The Pharma Innovation Journal 2022; 11(3): 642-647

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(3): 642-647

www.thepharmajournal.com
Received: 02-12-2021
Accepted: 12-02-2022

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Polysaccharide production and nitrate reduction by blackgram rhizobial strains relating to nitrogen fixing ability

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Abstract
Soil acidity is the one of the factors which restricts production of pulses by restricting nodulation, N fixation and limiting *Rhizobium* survival and persistence in soils. These constraints lead to sub optimal productivity of legume crops raised in acid soils; consequently it becomes inevitable to inoculate the crop with adequate effective *Rhizobium.* Therefore, the present study was undertaken to screen rhizobial isolates of black gram to find out most promising and low pH tolerant ones for enhancing black gram productivity in acid soil of Chhattisgarh. Out of a total of 106 black gram-*Rhizobium* isolates 12Screened promising, different acidity tolerant strains including one standard strain (IARI; Urd-10-B) were characterized under laboratory condition for their polysaccharide production and nitrate reduction. The correlation of these physiological properties with their nitrogen fixing ability was established through a field experiment conducted in 2016 with 14 treatments including 12 screened acid tolerant black gram rhizobial isolates, one national check (U-10-B) and one uninoculated –control in blackgram cv. KU-96-3. The rhizobial strains produced varying amounts of extracellular polysaccharides with different carbon sources ranging 177.44 mg l^{-1} to 286.19 mg l^{-1} in mannitol as C source. In general, all the strains produced maximum polysaccharide with mannitol as carbon source as compared to glucose. The strain RhiU3415 produced maximum amount of polysaccharide (286.19 mg l^{-1}) in mannitol, whereas the lowest amount of polysaccharide (177.44 mg l^{-1}) was formed by Rhi KU40. In glucose maximum polysaccharide i.e. 270.86 mg l^{-1} was produced by strain Rhi U3415 and the minimum amount was produced by Rhi U 3516 (141.63 mg l^{-1}). All 12 promising AT isolates showed a positive nitrate reduction test that the accumulation of nitrite was continuous after growth of rhizobial isolates in nitrate broth leading to a color change from creamy to red colour. Amount of nitrite accumulation (8.08 mM) was observed as maximum with the strain Rhi KU34 and the minimum amount (5.12 mM) was accumulated by the strain Rhi KU13when growth was occurred for 120 hours. On the basis of efficiency ratio at harvest, the strains Rhi KU34, Rhi KU40 and Rhi U3516seemed to be most efficient in N_2 fixation in symbiosis with black gram (Comparable to standard strain U- 10-B)followed by Rhi U137, Rhi KU20, Rhi U1511 and Rhi UKh2. The efficiency ratios of the strains at harvest showed a significantly positive correlation with nitrate reduction by AT strains measured as nitrite accumulation in broth at the end of 120 hours of growth. Whereas production of polysaccharides in different carbon sources was not positively correlated with efficiency. Hence the ability of Blackgram rhizobia to reduce nitrate may be taken as a test to screen effective strains.

Keywords: Black gram, rhizobial strains, Nitrate reductase, Polysaccharide production, Efficiency ratio

Introduction
The *Rhizobium*-legume symbiosis is superior to other nitrogen fixing systems due to its high potential. Different field studies have indicated that the legume seed inoculated with *Rhizobium* culture increased the crop yield from 20-80% and beneficial effect on the subsequent crop yield also observed significantly (Lalitha and Immanuel, 2013) [15]. Black gram / Urd bean (*Vigna mungo*) is the third most important pulse crop in India, it is a part of diet for millions of people and also provides a cheap source of protein. It is found to be less responsive in symbiotically N fixation in acid soil condition. Soil acidity is the one of the factors which restricts production of pulses by restricting root growth, nodulation, N fixation and limiting *Rhizobium* survival and persistence in soils (Appunu, 2009) [2]. A large portion of the potentially arable land in many regions of Chhattishgarh especially in Korba is acidic. Total geographical area of the state is 137.90 lakh ha, 35% of which is net sown area. The state has more than 20% of the area in acidic soil category. These constraints lead to sub optimal productivity of legume crops raised in acid soils; consequently it becomes inevitable to inoculate the crop with adequate effective *Rhizobium.*

