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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF NATIVE BACTERIAL ENDOPHYTES ISOLATED FROM GREEN GRAM (*VIGNA RADIATA* L.)

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Abstract

Ten endophytic bacterial isolates were recovered from native green gram varieties from four villages of Khordha district. Isolates were evaluated for their morphological, biochemical and functional (antibiotic sensitivity and sugar utilization pattern) characteristics. All the ten bacterial isolates were circular in shape, convex in elevation, regular in margin and were motile. Three (KHDK1, KHDEB1 and KHDEB4) of the isolates were opaque colonies while all other colonies were found to be translucent ones. Out of ten, seven were gram negative bacilli and three (KHDJ1, KHDR1 and KHDEB4) were gram positive bacilli. All the isolates were observed to have vigorous growth on media with 10% glucose as compared to each of them having 10% of sodium chloride and lactose. Five isolates (KHDJ1, KHDR1, KHDEB2, KHDEB3 and KHDEB5) found to reduce nitrate. All the isolates were found to be sucrose negative whereas all the ten isolates utilised dextrose and fructose as carbon source through both oxidative and fermentative pathway. Most of the strains were found to be resistant to Amphotericin and Penicillin except two (KHDC1 and KHDEB4 found susceptible to Penicillin). KHDEB1 and KHDEB5 were found most resistant to antibiotics among the ten isolated strains.Among the isolated strains KHDEB5 was found most eligible for further molecular characterization.

Key words: Endophytes, isolates, biochemical, antibiotic and sugar.

Introduction

Indian green revolution started in mid 1960s which enabled the food autonomy in country. However, excessive use of inorganic fertilizers and pesticides changed the traditional cultivation practices. The situation has become so alarming that now the role of microorganisms in development of sustainable agriculture is being realised. In order to increase the agricultural production, there has been a tendency to adopt high application rate of fertilizer and irrigation water, often together (Hussain and Jaloud, 1995). Crop plants are able to utilise about 50% of the applied nitrogen fertilizer while 25% is lost through leaching, volatilisation, denitrification etc. This causes not only anannual economic loss of three billion US$ but also pollutes the environment (Saravanan *et al*., 2008). On the other hand, biological nitrogen fixation (BNF) is a microbiological process which converts atmospheric nitrogen intothe readily accessible form to plants.

Green gram (*Vigna radiata* L.) also known as mung bean, is a well known pulse crop of India. Mungbean is digestible, high in protein (22-24%). It is rich in vitamins such as A, B, C, niacin and minerals such as potassium, phosphorus and calcium, which are necessary for human body (Rattanawongsa, 1993). Owing to all these characteristics it is a good substitute for animal protein and forms a balanced diet when it is taken with cereals. Although, this crop is capable of fixing atmospheric nitrogen through *Rhizobium* species living in root nodules, under our agro-ecological conditions, the nodulation of mungbean by native *Rhizobia* is poor and is a major cause for its lower yield. Further, inoculation of mungbean with *Rhizobium* spp. has shown increased plant height, leaf area, photosynthetic rate and dry matter production (Thakur and Panwar, 1995).

Although chemical fertilizers have played a significant role in green revolution, in appropriate and imbalanced uses of chemical fertilizers are affecting gradually the soil fertility, crop quality and to environmental degradation. Therefore, bioinoculation with *Rhizobium* in leguminous plants contributed for crop growth stimulation, a substitute for costly nitrogenous fertilizers (Tairo and Ndakidemi, 2013). Different field studies have indicated that the legume seed, inoculated with *Rhizohium* culture increased the crop yield from 20-80% and beneficial effect on the subsequent crop yield also observed significantly (Lalitha and Immanuel, 2013). On a global basis these symbiotic association between legume and *Rhizobium* may reduce about 70 million tons of atmospheric nitrogen to ammonia Per annum (Peoples *et al*., 1995).

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Material and Methods

The present study entitled "Biochemical and functional characterisation of native bacterial endophytes isolated from green gram (*Vigna radiata* L.)" was carried out in Department of Soil Science and Agricultural Chemistry. Fresh plump nodule samples of green gram (cv. OUM-11-15) were collected from Khordha district at 45 DAS (days after sowing). The collected nodules were surface sterilized with 75% ethanol and 0.1% mercuric chloride and washed thoroughly with distilled water. *Endophytic bacterial* strains were obtained by streaking the crushed root nodules on nutrient agar plates and incubated at 29.4°C (Aneja, 2003). Bacterial colonies were obtained after 2 days of incubation. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures of *endophytes*. Pure isolates were used for biochemical and antibiotic sensitivity tests.

Biochemical characterization of bacterial isolates

A series of biochemical tests were carried out to characte1rise the bacterial isolates as described by Cappuccino and Sherman (2002).

