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RHIZOBIUM RADIOBACTER : A UNIQUE MAIZE ENDOPHYTE WITH HIGH LEVEL OF STRESS TOLERANCE AND MULTIPLE PLANT GROWTH PROMOTING PROPERTIES

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Abstract

A unique naturally occurring bacterial endophyte BHU-4 was isolated from surface sterilized roots of the maize (*Zea mays* L.) plants north eastern part of India that exhibit a number of plant growth promoting properties. This isolate was identified as *Rhizobium radiobacter* (deposited in NCBI with accession number JN033549) showing 99.7% similarity when the phylogenetic analysis of the nucleotide sequence of 16S rRNA was done. It forms white mucilaginous colonies on yeast extract mannitol plates and the cells were rod shape with gram negative reaction. It grows in wide range of pH (4-10), temperature (4-45^{0}C) and salt concentration (0.1-10%) that provide an opportunity to survive in highly adverse conditions. High level of tolerance to nalidixic acid and ciprofloxacin, the inhibitors of DNA gyrase provides an addition attribute to this strain for survival under extreme temperature stress. Capability of producing high amount of phytohormone IAA (8.04µg ml^{-1}) and solubilising zinc (0.3mm h^{-1}) helps this strain to support the plant growth as plant growth promoting rhizobacteria. It was tested as bioinoculant for its effect on the growth of three cultivars of maize both under laboratory and glass house conditions. Plants grown under laboratory conditions exhibited significant increase in plant height (23.02 to 44.09%) and plant dry weight (15 to 25.73%) of inoculated plants over uninoculated (control) plants. Variability within the cultivars of maize in response to bacterial inoculation of BHU-4 was more visible when the same experiment was conducted in glass house. It was therefore concluded that due to presence of multiple plant growth promoting properties and high level of a number of abiotic stresses tolerance, this strain will be highly useful as bioinoculant sustainable cultivation of maize (*Zea mays* L.).

Key words: *Rhizobium radiobacter*, plant growth promoting properties, abiotic stresses tolerance, maize (*Zea mays* L.)

Introduction

Biofertilizers are important components of integrated nutrients management. They would play key role in productivity and sustainability of soil and also protect the environment as eco-friendly and cost effective inputs for the farmers. They are cost effective, eco-friendly and renewable source of plant nutrients to supplement chemical fertilizers. Biofertilizers are products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus, potassium, Zinc etc.) from unavailable to available form through biological processes performed by microbes. Salt stress is one of the abiotic stresses in worldwide that inhibit the crop's growth and productivity which is going to increasing day by day (Singh *et al*., 2019). These microbes support the growth of plants either living freely, in rhizosphere association, symbiotic association or through colonization of the interiors of plant root system as endophyte. Association of bacteria with many higher plants including cereals such as rice (Singh *et. al*., 2006) maize (Gutierrez-Zamora and Martinez-Romero, 2001) wheat (Biederbeck *et. al.,* 2000) etc. In most of the reports, these endophytes supported the growth of host plants through one or the either mechanisms which may be direct or indirect.

Occurrence of different species of rhizobia such as *Rhizobium leguminosarum* bv. *phaseoli, Rhizobium etli*, *Bradyrhizobium* sp. etc to develop natural endophytic association has been reported earlier that promotes the

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growth of the host cereal plants. Natural occurrence of *Rhizobium radiobacter* as endophyte of cereals is not reported so far from any part of the world. There was only one report about the presence of endophytic agrobacteria-like strains associated with different grain legumes from Tunisia (Saidi *et. al*., 2011).

Rhizobium radiobacter is an aerobic, gram-negative bacterium causing human systemic diseases that is found in soils of different part of world including India. The common name of this is *Agrobacterium tumefaciens* also named as *Agrobacterium radiobacter* belongs to the family Rhizobiaceae of alpha subclass of proteobacteria. Out of the eleven different strains of endophytic bacteria isolated from maize roots, two of them (BHU-4 and BHU-5) were identified as *Rhizobium radiobacter* based on the gene sequencing. In this study, the physiological and biochemical properties and its effect on plant growth of maize plants was determined.