So selection of effective Acid tolerant (AT) *Rhizobium* isolate is important for successful nodulation, enhancement of symbiosis and ultimately crop yield in acid soil(Gupta *et al*., 2005) [12]. Therefore, the present study was undertaken to screen rhizobial isolates of black gram to find out most promising and low pH tolerant ones for enhancing black gram productivity in acid soil of Chhattisgarh. But the usual procedure for screening the promising strains by inoculation trials is time consuming, expensive and subject to vagaries of nature. Therefore, attempts have been made by different workers to find out suitable methods for determination of efficiency of rhizobial strains from their easily determinable physiological and biochemical characteristics. (Evan, 2005; Wang *et al*., 2012) [9, 26]. Further to find out the relationship of N2-fixing efficiency of rhizobial strains with their easily determinable physiological and biochemical characteristics, namely acid production, ability to metabolize nitrates, and polysaccharide production. In view of the above facts, an attempt has been made in the present investigation to characterize black gram rhizobial isolates isolated from acidic soils of CG on the basis of their physiological properties like polysaccharide production and nitrate reduction and to study the correlations of these characteristics of the strains with their N2-fixation efficiency.

Materials and Methods

A total of 106 different black gram rhizobial isolates obtained from microbiology repository of Department of Agril. Microbiology, College of Agriculture, Raipur were used in the present study for screening on the basis of acidity tolerance behaviour in order to select microbial strains capable to survive and function in acidic soil.

All cultures were revived by inoculating in YEMA (Yeast extract mannitol agar) medium and maintained on (YEMA, pH: 7.0) medium under aseptic conditions. For liquid medium, agar was deleted from the composition of YEMA medium. The incubation temperature for all cultures was maintained at 28± 2^0C throughout the growth period of the organisms. Broth cultures were aerated by placing the flasks on rotary shaker at 140—160 rpm throughout the growth period. Desired volume of actively growing cultures of the test strains culture was prepared by inoculating the sterilized liquid medium at the rate of one per cent of total volume with the starter culture.

Screening of acid tolerant promising black gram-*Rhizobium* isolates

Loopful active culture of each isolates was inoculated into petriplates containing YEMA media of pH adjusted to 5.0 and in normal pH with replications. All the inoculated petriplates were incubated in incubator at 28± 2 ^{0}C for 2-4 days (Benson, 1990) [4]. Observations were recorded for survival and / or growth of inoculums. From above, those isolates of good survival at low pH (5.0) were further screened and confirmed for tolerance to different pH. *Rhizobium* isolates of Black gram were inoculated in YEMA broth adjusted to each level of pH, 4.5, 5, and 5.5 using HCl or NaOH. After completion of 3 days incubation period, survival of *Rhizobium* were recorded on YEMA media plate with pH (4.5, 5.0 and 5.5) by spot inoculation from log phase culture. Plates were incubated at 28 ^{0}C for 48-72 hours. Three replications were maintained (Graham and Parker, 1964) [11].

Screened promising 12 different acidity tolerant blackgram rhizobia strains including one standard strain (IARI; Urd-10-B) were characterized for their polysaccharide production and nitrate reduction under laboratory condition.

Production of polysaccharides

Each strain was tested for polysaccharide production in two carbon sources, namely mannitol and glucose (Damery and Alexander, 1969) [7]. The organisms were grown in 125 ml of liquid medium containing the carbon source under study in 500 ml flasks on a rotary shaker at 30 °C for five days. Following removal of cells by centrifugation, the culture supernatant was mixed with two volumes of acetone in cold. The crude polysaccharide- containing preparation that developed was collected by centrifugation at 3500 rpm for 30 minutes. Weights of the preparations were determined after drying at 80 °C. The results were expressed as milligram of polysaccharide per litre of medium.

