were treated with endophytic isolate. These isolates improved the seedling vigour and length when compared to control and the maximum increase was observed in seeds treated with REB-4 isolates (Fig 1). Endophytic bacteria have been reported to enhance seedling vigor and growth in different plant systems such as banana (Karthik *et al*., 2017) and *Withania* (Mishra *et al*., 2018). However, the isolates did not enhance the germination percentage with respect to control, which ranged around 85-88% (Fig 1).

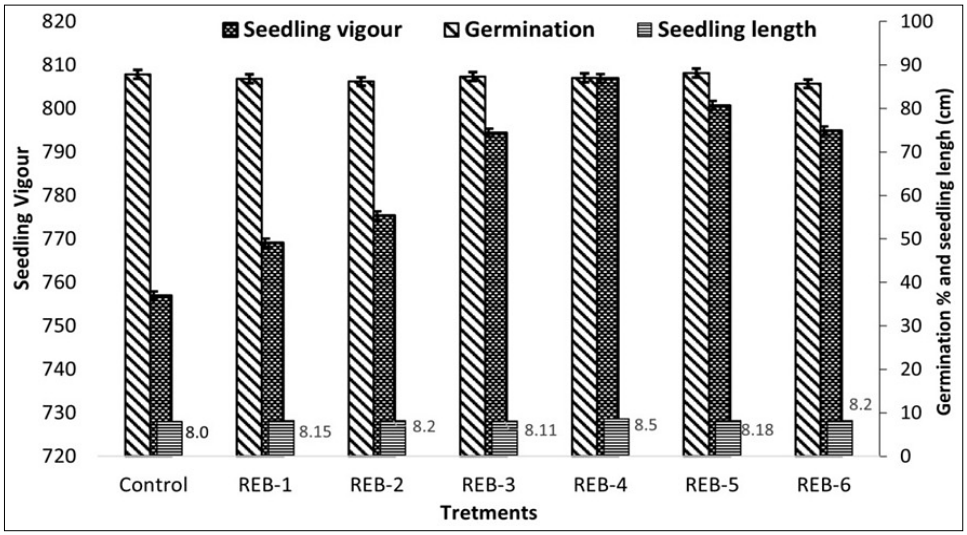


Fig 1: Effect of endophytic isolate treatment on physiological growth parameters of Berseem seedlings.

Bacterial endophytes produced cell wall degrading enzymes and exhibited antagonistic effect on fungal pathogens

Cell wall degrading enzymes that catalyse the hydrolysis of polymers present in plant cell wall are secreted by bacterial endophytes to gain entry into host plant tissue. The activity of these enzymes was observed in all endophytic isolates however, the amount of enzymes produced significantly varied (Supplementary Table 1). The highest enzyme activities were recorded in REB-4 (17.70 U/ml: cellulase, 3.0 U/ml: glucanase, 3.34 U/ml: chitinase). The lowest cellulase and glucanase activity were recorded in REB-1 (11.34 U/ml, 2.13 U/ml respectively) while lowest chitinase activity was observed in REB-5 (2.07 U/ml). These results indicated the capability of isolates to penetrate and colonize the host tissue *via* secretion of various lytic enzymes.

All the bacterial endophytes showed antagonistic activity against fungal pathogens that could be due to their ability of producing chitinase along with other hydrolytic enzymes. However, a common observation was that REB-4 showed greater antagonistic effects on growth of most of the pathogens showing maximum inhibitory effect on *Sclerotinia sp.* and least on *Alternaria alternata* (Table 1). Chitinase is an enzyme that hydrolyses chitin, the polymer present in fungal cell wall. Hence, the anti-fungal activities of bacterial isolates have been attributed to their capability of producing chitinase enzyme along with other bioactive compounds (Khan *et al*., 2018).

