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Exploring the Possibilities of Using *Bradyrhizobium japonicum* as a Nitrogen Fixing Bioresource in Soybean Cultivation in *Purna*-river Basin

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ABSTRACT

The arid and rainfed black cotton to alluvial soils of *Purna* river basin from *Varhad* region of Maharashtra were screened primarily for the presence of root nodulating nitrogen fixing bacteria in the roots of soybean [*Glycine max* (L.) Merrill]. The selected isolates were further subjected for secondary screening using cultural, microscopic and biochemical characterization. This revealed the roots were infected by bacteroids that developed colonies over YEMA and got discriminated from its common contaminants. The growth on differential medium differentiated it into slow and fast growers. The growth at various physical parameters like temperature and pH gave the tolerance to these bacteria at different levels. The standard biochemical characterization of these isolates leads to confirm that the selected isolates belonged to the species of *Bradyrhizobium japonicum* that can be further studied for its nitrogen fixing efficiency to derive its bioresource potential to be used as a nitrogenous biofertilizer in the commercial soybean cultivation of western *Vidarbha*.

Keywords: Soybean, *Glycine max*, *Bradyrhizobium japonicum*, bioresource, bioinoculants, *Purna* river basin.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the most important grain Legume crops in the world. Maharashtra, Madhya Pradesh and Rajasthan are the main soybean producing states of India. Soybean possess high nutritional value as it contains almost 40–42% proteins and 18–22% cholesterol free oil comprising of 85 % Unsaturated fatty acid (Meghvansi *et al*., 2010). Because of 40-45% protein contents in its seeds, soybean requires high nitrogen supply (Jaiswal *et al*., 2016). Soybean's roots act as a host for the nitrogen fixing bacteria like *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*; those can obtain more than 50 % of their nitrogen needs through biological nitrogen fixation (Leggett *et al*., 2017). Symbiotic nitrogen fixation (SNF) resulting from a mutual beneficial interaction between soybean roots (macro-symbiont) and bacteria (*Bradyrhizobia*, micro-symbiont) provides about 60-70% of total nitrogen requirement of the plant (Jaiswal *et al*., 2016). Biological nitrogen fixation in soybean roots improves the soil health and fertility through enrichment of soil nitrogen and reducing the soil C/N-ratio besides it also ameliorates the productivity of other crops (Jaybhaye, 2016). Soybean fixes about 49-130 kg/ha nitrogen and saves about 15-40% chemical fertilizer (Dinkwar *et al*., 2020, Ciampitti and Salvagiotti, 2018).

In agriculture, perhaps 80% of the biologically fixed nitrogen comes from symbiosis between leguminous plants and bacteria of family *Rhizobiaceae*. The family *Rhizobiaceae* currently involves six genera: *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium* and *Bradyrhizobium* which are collectively referred to as *Rhizobia* (Datta *et al*., 2015). Members of the genus *Rhizobium* are known for their ability to develop symbiotic Association with leguminous plants. They elicit

the formation of specialized organs called nodules, where bacteria can fix nitrogen to ammonia and make it available for the plants (Niste *et al*., 2015).

Genus *Bradyrhizobium* with a single species, *B. japonicum* was proposed as symbiont of soybean (Jordan, 1982). It is a dominant inhabitant of soybean root nodules. The association between Rhizobia and soybean is symbiotic, where the plant provides energy, carbohydrates and micro-nutrients to the bacteria and in turn bacteria use that energy to capture atmospheric nitrogen and make it available to the plant.

By knowing the importance of these bacteria in soybean, this study is aimed to isolate *Bradyrhizobia* from soybean root nodules from the black cotton/clay soils of *Varhad* (western *Vidarbha*) region of Maharashtra State of India and characterize and identify the different isolates of *Bradyrhizobium* on the basis of cultural, morphological and biochemical characteristics and provide the cultures in hand for its subsequent studies for the use as nitrogenous biofertilizers during *kharif* season of soybean cultivation to improve yield, productivity, fertility and sustainability.

