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BIOCHEMICAL CHARACTERIZATION OF *RHIZOBIUM* ISOLATES OF BLACK GRAM FOR SELECTION OF EFFECTIVE INOCULANTS

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KEYWORDS

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ABSTRACT

A laboratory experiment was carried out involving characterization of *Rhizobium* isolates of black gram with an objective to select suitable microbial inoculants for black gram in acidic soil condition in Chhattishgarh. A total of 34 *Rhizobium* isolates of Black gram were screened as stress tolerant especially to low pH (5.0) and high temperature (50^{0}C) and their morphological, cultural and biochemical characteristics were studied. On YEMA medium, the colonies were circular, whitish pink, convex, entire and opaque. The bacterial cells were rod shaped, aerobic and gram negative. All of them were positive for catalase and amylase tests. All isolates except Rhi-U3415, Rhi-U3550, Rhi-U3544, Rhi- Anj-10 and Rhi-U3545 showed positive reaction for ammonia production from urea. While Rhi-KU34, Rhi-KU154, Rhi-KU190, Rhi-KU8, Rhi-KU119 and Rhi-U3517 isolates were unable to hydrolyze the gelatin. Colony characterization, survivality and a series of biochemical tests revealed that among the isolates tested, 12 *Rhizobium* isolates of black gram were identified as potential stress tolerant (both thermal tolerant (50^{0}C) and acid (pH = 5.0) tolerance) which would be a better choice for inoculation of Black gram in acidic soils of Chhattishgarh and can be further easily immobilized onto carrier like charcoal powder which can be applied as biofertilizer.

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INTRODUCTION

Approximately 65% of the applied mineral nitrogen is lost from the plant-soil system, through gaseous emissions, runoff, erosion and leaching. With growing environment related concerns, various alternatives are being emphasized to reduce the dependence on N fertilizer for plant nutrients. It is in this context, that the use of the nitrogen fixing bacteria in agricultural practices is gaining importance. It provides the major biological source of fixed nitrogen in agricultural soils. Most researches results indicate that *Rhizobium* inoculation is promising biofertilizer because it is cheap, easy to handle and improves plant growth (Kumar et.al,2013 and Bhagat, et.al, 2014). The introduced strain must compete with highly adopted indigenous rhizobia for legume nodulation under specific soil conditions. Many biotic and abiotic factors affect the persistence of symbiotically effective introduced rhizobial strain in soil (Appunu, 2009). Soil acidity and high temp limits symbiotic N fixation by limiting *Rhizobium* survival and persistence in soils as well as reducing nodulation (Appunu, 2009). Low soil pH does not allow the rhizobial cells to survive in adequate numbers; consequently it becomes inevitable to inoculate the crop in adequate rhizobium. Selected symbiotically efficient rhizobia are to be used as inoculants to increase biologically fixed N_2 under field conditions.

Black gram (*Vigna mungo*) is the third important pulse crop of India. Black gram seeds are highly nutritious containing higher amount of protein (24-26%) and are reported to be rich in Potassium, phosphorous and calcium with good amount of sodium. Inoculation of *Rhizobium* sp. causes a greater increase in growth, yield and nodulation (Sam and Immanuel, 2013). Soil microorganisms produce quite a number of extra cellular enzymes to decompose the complex organic matter. (Kathiresam and Selvan, 2006, Richard et al., 2007). As per particular agroclimatic condition, effective bioinoculants of *Rhizobium* isolates should be developed which indicates that there is an urgent need of selection of effective strains for their ability to survive and nodulate in acid soil under high temperature condition that commonly prevails in Chhattishgarh (Gupta et.al,2005). Therefore the present investigation was carried out in laboratory to characterize of Rhizobial species of Black gram on the basis of bio chemical and morphological characters with the objective to find out the most effective isolates in order to biofertilization of Black gram in acid-soil conditions of Chhattishgarh.