Biochemical characterization and molecular identification of the most efficient endophytic isolate

Based on the performance of different isolates REB-4 was characterized further as it was the best performing isolate. The colony of this isolate showed a pale cream colour and was identified as Gram negative (Fig 2). The bacterial isolate was characterized biochemically and it showed positive result for citrate utilization and glucose whereas it gave negative results for lysine, ornithine, TDA, nitrate reduction, hydrogen sulfide production tests (Supplementary Table 2). Results from 16S rRNA gene sequencing revealed 99% similarity with *Pseudomonas fluorescens*. Data obtained after 16S rRNA sequencing is available at the NCBI GenBank database with accession number (MF326214).

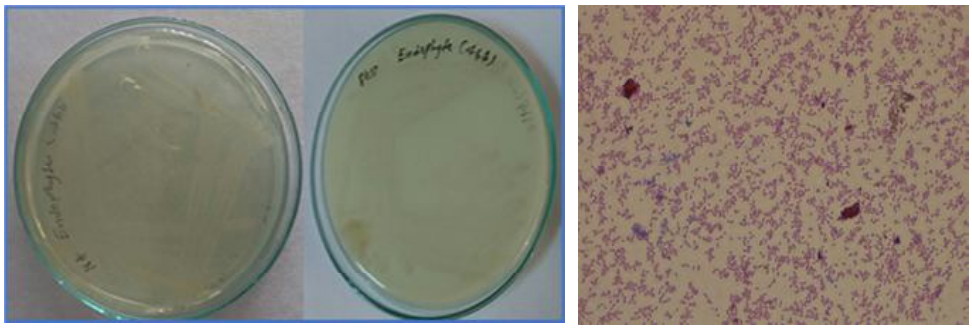
Many isolates of genus *Pseudomonas* exist within the plant as endophytes and actively interact with the host plant for the benefit of both organisms (Chung *et al.,* 2008). The strains of *Pseudomonas fluorescens*, *P. corrugata* and *P. putida* have been most frequently isolated as endophytes and rhizospheric bacteria (Garbeva *et al*., 2001; Suzuki *et al*., 2003). Further, two isolates of *P. oleovorans* have been identified as effective endophytes towards seedling growth enhancement and organic seedling production among tomato cultivars (Thomas and Upreti, 2016). *Pseudomonas fluorescens* in combination with *Bacillus amyloliquefaciens* has been reported to augment vigor index and reduce plant mortality upon *Alternaria* infection (Mishra *et al*., 2018). The results of our study are in line with these earlier reports and suggest that *Pseudomonas fluorescens* could be used for

Table 1: Antagonistic activities observed in different endophytic isolates against seed and soil borne pathogenic fungi.

Pathogens	Root endophytic bacterial isolates					
	REB-1	REB-2	REB-3	REB-4	REB-5	REB-6
Rhizoctonia sp.	17.78±4.43	13.97±2.27	39.91±7.51	43.93±3.60	12.99±2.43	40.22±0.87
Sclerotinia sp.	30.93±4.09	22.75±3.60	51.95±4.86	49.52±6.85	25.15±5.72	40.67±2.96
Fusarium sp.	40.99±0.49	32.73±5.62	44.16±5.06	48.23±6.50	48.23±6.50	46.73±4.60
Aspergillus flavus	18.19±8.45	17.29±8.03	22.96±1.39	38.54±7.48	12.90±11.40	46.00±0.32
Aspergillus niger	36.38±0.67	33.53±4.29	38.19±1.96	41.61±6.46	28.16±2.42	38.19±1.96
Alternaria alternata	11.04±3.60	17.72±4.64	30.02±1.00	36.55±5.45	17.90±5.72	37.69±3.83
Curvularia lunata	19.21±2.54	16.65±5.10	21.67±9.13	37.31±9.20	21.78±5.13	31.41±2.85
CD (5%)	7.55	8.97	9.35	N/A	11.09	5.06
SE (m)	2.46	2.93	3.05	3.86	3.62	1.65

Supplementary Table 1: Quantitative estimation of PGP traits and enzymatic activity exhibited by endophytic isolates.

