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Isolation and Evaluation of Native *Rhizobium* Isolates for Urdbean (*Vigna mungo* L.) from Acid Soils

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ABSTRACT

A Greenhouse experiment was conducted under sterilized sand culture conditions at the Department of Agricultural Microbiology, IGKV, Raipur during Kharif, 2014-15 with Urdbean crop using different soil inoculums collected from various parts of Korba District of Chhattisgarh with a view to screen out low pH tolerant isolates. The pH range of these soils was 4.0 to 7.0 out of which 76 % of soil samples fall under pH 4.0-6.0. By plant infection method, 182 root nodulating bacteria obtained, out of which, a total of top 46 isolates were screened out as per evaluated by their plant assay test on the basis of growth performance like shoot biomass accumulation, nodulation no. and native soil pH Among 46 isolates, 12 isolates were identified as superior and were temperature (50°C) and acidity (5.0pH) tolerant showing nodule no. 35-21 plant $^{-1}$and shoot dry matter accumulation of 1.53-1.17 g plant $^{-1}$. Isolates Rhi-Ku187, Rhi-Ku34 and Rhi-Ku166 were found to be potent low pH (4.0pH) tolerant and also showed survivality at 45°C temperature. Rhi-Ku187 accumulated highest shoot dry matter of 1.533g plant $^{-1}$, showing plant height of 44.90cm and nodule no. plant^{-1} (33). and Rhi-ku34 was found maximum no. of nodule of 35.00 plant $^{-1}$. Colony characterization, survivality and growth performance of urdbean revealed that native isolate Rhi-Ku187 was considered most effective followed by Rhi-Ku 34 which would be a better choice for inoculation of of urdbean crop in an acidic soil condition of Chhattisgarh.

Key words: *Urdbean, Acid tolerant, Temperature tolerant, Rhizobium, Nodulation.*

INTRODUCTION

Urdbean (*Vigna mungo* L.) is one of the important pulses crop, grown throughout the country and it is the third important pulse crop in India. Among the *Kharif* pulses, urdbean is one of the leading pulse crops of Chhattisgarh. Urdbean seeds are highly nutritious containing higher amount of protein (24-26%) and are reported to be rich in potassium, phosphorus and calcium with good amount of sodium.

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The crop is resistant to adverse climatic conditions and improves the soil fertility by fixing atmospheric nitrogen in the soil[14]. Inoculation of *Rhizobium* culture in legumes increased the crop yield from 20-80% and leaving beneficial effect on the subsequent crop yield. Similar to other pulses, urdbean (*Vigna mungo* (L.), being a legume, it enriches the soil nitrogen and has relatively short duration. It is a part of diet for millions of people in these countries and also provides a cheap source of protein. In Chhattisgarh, it occupies an area of 93.46 thousands hectare (*kharif*) and 3.46 thousands hectare (*rabi*) with production of 29.00 and 0.93 thousands tones, respectively and average productivity of 310 and 269 kg ha^{-1} respectively[2]. The low productivity per unit area of this crop is due to many reasons including low and mostly ineffective populations of root nodule bacteria, application of sub optimal nutrition and poor fertility status soils.

Economic success of these legumes depends upon symbiotic nitrogen fixation, so seed inoculation with appropriate strains of *Rhizobium* is important to improve symbiotic nitrogen fixation which requires survival and establishment of inoculated rhizobia in the soil environment[4]. However, many biotic and abiotic factors affect the persistence of symbiotically effective introduced rhizobial strain in soil[12,17]. Moreover, soil acidity is the one of the factors which restricts production of pulses through its impact on limiting *Rhizobium* survival and persistence in soils, as well as reducing nodulation. There is a good possibility to increase pulse production by exploiting better colonization of the roots and rhizosphere through application of effective *Rhizobium* isolates. It has been observed that the properly screened local strains are more effective for a particular agro-climatic region than the strains imported from other places in general[5]. The state has more than 20% of the area in acidic soil category**;** a large portion of the potentially arable land in Korba especially is acidic. Extent of acid soils in Chhattisgarh is 10.84 m ha (6.45 m ha of pH < 5.5 and 4.39 m ha of pH 5.5- 6.5). Therefore a search for low pH and high temperature tolerant *Rhizobium* isolate of urdbean especially in acidic soil condition of Chhattisgarh was carried out through systematic screening of the urdbean-*Rhizobium* germplasm. Hence, the present investigation was conducted with objectives of isolation and evaluation of native *Rhizobium* isolates of urdbean from acidic soils of Korba District of Chhattisgarh in order to search effective acid tolerant *Rhizobium* isolates for better biological nitrogen fixation under low input and eco-friendly technology for sustainable legume production.