Ability to produce nitrite (Nitrate reductase test)

In order to study nitrate reduction by different isolates, accumulation of nitrite in the growth medium was observed. This study signifies the ability to produce nitrate reductase enzyme that hydrolyze nitrate to nitrite. For this Nitrate broth was inoculated with a heavy growth of test organism using aseptic condition followed by incubation at 35 ^{0}C for 48 hours. Then one dropperfull of sulfanilic acid and one dropper full of α-naphthylamineto each broth were added. A colour change to red indicates positive nitrite accumulation test.

Further 1.01 g KNO3 was added per liter of YEM broth to give a final nitrate concentration of 10 mM per liter. Samples were drawn from the growing cultures at different time intervals and nitrite content was determined in the samples by modified Shinn technique (Tanner and Anderson 1964) [24].

Correlation studies

The correlation of these physiological properties with their nitrogen fixing ability was established through a field experiment. A field experiment was conducted at the research field of KVK, Korba, Indira Gandhi Krishi Vishwa Vidyalaya, Raipur with 14 treatments including 12 screened acid tolerant black gram rhizobial isolates, one national check (U-10-B) and one uninoculated –control in blackgram cv. KU-96-3 during *kharif* season. The experiment was designed in RBD with three replications. Seeds of individual plots were inoculated with freshly prepared carrier based culture of the test strains. The plant samples collected at 60 DAS were analyzed for nitrogen content following modified Kjeldahl method. The efficiency ratio of the strains was calculated as the ratio of N-uptake by shoot in inoculated to that in un-inoculated control plant (Pant and Katiyar 1994) [19].

All the quantitative data in laboratory experiments on characterization of the isolates were conducted in completely randomized design (CRD) with three replications while data generated in field experiment were analysed in RBD.

Results and Discussion

Assay of acidity tolerance behavior of black gram - *Rhizobium* isolates

All 106 *Rhizobium* isolates of black gram were revived on YEMA agar plates and were maintained on slopes of YEMA medium. Study on tolerance behavior to low pH (5.5) revealed that 38 isolates out of 106 were found to form colonies whereas 68 isolates showed no growth at all. As per

colony morphology on YEMA, It was found that favorable growth was there in 10 isolates(+++) followed by 19 isolates (++) and then 9 isolates (+) signifying that these isolates were acid (pH 5.5) tolerant. As per confirmation of acidity tolerance study, thirty eight isolates were grown at different pH of YEMA media (4.5, 5.0 and 5.5) to find out most potent acidity tolerant ones. All thirty eight isolates showed colony growth at 5.5 pH, however at 4.5 pH, 12 isolates were more promising ones (+++) over others which showed favourable growth, 2 isolates showed moderate growth(++), 10 showed poor colonies(+) and 14 isolates did not develop any colony growth(-). Out of 38 isolates as per favourable growth at 4.5 pH, 12 promising isolates (Rhi Ku13, RhiKu20, Rhi Ku34, Rhi Ku40, Rhi Ku50,Rhi Ku187,Rhi U137, Rhi U1511, Rhi U3415, Rhi U3441, Rhi U3516 and RhiUKh 2) were screened out to be superior for acid tolerant.

Soil acidity limits *Rhizobium* survival and persistence in soils and its subsequent root colonization, infection and nodule activity (Graham *et al*., 1994) [10]. pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow the best at neutral pH i.e. 7. (Kucuk *et al*., 2006; Baoling *et al*., 2007) [14]. As here in our experiment 12 *Rhizobium* isolate of black gram were the most potent acidity tolerant isolates and survive as low as pH 4.5 showing favorable good and prominent growth. Hence obviously they were screened as promising acid tolerant ones.

This work is in line with the study which involved the best described AT rhizobial strain is *Rhizobium tropici* CIAT899 (*R.tropici*. UMR 1899) [10] which was isolated from bean nodules (Graham *et al*. 1982) and produced isolated colonies on agar medium of pH 4.0 (Graham *et al*., 1994; Hungria *et al*., 2003) [10]. This strain has been used successfully as inoculant in acid soils.