Gram's staining: Bacterial smear was made on a clean glass slide. The smear was air dried and heat fixed. The slide was then flooded with crystal violet (primary stain) for 60 seconds followed by iodine (mordant) for 60 seconds and then rinsed with decolourizer and immediately washed off by distilled water. Safranine (secondary stain) was added to the slide for 30 seconds and rinsed off with distilled water. Slide was blot dried and observed under microscope.

Indole test: Peptone water was sterilized by dispensing into the tubes, inoculated with bacterial culture and incubated for 24 hrs at 37°C. Kovac's reagent was used as indicator. Appearance of red colour on the surface of the medium indicated a positive test.

MR (Methyl Red) test: MR-VP medium was prepared. Bacterial culture was inoculated for 24 hrs at 37°C. After incubation methyl red solution was added. Development of bright red colour indicated positive test.

VP (Vogues-Proskauer) test: MR-VP medium was prepared. Bacterial culture was inoculated for 24 hrs at 37°C. After incubation, α- napthol solution was added followed by 0.2 ml of 40% potassium hydroxide (KOH). A change in colour of the medium to crimson red indicated positive test.

Citrate utilization test: Simmons Citrate Agar slant was inoculated with fresh cultures, incubated for 24 hours at 37°C. Appearance of blue colour indicated positive reaction, while a negative result obtained with no colour change.

TSI test: TSI agar slant was prepared and incubated at 37°C for 24 hrs. Cultural results were interpreted as follows:

Slant	Butt	Inference
Red	Yellow	Only glucose fermentation
Yellow	Yellow	lactose and/or sucrose fermentation
Red	Red	No fermentation
-	Black	H_2S production
-	Bubbles	Gas production

Mannitol Motility test: Butts of Mannitol Motility Agar medium were prepared and inoculated by stabbing the fresh bacterial culture. Mannitol utilization was observed by yellow colouration and motility was confirmed by diffused growth in the butt along the stabbing line.

Anaerobic growth: Fresh culture of the isolate was streaked on Anaerobic Hiveg Agar medium and incubated for 24 hrs at 37°C. Growth on the medium indicated positive result.

Growth at 10% sodium chloride (NaCl): The isolates were tested on Nutrient Agar (NA) medium added with 10% NaCl to detect their tolerance to salinity. The isolates were streaked and incubated for 24 hrs at 37°C. Growth on the medium indicated positive result.

Growth at 10% Glucose: The isolates were tested on NA medium added with 10% Glucose to detect their tolerance to salinity. The isolates were streaked and incubated for 24 hrs at 37°C. Growth on the medium indicated positive result.

Growth at 10% Lactose: The isolates were tested on NA medium added with 10% Lactose to detect their tolerance to salinity. The isolates were streaked and incubated for 24 hrs at 37°C. Growth on the medium indicated positive result.

Functional Characterisation

Sugar utilization pattern: The test was performed on Oxidation Fermentation basal medium to study the oxidative and fermentative mode of metabolic degradation of various sugars by the isolates. Yellow coloration of the medium (inside the test tube) was considered positive while negative test was indicated by no colour change in the medium. The sugar discs used in the study were sucrose, dextrose, mellibiose, inulin, rhamnose, inositol, fructose, lactose, sorbitol, adonitol, salicin, raffinose, cellobiose, trehalose, galactose, dulcitol, mannitol and arabinose.

Antibiotic sensitivity test: The isolates were screened for antibiotic resistance following Kirby- Bauer disc diffusion method (Bauer *et al.*, 1966). The bacterial isolates were revived in Nutrient broth and 24 hours fresh culture was swabbed on Mueller Hilton HiVeg Agar plates. The swabbed plate was exposed to antibiotic discs *viz*; streptomycin, amikacin, polymyxin-B, amphotericin-B, ciprofloxacin, penicillin-G, tetracycline, chloramphenicol, vancomycin, erythromycin, neomycin and bacitracin. The plate was incubated at 30°C for 24 hours. After incubation, the diameters of the zones of inhibition were measured by a zone scale.

Result and Discussion

From the collected plant samples (Table 1) root nodules were separated and bacterial endophytes were isolated. Among the isolated endophytic rod shaped bacteria three (KHDJ1, KHDR1 and KHDC1) each from PDM54, SML668 and Pusa Baisakhi respectively, two (KHDK1 and KHDK2) from PDM54 while five (KHDEB1, KHDEB2, KHDEB3, KHDEB4 and KHDEB5) from OUM-11-15 variety of green gram were isolated and preserved for further characterization (Cappuccino and Sherman, 2002).