Materials and Methods

Isolation and identification of bacterial endophyte

Maize roots were collected at the age of 15 days of sowing. Roots were cleaned thoroughly with tap water, rinsed with sterile distilled water and cut into 2-5cm long pieces. Five grams of root pieces of each sample were transferred to a sterile 250ml Erlenmeyer flask containing 50ml of sterile water, shaken for 15 minutes, and washed six times in 50ml of sterile distilled water. Pieces of root were then aseptically transferred to another sterile 250ml Erlenmeyer flask and surface sterilized as follows. The root samples were placed in 95% ethanol for 1 minute and then washed with sterile distilled water. In second step, root pieces were sterilized with 0.1% $HgCl_2$ for 5 minutes, and washed six times with sterile distilled water (Chaintreuil *et. al*., 2000). Root pieces were macerated and the slurry was filtered through sterile cotton wool (Lindberg and Granhall, 1984). The isolation and purification were carried out in different media such as Yeast-extract-manitol (YEM) medium of Vincent (1970), Luria Broth (LB) medium of Bertani (1951), Azelic Acid medium of Santos *et al*., (2001) and King's B medium of King *et. al*., (1954) with 1.5% agar-agar was used to solidify the medium whenever required for this study. 0.1ml suspension of sample was spread on these media and incubated at 28^0C for 48-72hr. Well developed colonies were selected and stored in the same media at 15^0C.

Table 1: Morphological, biochemical characterization and similarity of root-associated endophytic bacterial strain based on 16S rDNA sequencing.

Sl. No.	Characters	
1	Isolate	BHU-4
2	Highest similarity (99.7%)	*Rhizobium radiobacter*
3	16S rDNA (bp)	1,440
4	Accession No.	JN033549
5	Growth medium	YEM Yes
6	Colony morphology	White mucilaginous
7	Pigmentation	No
8	Colony shape	Rod shape
9	Gram reaction	Negative
10	Zinc solubilization (mm h^{-1})	ZNO 0.30
11	Available zinc (mg l^{-1})	
	released by bacteria in	ZNO 17.71
	broth medium	
12	IAA production (µg ml^{-1})	8.04
13	pH	4 to 10
14	Temperature (^{0}C)	5 to 45
15	NaCl (%)	0.1 to 10
16	Location of isolate	North-Eastern part of
		Utter Pradesh, India

Gram's reaction

Bacterial endophytes were tested for Gram's reaction, colony characteristics and cellular morphology. For Gram's reaction, smear was prepared on glass slide from exponentially growing cultures. Dried slides were stained with Gram's reagents and observed under light microscope (Lietz Ortholux-II, Germany). For the observation on colony characteristics and cell morphology, the cell of isolate was cultured on YEM plates.

Temperature tolerance

Bacterial growth at different temperature was determined on YEM agar plates inoculated with 10 µl of culture (± 10^9 CFU ml^{-1}) and incubated at temperatures 5^0C, 25^0C, 35^0C and 45^0C. Same experiment was performed in YEM broth and optical density measured at 420 nm after 7 days of incubation.

Salt tolerance

Tolerance to sodium chloride (NaCl) was determined by the growth on YEM plates supplemented with 0.1-10% NaCl after 7 days of incubation at 28^0C. Same experiment was also done in YEM broth by taking optical density at 420 nm after 7 days of incubation.

pH tolerance

Tolerance to extreme pH (ranging from 4 to 12) was tested in YEM medium. Hydrochloric acid (HCl) was used to adjust pH lower than 7 and NaOH was used to adjust pH above 7. 10 µl of exponential phase culture (± 10^9 CFU ml^{-1}) were used to inoculate in 30ml YEM broth. Optical density was measured after seven days of inoculation at 420 nm. Growth at different pH was also

Table 2: Effect of endophytic bacteria on three cultivars of maize under laboratory conditions.

Characters	Bacterial endophyte	Cultivar		
		MHM-2	DHM-117	Pro Agro-4212
Seed germination	Control	92.25	80.75	75.50
percentage	BHU4	94.25 (2.17)	91.25 (13.00)	93.00 (23.18)
Shoot length (cm)	Control	4.00	3.90	3.70
	BHU4	6.00 (50.00)	5.40 (38.46)	5.65 (52.70)
Root length (cm)	Control	4.63	3.60	3.45
	BHU4	5.00 (8.11)	4.60 (27.78)	4.85 (40.58)
Plant height (cm)	Control	8.00	7.40	7.35
	BHU4	11.00 (37.50)	10.00 (35.14)	10.00 (36.05)

* Values in parenthesis indicate the percent increase over control

measured in terms of colony forming units (CFUs ml^{-1}) by plating cell on YEM agar plates after suitable condition.