MATERIALS AND METHODS

The experiment was conducted in sand culture system in the green house of Dept. of Agricultural Microbiology, College of Agriculture, Raipur (Chhattisgarh) during *kharif* 2014-2015 with Urdbean using soil inocula as treatments. The experiment was laid out in CRD comprising 201 treatments (200 soil samples and one control i.e., no soil) replicated thrice to isolate root nodulating bacteria and to screen location specific acidity tolerant *Rhizobium* isolates for Urdbean through systematic screening of *Rhizobium* germplasm. 200 soil samples were collected to a depth of 6 inches from soil surface from different locations of district Korba, Chhattisgarh during January, 2014. From each collected soil samples, about 100 gm soil were air dried, processed and kept in a polythene bag for chemical analysis. Another about 25 gm soil was kept as such in polythene bag with properly tagged, sealed to prevent the moisture loss and stored in refrigerator at $4^{o}C$ for isolation purpose. Isolation of native *Rhizobium* from different soil samples was done by plant infection method[18]. Fine graded sterilized river sand was used to raise urdbean plants. Healthy seeds of urdbean (Varity: KU-96-3), were surface sterilized with 95 per cent ethanol and then in 0.1 per cent mercuric chloride solution for few seconds. The seeds were then washed thoroughly with double distilled sterilized water for at least five times. These were sown in sand culture system with different collected soil samples was used as

soil inoculums. Timely and uniform irrigation were provided with N free Mcknight seedling nutrient solution as and when required. At 45 DAS, plants were uprooted, plant height, biomass accumulation and nodulation were recorded in 3 randomly selected plants in each treatment at 45 DAS.

Isolation and characterization of urdbean-*Rhizobium* isolates

Rhizobium was isolated from fresh nodules relatively pink and healthy nodules situated on the tap root of harvested Urdbean crop at 45 DAS using YEMA media. The isolated *Rhizobium* was multiplied in the departmental laboratory. The expected rhizobial colonies which appeared were maintained in YEMA slants and incubated them initially for 2-3 days at 28°C to ensure enough growth of the bacteria without any contamination. These isolates were subjected to various biochemical tests like Gram staining, acidity and temperature tolerance.The colony characters *viz,* margin, elevation, size and colour were observed on agar medium and recorded. One ml of appropriate dilution of *Rhizobium* isolates was transferred into the petri plates containing YEMA with congored medium and the phenotype and growth pattern were observed after incubation. The Gram's reaction was carried out for classifying bacteria as gram positive / gram negative[1]. Thermal tolerances of isolates were carried out by subjecting inoculated YEM broth cultures to 55°C for 30 minutes in water bath. After thermal shocks, inoculums were observed for their survival and / or growth after inoculation of broth cultures into petriplates containing specific medium of 5.0 pH and incubation at 28± 2°C for 2-3 days[3].

Acidic tolerant- The ability of acidic tolerant *Rhizobium* isolates were tested on on YEMA medium having pH adjusted with using HCl and NaOH to 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. Observations for growth of inoculums were observed after 48 hours of incubation.