MATERIALS AND METHODS

Laboratory experiment was conducted involving 34 *Rhizobium* isolates of black gram present in culture deposits of Microbiology repository of Agricultural Microbiology Dept., IGKV, and Raipur. These isolates were screened as thermal (55^{0}C) and low pH (5.0) tolerance. Inoculated YEM broth cultures were subjected to 50 ^{0}C for 30 minutes in water bath and survival and / or growth of isolates were observed after inoculation of broth cultures into petriplates containing specific medium of pH 5.0 and incubation at 28 $\pm$ 2^{0}C for 2-6 days (Benson, 1990).

7 days old culture was used for morphology, colony characters and Gram's reaction. One ml of appropriate dilution of *Rhizobium* isolates was transferred into the petri

plates containing YEMA with congored medium .The phenotype and growth pattern were observed comprised colony morphology, including parameters like diameter, margin, elevation, form, transparency and colour (Aneja,2003). The Gram's staining technique was followed as suggested by (Graham and Parker,1964). The bacteria that appeared purple were referred to as Gram positive and those which appeared pink were described as Gram- negative (Aneja, 2003).

Biochemical Tests

All the collected samples were subjected to different biochemical tests viz, Starch hydrolysis Test, Gelatin liquefaction Test, Catalase Test. Liquefaction of gelatin was tested in gelatin in agar medium (Pohlman, 1931). The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3g/L beef extract,12g/L gelatin) and grown for 48 hours. On subjecting the growing cultures to low temperatures treatment at 4ºC for 30 minutes to 60minutes, the culture which produce gelatinase remains liquefied while others due to the presence of gelatin becomes solid.

Hydrolysis of starch was examined by streaking the organism on starch with nutrient agar and iodine solution (Graham and Parkar, 1964). Starch agar medium (5g/L peptone, 2g/L Potato starch, 3g/l beef extract, 15g/L agar, pH 7.0) were inoculated with culture and incubated at 29ºC temperature for 48 hours. Drop of iodine solution 0.1N were spread on 48 hours old culture grown on petriplates. Formation of blue colour indicated non - utilization of starch and vice-versa.

Urease enzyme was examined by inoculating culture in Christensen urea agar with phenol red as an indicator. After incubation the phenol red to turn to a deep pink colour. This is a positive reaction for the presence of urease. Failure of deep pink colour is evidence of negative reaction (Graham and Parkar, 1964).

Catalase activity was tested by adding 2-3 drops 3% H_2O_2 to a drop of matured broth of *Rhizobium* isolates to see whether any bubbles are evolved or not (Graham and Parkar, 1964). If catalase is present resulted in immediate formation of gas bubbles.

Promising isolates were repeated for their confirmation.

Bio-fertilizer preparation

Once the pure culture of *Rhizobium* has been established and confirmed , *Rhizobium* cells were immobilized on carriers, which is an inert material used for mixing broth . The carrier (charcoal) was powdered and dried in sun to get 5% moisture level. Then it is screened through 100-200 mesh sieves and neutralized by mixing with calcium Carbonate powder. The broth containing rhizobizal cells were mixed with carrier and kept in tray. The moisture content was maintained to about 35-40%. After proper mixing it is left for 2-10 days by covering the tray with polythene at 22-24ºC. During this period *Rhizobium* cells multiplied, a process called curing. Thereafter, *Rhizobium* inoculants can be used directly or packed and stored.

RESULTS AND DISCUSSION

Rhizobium isolates of black gram (Table 1) present in culture deposits of department and isolated from nodules of black gram grown with acidic soils of Korba District of Chhattishgarh had been screened for their stress tolerant ability especially to high temp and low pH in order to select microbial strains capable to survive and function in acidic soil condition of Chhattishgarh. The isolates were revived on YEM (Yeast extract mannitol), pH-7.0) agar plates and incubated at 29^{0}C (Aneja,2003). After two days of incubation, *Rhizobium* colonies were obtained. The isolates were maintained on slopes of YEMA medium.