Zinc solubilisation

Isolates were inoculated into specified liquid mineral salts medium (g/l) (Saravanan *et al.*, 2007) containing dextrose: 10.0; $(NH4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble zinc compound (ZnO: 0.1%; and Agar: 15.0g) and autoclaved at 121^{0}C for 20 minutes. Actively growing culture of spot-inoculated (5 µl) on the agar plates was incubated at 28^{0}C for 48hr. The clear halo zone around colony was recorded. Quantitative estimation of zinc solubilization was studied in 150 ml conical flasks containing 50 ml of liquid mineral salts medium. The broth was inoculated with 10µl of overnight grown bacterial inoculums and incubated for 72 hr at 160 rpm in an incubator shaker at 28^{0}C. After incubation, the culture broth was centrifuged and the concentration of zinc in the supernatant was estimated in atomic absorption spectrophotometer (GBC, Australia).

Rate of clearance (Solubilization mm/hr) = (Total area of clearance (mm) / (Time of inoculation)

Intrinsic antibiotic resistance

Resistance to antibiotics was tested on YEM agar plates containing the filter-sterilized (0.22mµ Millipore membrane) solutions of Ciprofloxacin and Nalidixic separately with various concentrations. Each plate was divided into six equal sectors and spot inoculation with 10µl of exponentially grown bacterial culture (± 10^9 CFU ml^{-1}) was done in triplicate. The plates were incubated at 28^{0}C for 7 days (Maatallah *et. al*., 2002).

Estimation of indole acetic acid (IAA) production by endophytic bacterial strains

IAA production was estimated by growing the bacterial strains in YEM broth supplemented with 100µg tryptophan ml^{-1}. Tubes were incubated at 28^{0}C for 48 hours with continuous shaking. Cultures was centrifuged at 10,000g for 15 minutes at 4^{0}C. IAA produced ml^{-1} culture was estimated by mixing 4ml of Salkowasky reagent (1ml 0.5 M $FeCl_3$ in 50ml of 35% perchloric acid) with 2ml. culture supernatant followed by measuring absorbance at 530 nm after 30 minutes (Gordon and Weber, 1951) The amount of indoles was determined by using the standard curve of IAA (10-100µg ml^{-1}).

PCR-RFLP analysis of 16S rDNA genes

PCR-RFLP analysis of 16S rDNA genes was done according to the method described by Singh (2013 Ph.D. thesis). The purified PCR products were sequenced in an automated sequencer in combination with a dye deoxy terminator cycle sequencing kit (Van Berkum *et. al*., 2003). The 16S rDNA sequences were compared to the Gene Bank database by using the algorithm BLASTN (Altschul *et. al*., 1997) to identify the most similar 16S rDNA sequences. The sequences were multiple aligned using CLUSTAL W algorithm, version 1.8 (Thompson *et. al*., 1994), with a set of sequences of representatives of the most closely related genera identified.

Assessment of effectiveness of bacterial endophyte with maize plants

Laboratory plant growth experiment

Healthy and bold maize seeds of maize cultivars MHM-2, DHM-117 and Pro Agro-4212 were surface sterilized by treatment with 0.2% acidified mercuric chloride for 3-5 minutes and then rinsed five times with sterilized distilled water and subsequently 70% ethanol for 3 minutes, rinsed three times with sterilized distilled water as described earlier. About 25 surface sterilized seeds were soaked in bacterial culture for 15-30 minutes and rest 25 surface sterilized seeds were untreated to act as control. Both treated and untreated (control) seeds were placed on agar (1%, w/v) plates under aseptic condition at room temperature for germination.

Germinated seeds of maize were transferred into wide mouth (38×200mm) culture tubes containing agar slants of Thorton's (1930) medium. Seedlings were grown for 10 days with regular watering. Data on plant growth parameter such as shoot length and root length and analyzed.

Glass house plant growth experiment

For the evaluation of effectiveness of bacterial endophytes on maize plants, seeds were surface sterilized and allowed to germinate for two days in sterile Petri

dishes as described earlier. Maize seedlings (approximately 1cm in length) were transferred to Petri dishes containing exponentially growing bacterial cells and incubated for one h at room temperature. Three seedlings (inoculated and uninoculated) were transferred to each plastic pot (15cm diameter and capacity to hold 2kg of mixture of sand and soil mixed in a ratio of 1:2 wt/wt). For preparation of sterile soil, field soil was autoclaved twice for 20 minutes at 120^0C with a 24hr interval. Treatments were arranged in a factorial experiment based on completely randomized design. Seedlings were irrigated every 4th day with nitrogen-free plant nutrient solution (Thornton's, 1930). After 35 days, the plants were carefully removed from the sand and soil mixture. The roots were excised and gently washed with tap water to eliminate sand and clay particles and dried with a paper towel to remove excess water and data recorded on various plant growth parameters.