Ability to withstand against stressed conditions is an important factor which determines the growth and survival of microorganism in soil (Walpola *et al*.,2014) [25]. The findings of present investigation are close to findings of Alexander and Oliveira, 2013) [1].

Physiological characterization of screened promising acid tolerant black gram *Rhizobium* isolates

Physiological characterization of 12 screened promising AT black gram *Rhizobium* isolates and one standard strain (IARI; Urd-10-B) were carried out.

Polysaccharide production

Acid tolerant black gram rhizobial isolates were tested for production of extracellular polysaccharides (EPS) in liquid media containing mannitol and glucose as carbon source. It was evident from the data Table 1 that there was a significant variation in extracellular polysaccharide production among strains irrespective of carbon source. However, irrespective of the strains, the highest amount of polysaccharide was produced in case of mannitol as compared to glucose as carbon source. The strain RhiU3415 produced maximum amount of polysaccharide (286.19 mg l^{-1}) in mannitol, whereas the lowest amount of polysaccharide (177.44 mg l^{-1}) was formed by Rhi KU40. In glucose maximum polysaccharide i.e. 270.86 mg l^{-1} was produced by strain Rhi U3415 and the minimum amount was produced by Rhi U 3516 (141.63 mg l^{-1}). Maximum amount of polysaccharide (278.53 mg litre^{-1}) was formed by the strain Rhi KU3415 considering the mean of the amounts produced in media of different carbon sources. The highest amount of polysaccharide 286.19 mg l^{-1} was produced by the strain Rhi KU3415 in Mannitol as carbon source, whereas the lowest amount of polysaccharide (177.44 mg l^{-1}) was formed by Rhi KU40 in glucose as carbon source. Perusal of data revealed that amounts of polysaccharides produced were significantly different in all strains over control.

The low amount of polysaccharide production may be attributed to the more effective utilization of carbohydrates by those strains due to fast growing. The variation in the amount of polysaccharides formed in different carbon sources indicated that black gram rhizobia examined in this study were able to utilize glucose most effectively followed by mannitol as the carbon source. EPS production by isolated *Rhizobium* species not only beneficial to the host plant and the symbiont, but may also important in N fixing bacteria to resist oxygen diffusion throughout the nodule cells as the nitrogenase enzyme responsible for N fixation is extremely oxygen sensitive (Mukharjee *et al*., 2010). *Rhizobium* strains were able to utilize glucose and sucrose more efficiently than norma lYEM medium (Kucuk *et al*., 2006) [14].

Table 1: Production of extracellular polysaccharide by acid tolerant blackgram rhizobial isolates under different carbon sources

Sl. No.	Isolates	Amount of crude polysaccharides (mg l^{-1})		
		Mannitol	Glucose	Mean
1	U-10-B	184.52	168.23	176.38
2	Rhi KU13	240.19	228.67	234.43
3	Rhi KU20	225.30	214.33	219.82
4	Rhi KU34	207.41	184.01	195.71
5	Rhi KU40	177.44	148.33	162.89
6	Rhi KU50	260.12	238.41	249.26
7	Rhi KU187	275.33	258.47	266.90
8	Rhi U137	191.67	182.77	187.22
9	Rhi U1511	214.36	192.45	203.41
10	Rhi U3415	286.19	270.86	278.53
11	Rhi U3441	272.35	255.49	263.92
12	Rhi U3516	182.33	141.63	161.5
13	Rhi UKh2	214.33	196.76	205.55
Mean		224.73	206.19	-
S.Em(±)		2.076	1.902	-
CD(P=0.05)		6.036	5.529	-

Nitrate Reduction test

Nitrate reduction by the various promising acid tolerant isolates was observed by adding reagents sulfanilic acid and alpha-naphthylamine after inoculation and incubation of cultures in nitrate broth for 24 to 48 hours. As the nitrite accumulation occurs in the growth medium, the colour of the medium changed from creamy to red colour. The results are presented in Table 2.The data indicated all isolates showed a positive nitrate reduction test that the accumulation of nitrite continuously after growth of rhizobial isolates in nitrate broth leading to a color change to red. (Cappucino and Sherman, 1992) [5]. If the organism has reduced nitrate to nitrite, when sulfanilic acid is added, it will react with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction. The accumulation of nitrite increased continuously up to 120 hours and then started decreasing till the end of 144 hours of

observation (Table 2). Amount of nitrite accumulation (8.08 mM) was observed as maximum with the strain Rhi KU34 and the minimum amount (5.12 mM) was accumulated by the strain Rhi KU13when growth was occurred for 120 hours.