Temperature tolerant- The survival of the rhizobial isolates at high temperature was determined by incubating grown overnight culture at 28± 2°C on a reciprocal shaker. These cultures were inoculated on petriplats containg YEMA media. The plates were incubated at 25, 30, 35, 40, 45 and 50 ^{o}C for 2-3 days.All observations recorded were statistically analyzed using analysis of variance (ANOVA) for completely randomized design (CRD).

RESULTS AND DISCUSSION

A survey was conducted in the months Jan-Fab of the year 2014 in Korba District, Chhattisgarh. About 200 soil samples were collected at 0-15cm soil depth from important urdbean growing areas of total 67 villages of four blocks of district Korba with the purpose of isolation of native *Rhizobium* isolates of urdbean (Table-1). The study was aimed to access the degree of Biomass accumulation and acidity as well as temperature tolerance behavior of local isolates for Chhattisgarh. Because during summer season surface soil temperature of Chhattisgarh plains region raises more than 50°C, resulting in destruction of rhizobial population and almost sterilization of surface soil[6]. By plant infection method in green house condition under sand culture for presence of root nodule bacteria of urdbean showed that 09 per cent soil samples did not have native *Rhizobium* of this legume. Hence, 182 isolates obtained were evaluated by their plant assay test in sand culture grown urdbean under controlled condition to screen the promising ones. 46 topmost Urd-*Rhizobium* isolates were selected on the basis of performances of crop showing higher shoot biomass accumulation and maximum nodulation (>20 no. of nodules per plant) and native to soil samples showing pH < 5.80.

Chemical properties of collected soil samples

The pH range of soil samples collected from various part of Korba District, Chhattisgarh was 4.0 to 7.0 of which 76 % of soil samples fall under pH 4.0-6.0, whereas EC varied from 0.02-0.2 dS m^{-1}. Out of 200 soil samples, 86.5% soil samples fall under E.C. range from 0.02-0.08 dS m^{-1} and 13.5% soil samples fall under E.C. range from 0.08-0.2 dS m^{-1} (Table-2).

Isolation and Characterization of the promising Urd-*Rhizobium* isolates

In this present study, Out of 182 strains of *Rhizobium* bacteria were isolated from the root nodules of *Vigna mungo*, using yeast extract mannitol agar media. Colonies showing different morphological characteristics on the plates were selected for further characterization.

Colony morphology: *Rhizobium* isolates from Urdbean crops raised by using various soil inoculums as treatments produced translucent, nearly round and gummy colonies which were Convex in elevation having nearly entire margin varied in size between 1.5 to 2.00 mm.

Biochemical characteristics: After the gram-staining the bacteria assumed a red colour which indicated that it was a Gram –ve bacteria. Gram staining of the cultured isolate was done to provide information as presumptive tests of the isolates.