Biochemical characterization of the endophytic bacterial isolates

A series of biochemical tests characterisation of the ten isolates (Table 2). Out of ten seven were gram negative bacilli and three (KHDJ1, KHDR1 and KHDEB4) were gram positive bacilli. Six isolates could utilized glucose in TSI medium while rest (KHDR1, KHDC1, KHDEB3 and KHDEB5) four couldn't utilized. All the isolates were observed to have vigorous growth on media with 10% glucose as compared to each of them having 10% of NaCl and 10% lactose. Four (KHDR1, KHDC1, KHDEB3 and KHDEB5) isolates produced H_2S while none of them produced gas. Three (KHDK2, KHDEB1 and KHDEB4) produced acid. Eight isolates were methyl red positive and two (KHDJ1 and KHDEB2) were negative. Five isolates (KHDR1, KHDK1, KHDK2, KHDEB2 and KHDEB4) were voges-proskauer positive. Seven isolates were indole positive while only three (KHDJ1, KHDR1 and KHDEB1) found negative. Only four isolates (KHDK1, KHDK2, KHDC1 and KHDEB4) showed positive growth on anaerobic agar medium. Five isolates (KHDJ1, KHDR1, KHDEB2, KHDEB3 and KHDEB5) found to reduce nitrate. These findings corroborate with the results of Michael (2006), Singh (2008) and Erum (2008) who also reported isolation and characterization of root endophytes.

Functional Characterisation of the endophytic bacterial isolates

Sugar utilisation pattern (oxidation and fermentation) and antibiotic sensitivity pattern were carried out for functional characterisation of the ten isolates. All the isolates were found to be sucrose negative. Two (KHDJ1 and KHDEB4) of the isolates could only utilized lactose. Further, it was observed that, all the ten isolates utilised dextrose and fructose as carbon source through both oxidative and fermentative pathway. Most of the strains were found to be resistant to Amphotericin and Penicillin except two (KHDC1 and KHDEB4 found susceptible to Penicillin) whereas all of them were found susceptible to Tetracycline. As depicted in table 3 endophytic strains KHDEB1 and KHDEB5 were found most resistant to antibiotics among the ten isolated strains. This shows a significant degree of geographical sympatry among the two (KHDEB1 and KHDEB5) isolates (Singh *et al.*, 2013).

Conclusion

Among the isolated bacterial endophytes KHDEB5 strain was found to be more efficient in both biochemical and functional characterization. Further experimentation on the strain for plant growth promoting activities and molecular characterisations can be beneficial for its bioinoculation and crop improvement.

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Table 1: Plant sample collection from locations of Khordha District

Locations	Varieties of Green gram	Isolates	Gram's reaction	Shape
Jagiribadi	PDM54	KHDJ1	-ve	rod
Routpada	SML668	KHDR1	-ve	rod
Katakpatna	PDM54	KHDK1	-ve	rod
		KHDK2	+ve	rod
Chhima	Pusa Baisakhi	KHDC1	+ve	rod
Agronomy Research Field OUAT Bhubaneswar	OUM-11-15	KHDEB1	-ve	rod
		KHDEB2	-ve	rod
		KHDEB3	-ve	rod
		KHDEB4	+ve	rod
		KHDEB5	-ve	rod

Table 2: Biochemical tests of the endophytic bacterial isolates

Isolates	Anaerobic growth	Growth at 10% NaCl	Growth at 10% Glucose	Growth at 10% Lactose	Motility	Methyl red	Voges–proskauer	Citrate
KHDJ1	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve
KHDR1	-ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve
KHDK1	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve
KHDK2	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
KHDC1	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve
KHDEB1	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
KHDEB2	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
KHDEB3	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve
KHDEB4	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
KHDEB5	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve

Table 3: Antibiotic sensitivity pattern of endophytic bacterial isolates

Isolates	E15	AP50	B10	CIP5	PB100	T	P10	N30	AK30	S10
KHDJ1	MR	MR	R	MS	R	MS	MR	R	MS	S
KHDR1	MS	MR	R	MS	MS	S	R	MR	MS	MS
KHDK1	MR	R	MS	S	MS	S	R	S	S	MS
KHDK2	S	R	MS	S	MS	S	R	MR	R	MS
KHDC1	S	R	R	S	MR	S	S	MS	MS	S
KHDEB1	MS	R	R	S	R	MS	R	MR	MS	S
KHDEB2	MS	R	MS	S	R	MS	R	R	MS	MS
KHDEB3	MS	R	R	S	MS	MS	R	MS	MS	MS
KHDEB4	S	R	MS	S	MS	S	S	MS	S	S
KHDEB5	S	R	R	S	MS	MS	R	MS	R	MS

***R -** Resistant (<5 mm), **MR** - Moderately resistant (>5 to 10 mm), **MS** - Moderately susceptible (>10 to 20 mm) and **S** - Susceptible (> 20 mm)
E15 - Erythromycin, **AP50** - Amphotericin-B, **B10** - Bacitracin, **CIP5** - Ciprofloxacin, **PB100** - Polymyxin-B, **T** - Tetracycline, **P10** - Penicillin-G, **N30** - Neomycin, **AK30** - Amikacin, **S10** - Streptomycin