The initial increase in nitrite level in the growth medium up to certain extent and then decrease might be due to the inability of the rhizobial strains to metabolize nitrite in presence of high concentrations of nitrate. Similar observation of inability of rhizobia to utilize nitrite in the presence of nitrate or ammonia was reported earlier in *R. trifolii* strains (Pant and Gangwar 1984). Nitrate reduction test is used for the differentiation among isolates on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3) to nitrite (NO_2^-).There are several publications demonstrating that enhancement of N_2 fixation is due to the presence of active NR in bacteroids. (Chamber-Perez *et al.*, 1997; Serrano & Chamber, 1990) [6, 23].

Table 2: Reduction of nitrate by the promising black gram rhizobial isolates expressed as mM nitrite accumulated per 10 mM nitrate at different time intervals.

Sl. No.	Black gram rhizobial isolates	Nitrate reductase test	Nitrite accumulation (mM/NO2)				
			Time intervals (hours)				
			72	96	120	144	mean
1	U-10-B	+ve	3.12	3.87	5. 34	4.87	4.87
2	Rhi KU13	+ve	3.06	4.21	5.12	4.67	4.67
3	Rhi KU20	+ve	3.23	4.33	6.02	5.84	5.84
4	Rhi KU34	+ve	3.65	6.12	8.08	7.77	7.77
5	Rhi KU40	+ve	3.64	5.87	7.87	7.63	7.63
6	Rhi KU50	+ve	3.13	5.12	6.34	6.12	6.12
7	Rhi KU187	+ve	3.16	5.06	7.13	6.75	6.75
8	Rhi U137	+ve	3.56	6.11	7.43	7.01	7.01
9	Rhi U1511	+ve	3.42	5.89	7.24	6.82	6.82
10	Rhi U3415	+ve	3.12	5.78	7.04	6.63	6.63
11	Rhi U3441	+ve	3.24	6.54	7.82	7.21	7.21
12	Rhi U3516	+ve	3.65	6.74	7.94	7.54	7.54
13	Rhi UKh2	+ve	3.44	6.12	7.65	7.43	7.43
	S.Em ±	-	-	-	0.103	-	-
	CD (*P* = 0.05)	-	-	-	0.310	-	-

Nitrogen fixation efficiency of the test black gram-*Rhizobium* isolates: The relative effectiveness of the isolates to fix atmospheric nitrogen was assessed indirectly by computing the efficiency ratio (Table 3) of the isolates at 60 days of plant growth. The efficiency ratio was calculated as the ratio of N-uptake by shoot in inoculated to N uptake by shoot in uninoculated control plant at harvest (Pant and Katiyar, 1994; Patra *et al*., 2010) [20, 21]. On the basis of efficiency ratio, it was observed that some AT isolates performed consistently better than other isolates in field under acidic soil condition. (Table 3).