Treatments	PS (µg/ml)	IAA (µg/ml)	GA (µg/ml)	Cellulase (U/ml)	β-1, 3 glucanase production (U/ml)	Chitinase (U/ml)
REB-1	65.30^{ab}±2.35	36.37^{cd}±0.72	12.37^{bc}±0.60	11.34^{b}±0.50	2.13^{a}±0.35	2.58^{b}±0.19
REB-2	$57.63^{b\pm}$3.26	33.24^{d}±1.92	9.40^{c}±0.69	16.63^{a}±1.47	2.34^{a}±0.22	2.14^{b}±0.055
REB-3	61.90^{b}±1.75	43.03^{ab}±1.06	12.43^{bc}±1.30	14.92^{ab}±1.82	2.52^{a}±0.24	2.17^{b}±0.20
REB-4	75.23^{a}±2.34	47.70^{a}±2.10	16.53^{a}±0.74	17.70^{a}±0.75	3.00^{a}±0.096	3.34^{a}±0.073
REB-5	66.63^{ab}±4.39	43.10^{ab}±1.15	14.50^{ab}±0.88	12.99^{ab}±1.55	2.17^{a}±0.32	2.07^{b}±0.25
REB-6	62.63^{b}±2.30	41.70^{bc}±1.41	14.80^{ab}±0.24	16.22^{a}±1.29	2.59^{a}±0.13	2.47^{b}±0.13
CD (5%)	5.13	2.65	1.77	2.363	0.44	0.302
SE (m)	1.64	0.85	0.57	0.758	0.141	0.097



a b

Fig 2: Pure culture (a) and Gram staining (b) of REB-4 isolate.

Supplementary Table 2: Characterisitics and molecular identification of the most efficient endophytic strain (REB-4).

Morphological characteristics	
Colony colour	Pale cream
Gram staining	Negative
Biochemical characteristics	
Citrate utilization	+
Lysine	-
Ornithine	-
Urease	v
TDA	-
Nitrate reduction	-
Hydrogen sulphide production	-
Glucose	+
Adonitol	nd
Lactose	v
Arabinose	v
sorbitol	nd
Molecular identification	
16S rRNA gene homology	*Pseudomonas fluorescens*
Percent similarity (%)	99
Accession number at GenBank database, NCBI	MF326214

+ = Positive (>90%); - = Negative (>90%); v = 11-89%; nd = No data available.

enhanced production of berseem and potentially as a biocontrol agent. This could be highly beneficial for farmers as berseem seed production has been suggested to be more beneficial than rice or wheat production, if the benefit to cost ratio is considered (Vijay *et al*., 2017).

CONCLUSION

Based on the results of the current study, we can conclude that the berseem roots are a rich source of endophytes that could solubilize phosphate and produce plant growth regulators such as indole acetic acid and gibberellins. They could also improve host plant's physiological growth parameters such as seedling vigor and length indicating their plant growth promoting properties. In addition, all the bacterial isolates exhibited *in vitro* antagonistic effects against seed and soil borne pathogens suggesting their application as biocontrol agents. However, their efficacy needs to be proved under field conditions in future studies. Moreover, the best performing isolate (*Pseudomonas fluorescens*) exhibiting high levels of all desirable traits can be used as a seed priming agent in berseem seed production after methodological validation.

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Authorship contribution statement

Manjunatha N: Isolation of endophytes, to test biocontrol ability of endophytes, data analysis and editing of manuscript; Sanjay Kumar: Analysis of research data of seedling parameters; Rana M: molecular identification of endophyte; Tomar M: growth promotion and enzyme studies; Srinivasan R: morphological and biochemical characteri zation; Agarrwal R: compilation of data and manuscript writing.

Conflict of interest

On behalf of all the authors I declare that there are no conflict of interest.

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