Result

Out of the eleven endophytic bacteria isolated from maize plants collected from different locations of eastern part of India, two (BHU-4 and BHU-5) were identified as *Rhizobium radiobacter* after 16S rDNA gene analysis and gene sequencing. These were deposited in NCBI with the accession number JNO33552 and JNO33553 respectively. Since many of the properties were similar among both strains, BHU-4 was used further for detailed physiological and biochemical studies. This isolate showed high level of intrinsic resistance to various antibiotics and showed growth at 600µg ml^{-1} of ciprofloxacin, nalidixic acid, neomycin, kanamycin and ampicilin. It also showed survival to different stresses such as pH ranging from highly acidic to (4) to highly alkaline (12), sodium chloride (0.1-10%) and temperature 5-45^0C (Table 1). Thus this strain have many unique characters which placed it with different identity.

It produces 8.04µg ml^{-1} of IAA that helped in accelerating the plant growth. When it is present in the broth medium, it releases 17.70 mg l^{-1} of available form of zinc from the ZnO (used as the substrate for estimation of zinc solubilisation). BHU-4 failed to solubilise phosphate and express nitrogenise activity.

Effect of inoculation of BHU-4 on growth promotion of maize plants grown under controlled laboratory conditions and in glass house:

Three cultivars of maize *viz.,* MHM-2, DHM-117 and Pro Agro-4212 were used to test the effect of inoculation of BHU-4 (*R. radiobacter*) on the different plant growth parameters under controlled laboratory conditions and glass house (Table 2). There was a significant effect of inoculation of this endophytic bacterium. Plant height of the inoculated plants were significantly high (35.14 to 37.5%) as compared to uninoculated (control) plants; shoot length was comparatively more affected than root length. Since the growth of the plants was limited in glass tubes, the same experiment was repeated in bigger plastic pots placed in glass house. Response of inoculation among the cultivars was much clear when these were grown in soil in pots (Fig. 1, 2 and 3). Response of inoculation on shoot length was highest in Pro Agro-4212 and least in MHM-2 (Table 3). However, accumulation of dry matter and chlorophyll content was highest in MHM-2 as compared to others and to control.

Table 3: Effect of endophytic bacteria with three cultivars of maize grown under glass house conditions.

Characters	Bacterial endophyte	Cultivar		
		MHM-2	DHM-117	Pro Agro-4212
Shoot length (cm)	Control	41.75	39.00	37.75
	BHU4	51.25 (22.75)	53.75 (37.82)	54.75 (45.03)
Root length (cm)	Control	21.25	16.00	18.75
	BHU4	26.25 (23.53)	25.50 (59.38)	24.50 (30.67)
Plant height (cm)	Control	63.00	55.00	56.50
	BHU4	77.50 (23.02)	79.25(44.09)	79.25 (40.27)
Shoot dry weight (g)	Control	2.12	2.00	2.00
	BHU4	2.91 (37.15)	2.29 (14.63)	2.33 (16.45)
Root dry weight (g)	Control	1.30	1.20	1.00
	BHU4	1.39 (3.27)	1.39 (10.00)	1.27 (25.25)
Plant dry weight (g)	Control	3.42	3.20	3.00
	BHU4	4.30 (25.73)	3.68 (15.00)	3.60 (20.00)
Chlorophyll content	Control	10.23	14.63	9.98
	BHU4	16.60 (62.35)	16.23 (10.94)	15.60 (56.39)

* Values in parenthesis indicate the percent increase over control

Discussion

A number of bacterial endophytes that shows plant growth promotion properties have been reported in the past from the different plants including cereals, pulses and vegetable crops. Bacterial endophytes isolated and characterized with respect to various mechanisms of plant growth promotion parameters are *Pseudomonas, Burkholderia, Bacillus, Rhizobium* (that includes *R. Leguminosarum bv. trifolii, R. Etli, R. leguminosarum bv. phaseoli* and *Bradyrhizobium*) *Acinetobacter, Pantoea, Vibrio* and many more. Majority of them were capable of producing plant growth hormones particularly IAA. In addition to this some of these bacteria were