Thermal tolerance and Acid tolerant: All 46 *Rhizobium* isolates of urdbean were subjected to thermal shock at 50^0C for 30 minutes and then inoculated on YEMA media of pH adjusted to 5.0. After incubation it was found that favorable growth was there in 17 isolates signifying that these isolates were both thermal (55^0C) and acid (pH 5.0) tolerant Whereas 29 isolates showed no growth at all (-) (Table-3). Further out of 17 isolates as per colony morphology and growth at 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5pH. All of the 17 *Rhizobium* isolate were further subjected to survival at different temperature. Out of 12 promising isolates (Rhi-Ku13, Rhi-Ku17, Rhi-Ku20, Rhi-Ku27, Rhi-Ku34, Rhi-Ku40, Rhi-Ku50, Rhi-Ku119, Rhi-Ku154, Rhi-Ku166, Rhi-Ku187 and Rhi-Ku194) were screened out to be superior for low pH and high temperature tolerant, whereas 5 isolates (Rhi-Ku3, Rhi-Ku4, Rhi-Ku8, Rhi-Ku24 and Rhi-Ku190) showed poor colonies as compared to previous ones (+). Out of 12 stress tolerant isolates, three isolates showed most tolerant ones i.e., Rhi-Ku187, Rhi-Ku34 and Rhi-Ku166. These results are in agreement with the reported of Lalitha and Sam[11],Study related to acidity tolerant behavior of *Rhizobium* isolates, showed low pH tolerance up to pH 4.5. Similar observations were recorded by Segura[16] while screening of acidity tolerant *Rhizobium* strains. He observed that out of fifty isolates, five strains of *Rhizobium* tolerance to acidity upto pH 4.5 and 5.0 in liquid and solid culture, while Kulkarni *et al*.[10] mentioned that rhizobia strains survived at 50 °C and 65 °C on YMA at pH 7 for up to 2 and 4 hours and rhizobial isolated from *C. arietinum*, which grew at 45 °C[13]. Rai *et al*.[15] found that 2 fast growing isolates out of 27 isolates were able to grow at pH 4. Out of 27 *Rhizobium* isolate of black gram were the acidity tolerant and survive as low as pH 4.5, but 6 to 7 was found most suitable pH for growth.Hence identification of stress tolerant crop beneficial microbes is certainly useful in order to formulate those cultures which are able to survive / persist for longer period and work more efficiently under climatic conditions of Chhattisgarh Plains. The study related to stress tolerant (especially high temperature and low pH) behavior of these 46 isolates revealed that 12 isolates performed better with respect to shoot biomass accumulation and nodulation pattern and were also tolerant to low pH and high temperature.

Morphological growth parameters of urdbean as affected by promising acid tolerant *Rhizobium* isolates native to soil inoculums:

Plant height: The plant height of urdbean at 45 DAS increased significantly over control due to different promising *Rhizobium* isolates native to soil inoculums (Table-4). Plant height increased significantly from 25.20 to 44.90cm due to different promising *Rhizobium* isolates. The highest plant height was associated with local isolate Rhi-Ku187 (44.90cm) followed by Rhi-Ku119 (44cm) and Rhi-Ku13 (42.78cm). However significantly lowest plant height (25.20cm) was recorded with uninoculated plants. These findings was clearly supported by Keneni *et al*.[9] who mentioned that growth of legume plants can be increased significantly by inoculation with effective acidic tolerant rhizobial isolates.

Plant Biomass: At 45 DAS, shoot dry weight showed significant increase over un-inoculated control plants (Table-4). Among inoculated treatments, crops raised with soil inoculums Rhi-Ku187 produced maximum shoot dry biomass 1.53g plant^{-1} followed by Rhi-Ku13 (1.41g plant^{-1}). The shoot dry biomass significantly increased from 0.88g plant^{-1} at control to 1.53g plant^{-1} through inoculation. Similarly, Hariharasudhan and Kalaiarasu[7] revealed the inoculation of *Rhizobium* to blackgram in acid soil significantly increased the growth parameters such as plant height and dry matter production and yield parameters.

Nodulation study: At 45 DAS, among soil inoculated treatments, Rhi-ku34 showed significantly maximum no. of nodules per plant 35.00 followed by Rhi-Ku 40 and Rhi-Ku20 showing nodule no. 34.00 and 33.00 per plant respectively. There was no nodule in uninoculated control plants (Table -4). This observation is found corroborate with that of Kalita *et al*.[8]. In present study inoculation of urdbean with acid tolerant promising *Rhizobium* isolates Rhi-Ku 187 and Rhi-Ku 34 from native inoculums could bring out significant effect so far nodulation, growth and dry matter production of crop concerned.