Table 1: Isolate number and origin from where *Rhizobium species* were collected

S.No	Isolate number	Origin
	Isolated from root nodules of black gram grown in sand culture system using different soil inocula collected from various parts of Korba District	
1	Rhi-KU34	Local, Korba District
2	Rhi- KU50	Local, Korba District
3	Rhi- KU154	Local, Korba District
4	Rhi-KU3	Local, Korba District
5	Rhi-KU20	Local, Korba District
6	Rhi-KU190	Local, Korba District
7	Rhi-KU40	Local, Korba District
8	Rhi-KU27	Local, Korba District
9	Rhi-KU13	Local, Korba District
10	Rhi-KU17	Local, Korba District
11	Rhi-KU187	Local, Korba District
12	Rhi-KU8	Local, Korba District
13	Rhi-KU4	Local, Korba District
14	Rhi-KU166	Local, Korba District
15	Rhi-KU194	Local, Korba District
16	Rhi-KU24	Local, Korba District
17	Rhi-KU119	Local, Korba District
	Present In culture deposits of Dept. of Agricultural Microbiology, IGKV, Raipur	
18	Rhi-U1511	Present In culture deposits
19	Rhi-U3510	Present In culture deposits
20	Rhi-U3517	Present In culture deposits
21	Rhi-URGKh2	Present In culture deposits
22	Rhi-U3494	Present In culture deposits
23	Rhi-U3415	Present In culture deposits
24	Rhi-U3536	Present In culture deposits
25	Rhi-U137	Present In culture deposits
26	Rhi-UTh-8	Present In culture deposits
27	Rhi-U3550	Present In culture deposits
28	Rhi-U3544	Present In culture deposits
29	Rhi-UAnj-10	Present In culture deposits
30	Rhi-U3441	Present In culture deposits
31	Rhi-U3487	Present In culture deposits
32	Rhi-U3545	Present In culture deposits
33	Rhi-U3505	Present In culture deposits
34	Rhi-U3516	Present In culture deposits

Rhi - *Rhizobium* sp, U – Urdbean, K- Korba District, Chhattishgarh

Pure 34 isolates were used to study the morphological, cultural and biochemical characteristics and all tests were performed in triplicates. Thirty four isolates were tested for confirmation to stress tolerant ability. All 34 *Rhizobium* isolates of black gram were subjected to thermal shock at 50^{0}C 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 these isolates signifying that these isolates were both thermal (50^{0}C) and acid (pH 5.0) tolerant. Superior growth of *Rhizobium* has been reported at neutral pH (7.0). Results

showed that cells were able to grow at pH 4.5, 5.0 and 5.5. Similar observations were made by (Kucuk *et al.*,2006 , Baling *et al.*, 2007). Further out of 34 isolates as per colony morphology and growth at 5.0 pH, 12 promising isolates (Rhi-U137, Rhi-U1511, Rhi-U3415, Rhi-U3441, Rhi-U3516, Rhi-RGKh 2 , Rhi-Ku13, Rhi-Ku20, Rhi-Ku34, Rhi-Ku40, Rhi-Ku50 and Rhi-Ku187) were screened to be superior for low pH and high temperature tolerant. Similar observations were recorded by (Segura, 1995) while screening of acidity tolerant *Rhizobium* strains. 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 Chhattishgarh Plains. The stress tolerant traits of these *Rhizobium* isolates are of potential value from the point of view of biofertilization of black gram crop in an acidic soil under high temperature condition of Chhattishgarh. Further these thirty four isolates were subjected to various biochemical analyses.

Table 2: Cultural & morphological characters of *Rhizobium* isolates

S.No	Characters	Observation
1	Shape	Circular
2	Size of colony	3.1 mm
3	Color	Whitish pink &Glistering
4	Elevation	Convex
5	Margin	Regular
6	Opacity	Opaque
7	Motility	motile
8	Bacterium	shape Rod
9	Oxygen demand	Aerobic
10	Gram's nature	Gram negative

Morphological Characters

In the present study, isolates (Table 2) were found to be having circular colonies with regular borders, convex, whitish pink in colour and glistering. *Rhizobium* isolates produced translucent, nearly round and gummy colonies which were having nearly entire margin varied in size between 1.5 to 2.00 mm. (Fig. 1). These findings are in line with Hussain et al., 2002, Deka and Azad 2006 who also isolated the *Sinorhizobium meliloti* from alfalfa with same characteristics.