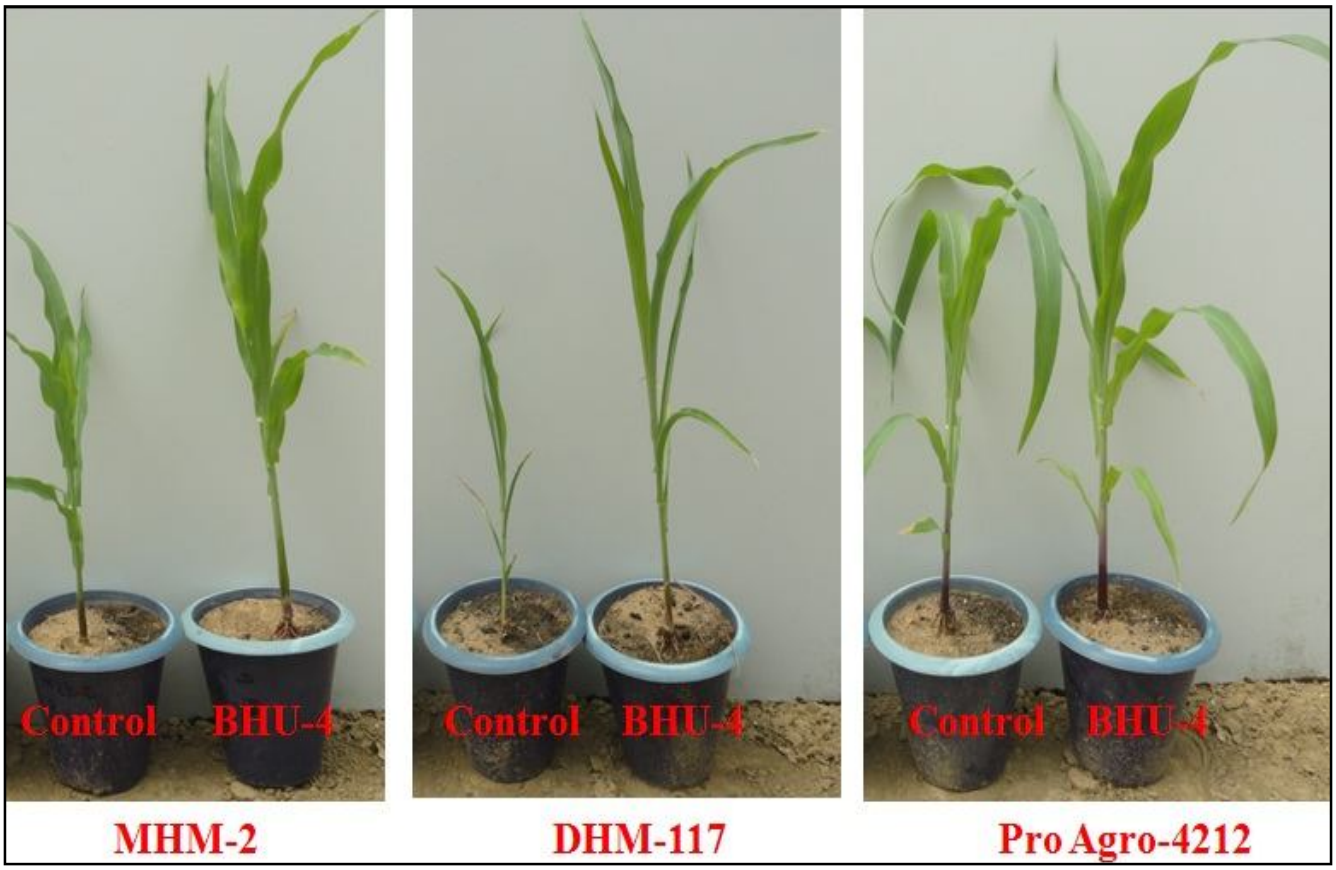

Fig. 1: Effect of endophytic bacteria on maize plants growth after 35 days of inoculation under glass house conditions.

capable of solubilising phosphate, potassium or they may be diazotrophs which can fix atmospheric nitrogen. A plant that can harness more nutrients either with high nutrient use efficiency or proliferated root system can response to the inoculation of these endophytes.

Rhizobium radiobacter BHU-4 reported in this paper has capability of producing of producing IAA that makes root system of inoculated plants to proliferate more than the control plants (Table 1) and thus these plants grew more vigorously. Capability of solubilising zinc by this strain also supported the plant growth. Response of inoculation with three cultivars of maize strongly supports the role of endophytic bacteria in plant growth promotion. Tolerance to various abiotic stresses such as temperature, pH and salt makes it more versatile for application in the field of different climatic zones. Earlier Saidi *et. al.,* (2011) reported the diversity of nodule endophytic *Agrobacterium* like strains associated with different grain legumes of Tunisia. However, based on the rigorous survey of literature, it can be said that this is the first report on occurrence of *Rhizobium radiobacter* as endophyte in maize, a cereal crop that can promote the growth of host plant.

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