Table 1: Isolation of native *Rhizobium* from Urdbean cultivated land of Korba District during 2014

Name of District	Name of Blocks under study	Name of Village surveyed	Total
Korba	Katghora	KVK, Lakhanpur, Suttra, Salora, Gopalpur, Dhelwadih, Ponsara, Arda, Gajra, Shyahimudi, Dhadpara, Nawagaon, Manjhidera, Lotlota, Chhuri, Agarkhar, Surakhachhar, Balgi, Latakhar, Amjhar, Pandripani, Bancher, Jenjra, Mudapar, Hukra, Katghora, Pakhdeva, Lohanpur, Hachidara, Urela, Mohanpur, Dongari, Vijaypur, Chhalkachhar, Rayal, Amerpur, Khalari, Basantpur, Supetpara, Ranjana, Beltikri, Pondi, Yamunanagar, Kusmunda, Devgaon,	45
	Korba	Urga, Jatapara, Rajivnagar, Ayodhyapuri, Bhaisma,	05
	Kartala	Barpali, Saragbundiya, Pachpedi, Madwarani, Turbarpur, Jamnipali, Kotari, Bhatapara, Tuman, Sohagpur, Mahuwadih, Saraipali, Kharwani, Navalpur	14
	Pali	Chaitma, Kerakachhar, Basibar	03
Total	04		67

Table 2: Properties of collected soil samples of Korba District tested for Urdbean - *Rhizobium*

S. No.	Parameters	Range	No. of soil samples
1	pH (1:2.5)	4.0-4.5	03
		4.5-5.0	30
		5.0-5.5	57
		5.5-6.0	62
		6.0-6.5	42
		6.5-7.0	06
2	E.C. (dS m^{-1})	0.02-0.04	25
		0.04-0.06	89
		0.06-0.08	59
		0.08-0.1	15
		0.1-0.2	12
• **76% soil samples fall under pH range 4.0-6.0** • **24% soil samples fall under pH range 6.0-7.0**			
• **86.5% soil samples fall under E.C. range 0.02-0.08 dS m^{-1}** • **13.5% soil samples fall under E.C. range 0.08-0.2 dS m^{-1}**			

Table 3: Characterization of promising stress tolerant Urdbean-*Rhizobium* isolates

Name of isolates	Gram staining		pH				Temperature				
		4.0	4.5	5.0	5.5	6.0	6.5	30 °C	35 °C	40 °C	45 °C	50 °C
Rhi-Ku3	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku4	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku8	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 13	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 17	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 20	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 24	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 27	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 34	-ve	+	+	+	+	+	+	+	+	+	+	-
Rhi-Ku 40	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 50	-ve	-	-	+	+	+	+	+	+	+	-	-
Rhi-Ku 119	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 154	-ve	-	-	+	+	+	+	+	+	+	-	-
Rhi-Ku 166	-ve	+	+	+	+	+	+	+	+	+	+	-
Rhi-Ku 187	-ve	+	+	+	+	+	+	+	+	+	+	-
Rhi-Ku 190	-ve	-	-	+	+	+	+	+	+	-	-	-
Rhi-Ku 194	-ve	-	-	+	+	+	+	+	+	-	-	-

- \+ indicate growth performance
- \- No growth was found

Table 4: Influence of promising acidity tolerant native *Rhizobium* isolates of Morphological growth parameters and nodulation of sand culture grown urdbean at 45 DAS

Name of isolates	Plant height (cm)	Dry weight of shoot (g plant^{-1})	No. of nodules (plant^{-1})
Control	25.20	0.88	Nil
Rhi-Ku 13	42.78	1.41	32.50
Rhi-Ku 17	31.70	1.21	29.00
Rhi-Ku 20	39.85	1.28	33.00
Rhi-Ku 27	38.95	1.21	30.00
Rhi-Ku 34	39.15	1.22	35.00
Rhi-Ku 40	37.80	1.19	34.00
Rhi-Ku 50	38.15	1.26	28.50
Rhi-Ku119	44.00	1.21	26.00
Rhi-Ku154	33.35	1.17	20.50
Rhi-Ku166	42.05	1.18	21.00
Rhi-Ku187	44.90	1.53	32.50
Rhi-Ku194	37.95	1.17	31.50
SEm±	1.058	0.04	0.635
CD(5%)	3.093	0.11	1.858

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