Table 3: Biochemical characterization of blackgram-*Rhizobium* isolates

	Growth in different pH after subjected to thermal shock at 50^{0}C for 30 minutes	Extra-cellular enzymes			
	5.0pH	Catalase	Amylase	Urease	Gelatinase
Rhi-KU34	+ +	+	+	+	-
Rhi- KU50	+ +	+	+	+	+
Rhi- KU154	+	+	+	+	-
Rhi-KU3	+	+	+	+	+
Rhi-KU20	+ +	+	+	+	+
Rhi-KU190	+	+	+	+	-
Rhi-KU40	+ +	+	+	+	+
Rhi-KU27	+	+	+	+	+
Rhi-KU13	+ +	+	+	+	+
Rhi-KU17	+	+	+	+	+
Rhi-KU187	+ +	+	+	+	+
Rhi-KU8	+	+	+	+	-
Rhi-KU4	+	+	+	+	+
Rhi-KU166	+	+	+	+	+
Rhi-KU194	+	+	+	+	+
Rhi-KU24	+	+	+	+	+
Rhi-KU119	+	+	+	+	-
Rhi-U1511	+ +	+	+	+	+
Rhi-U3510	+	+	+	+	+
Rhi-U3517	+	+	+	+	-
Rhi-URG2	+ +	+	+	+	+
Rhi-U3494	+	+	+	+	+
Rhi-U3415	+ +	+	+	—	+
Rhi-U3536	+	+	+	+	+
Rhi-U137	+ +	+	+	+	+
Rhi-UTh-8	+	+	+	+	+
Rhi-U3550	+	+	+	-	+
Rhi-U3544	+	+	+	—	+
Rhi-UAnj-10	+	+	+	-	+
Rhi-U3441	+ +	+	+	+	+
Rhi-U3487	+	+	+	+	+
Rhi-U3545	+	+	+	-	+
Rhi-U3505	+	+	+	+	+
Rhi-U3516	+ +	+	+	+	+