During field condition the isolate, Rhi U3516 registered the highest efficiency ratio which was followed by Rhi KU34 and Rhi KU40 while Rhi U3415 while least efficiency was found with respect to performance in acid soil. However some strains consistently better (Rhi U3516, Rhi KU34 and Rhi KU40) in the field experiment and some strains consistently perform Rhi KU187 and Rhi U3415 least. The strains were classified as most efficient, efficient, moderately efficient and less efficient provided the per cent increase in N uptake by inoculated plant over control was more than 60 per cent, within 50 to 60 per cent, within 30 to 50 per cent and less than 30 per cent respectively (Pant and Katiyar, 1994; Patra *et al*., 2010,) [20, 21]. Similar type of efficiency classes as per N fixation efficiency of soyabean Rhizobium strains were carried out by Patra *et al*. (2010) [21]. In other words, the strains with efficiency ratio more than 1.60, within 1.50 to 1.60, within 1.30 to 1.50 and less than 1.30 were classified as most efficient, efficient, moderately efficient and less efficient respectively. Hence in the present investigation on the basis of efficiency ratio during field study, the strains U-10–B, Rhi KU34, Rhi KU40, Rhi U137 and Rhi U3516were found to be most efficient (Comparable to standard strain U- 10-B) whereas the strains Rhi KU20, Rhi KU50, Rhi U1511 and Rhi UKh2 were efficient, the strains Rhi U13, Rhi U187, Rhi U3415 and Rhi U3441 were moderately efficient and no strain was less efficient. However the isolate Rhi U3516 performed best and followed by the isolates Rhi KU34 and Rhi KU40 on growth and yield of black gram in acid soil condition. Patra *et al*. (2010) [21] also showed the N uptake by shoot and grain of soybean were increased with inoculation of effective strains as compared to others and stressed the validity of these parameters to be used as indicator of N fixation efficiency of the inoculated strain.

Table 3: Response of black gram to inoculation with promising *Rhizobium* isolates in acid soil: Effect on N uptake by shoot and N fixing efficiency ratio of strains *

	Strains	N- uptake by shoot (mg plant^{-1})	Efficiency ratio of strains*
T_1	Control	46.91	-
T_2	U-10-B	77.73	1.657
T_3	Rhi KU13	67.83	1.446
T_4	Rhi KU20	73.68	1.571
T_5	Rhi KU34	81.44	1.736
T_6	Rhi KU40	78.94	1.683
T_7	Rhi KU50	70.56	1.504
T_8	Rhi KU187	64.18	1.368
T_9	Rhi U137	76.37	1.628
T_{10}	Rhi U1511	71.45	1.523
T_{11}	Rhi U3415	64.15	1.367
T_{12}	Rhi U3441	66.44	1.416
T_{13}	Rhi U3516	81.97	1.747
T_{14}	Rhi UKh2	74.31	1.584
	Mean	71.14	
	S.Em.±	1.062	
	C.D.(P=0.05)	3.087	

$$\text{* Efficiency ratio} = \frac{\text{N-uptake by inoculated plant}}{\text{N-uptake by uninoculated plant}}$$

Table 4: Correlation of physiological properties of promising *Rhizobium* isolates with efficiency ratio.

Sl. No.	Physiological properties	Correlation coefficients (r)
1.	Nitrite accumulation at the end of 120 hours of growth	0.714*
3.	Polysaccharide production in 120 hrs with mannitol as carbon source	-0.809*
4.	Polysaccharide production in 120 hrs with glucose as carbon source	-0.792*

* Significant at 5% probability.

Production of polysaccharides by the strains in different carbon sources was not positively correlated with efficiency ratio. Whereas the efficiency ratios of the strains at harvest showed a significantly positive correlation with nitrate reduction by AT strains measured as nitrite accumulation in broth at the end of 120 hours of growth (Table 4). A positive correlation between the nitrogen content in shoots and NR activity is in agreement with the suggestion that NR activity is somehow correlated with the nitrogen metabolism of the whole plant.(Luciñski *et al*, 2002).

It has been demonstrated that bacteroids could utilize ATP originating from nitrate respiration to fix nitrogen (O'Hara & Daniel, 1985) [18]. Such an adaptation would be especially profitable to bacteroids at times of stress condition of the root zone (Serraj *et al.*, 1999) [22]. It was also reported that nitrate reductases and nitrogenase share a Fe-Mo cofactor in the bacteroids (Evans and Russell, 1971) [8] and hence a positive correlation between N_2 fixation efficiency and nitrate reduction was possible.

Conclusion

The ability of Blackgram rhizobia to reduce nitrate may be taken as a test to screen effective strains whereas the differential degree of exo-polysaccharide production by the strains which was not significantly correlated with efficiency ratio could be helpful in characterization of different strains to study the diversity among blackgram rhizobia.

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