+ Positive reaction; - Negative reaction Characteristics

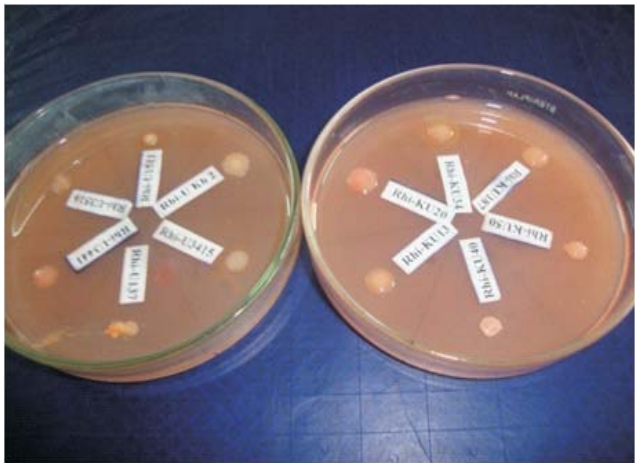

Figure 1: Colonies of promising *Rhizobium* isolates of Black gram on YEMA

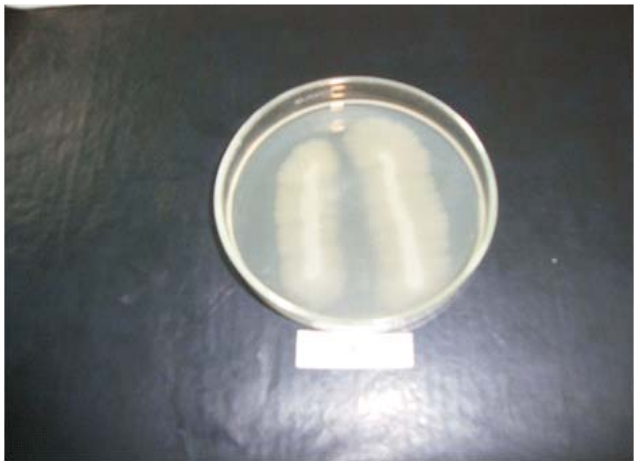

Figure 2: Assay of gelatinase enzyme

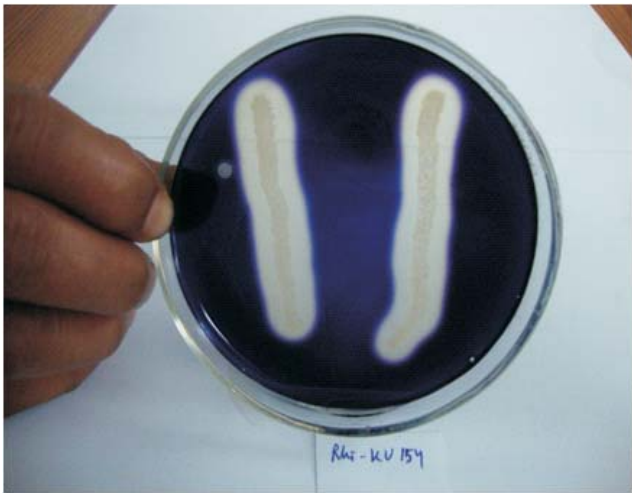

Figure 3: Assay of amylase enzyme

Figure 4: Assay of urease enzyme

All the positive samples were also streaked on Yeast Extract Mannitol (YEM) selective media for further confirmation. Similarly using Gram staining technique as described by Aneja, 2003 pink colored Gram negative rods were observed under light microscope. (Table-2). The authentication of the isolates was performed using sub culturing method.

Biochemical Character

All the isolates showed positive for catalase, amylase activity but some were negative to gelatinase and urease (Table 3). These results are in agreement with the report of (Kucuk et al, 2006) and show that the isolates are more related to fast-growing rhizobia. Biochemical characteristics among the selected isolates indicated that they were closely related to *Rhizobium* species (Sam and Immanuel, 2013).

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product as ammonia. The test was performed to determine capability of *Rhizobium* strains to produce urease enzymes or not. The presence of urease is detectible when the organisms are grown in a urea broth medium containing the pH indicator phenol red. Some of the isolates (Rhi-U3415, Rhi-U3550, Rhi-U3544, Rhi- Anj-10 and Rhi-U3545) were found negative for Urea hydrolysis tests and were not changed to dark pink colour after inoculation and incubation of urea broth as depicted in Fig.4 While in Gel liquefaction tests, the isolates Rhi-KU34, Rhi-KU154, Rhi-KU190, Rhi-KU8, Rhi-KU119 and Rhi-U3517 were found negative reaction (Table-3). In other isolates, rhizobial cells produced gelatinase enzymes as medium containing gelatin liquified when kept at 4^0C for 30 as well as 60 minutes (Fig. 2). The test was performed to determine capability of *Rhizobium* to produce gelatinase enzyme. Degradation of gelatin indicates the presence of gelatinase enzymes (Aneja, 2003). Our findings are in close agreement with Hassem et. al., 1998 who also characterized the rhizobium from soil and root nodules with the same biochemical tests.

Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to Iodine test, clear zones around the colonies were seen and colonies turned yellow in appearance, whereas blue colour appears on no growth area

(Fig.3). This indicates that the isolates have potential to hydrolyze starch present in the medium. The test was performed to determine capabilities of *Rhizobium* strains to use starch as carbon source (Oliveria et al.,2007). In presence of starch, the production of extracellular enzymes occurs indicating the potential of organisms to use starch as carbon source. De Oliveira, 2007 also observed that Rhizobium strains can utilize starch obtained from different sources. Results on catalase activity indicated that they were positive to catalase activity. These results are in agreement with the report of (Sam and Immanuel, 2013). Mahana *et al.,* 2000 reported catalase activity in some isolates from *Vigna mungo*.

Once the pure culture of *Rhizobium* has been established and confirmed for its various activities, the next step was conversion of rhizobia broth into a form which is easily used by farmers .

Rhizobium can easily immobilized onto carrier like charcoal powder so that inoculants can easily be handled, packed, stored, transported and can be applied as biofertilizers; however field trials are yet to be conducted in an acidic soil condition. Acidity tolerant *Rhizobium* sp. can be applied as biofertilizer to increase the yield of Black gram in an acidic soil environment and to create awareness among farmers to cultivate leguminous plants for better agriculture growth.

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