MATERIALS AND METHODS

Soybean plants from *kharif* season were uprooted carefully so that the intact roots can be obtained in the month of July 2019 from various locations in the *Varhad* region (Western *Vidarbha*, *Amravati*, *Akola* and *Buldhana* District from *Purna* river basin). The roots with healthy pinkish nodules were collected and preserved at lower temperatures of 4-7 ^{o}C, transported and processed further.

Soybean nodules were cut by knife from roots and maintained under saline. Pinkish, healthy and unbroken root nodules were selected, surface sterilised by a disinfectant 0.1 % (w/v) $HgCl_{2(aq)}$ for 4-5 min and subsequent treatment with 70 % (v/v) ethanol and then, thoroughly washed with sterile distilled water to remove the traces of disinfectants from the samples to avoid it's deleterious effect on Rhizobia. These nodules were crushed in saline (0.85 % (w/v) NaCl) to obtain rhizobial suspension for further isolation of root nodulating rhizobia (Vincent, 1970). Streak plate method was employed to isolate the nitrogen fixing and root nodulating bacteria on Congo red yeast extract mannitol agar (YEMA) with composition [Aqueous Media composition in % (w/v): Yeast extract 0.1, Mannitol 1, K_2HPO_4 0.05, $MgSO_4$ 0.02, NaCl 0.01, Congo red 0.0025, Agar 1.5, pH 6.8+0.2] and incubated at 28+2 ^{o}C for 2-7 days. After incubation, single well isolated colonies were selected and sub-cultured on YEMA for pure culture (Gachande and Khansole, 2011). These cultures were maintained as stock cultures in YEMA slants, preserved at lower temperatures 4-7 ^{o}C in refrigerators and sub-cultured with a period of 3-4 months for subsequent maintenance of stock.

These isolates were further characterized for identification and confirmation of those as a Bradyrhizobia. Cultural and morphological characterisation was done by obtaining colonies by spread plate technique by spreading an aliquot of 0.1 ml of bacterial suspension on YEMA (30^{o}C, 48h). The colony characteristics were recorded as shape, size, diameter, transparency, colour, consistency, mucous production and compared with standards of Bergey's Manual of Determinative Bacteriology (Half *et al*., 1994). The morphological traits of individual isolated cells were observed by compound light microscopy using micrometry, simple, Gram's, flagellar, spore, capsular staining and motility techniques (Salle, 1954).

The selection of nitrogen fixing symbiotic bacteria was done by differentiating the bacteria of interest with other undesired properties that were shown by the probable common contaminant *Agrobacterium* species while selecting Rhizobacteria from Congo red YEMA plates depending upon colony characteristics and acquiring the colour of the dye Congo red by both the organism of interest and disinterest (Maruekarajtinplenga *et al*., 2012, Kaur *et al*., 2012).

For obtaining the slow growing cultures to further study, utilise and exploit in agriculture and discriminating it from fast growing cultures; the cultures were streaked on BTB agar [YEMA without addition of Congo red, supplemented with 0.25% v/v bromo-thymol blue dye (0.5 % (v/v) ethanol stock)] plates for 28 ± 2 °C for 2-7 days and obtaining results of slow-growing isolates giving blue colour reaction whereas fast-growing isolates giving yellow colour Reaction (Somasegaran and Hoben, 1994).

The climatic temperature variation occurs in the region of the study. Equally, the soil pH varies depending upon location, natural and anthropogenic inputs from neutral to alkaline. For testing the feasibility of the isolates at varying temperature and pH, effect of these physical parameters were checked by growing isolates on YEMA and YEM-Broth at different temperatures ranging from 10°C to 40°C with an interval of 5°C and cultivating these isolates at different pH of YEMA and YEMB of 2, 3, 4, 5, 6, 7, 8 and 9 and incubating all for 72 h (Pawar *et al*., 2014).

The other biochemical tests were performed by following standard methods and results were recorded. Thus, catalase test (loopful of bacterial suspension dipped into 10 % (v/v) H_2O_2 to find effervescence, Graham and parker, 1964), citrate utilization test (citrate as a carbon source by replacing mannitol with sodium citrate and adding bromo-thymol blue dye and incubation at 30°C

for 48 h; Koser, 1923), gelatine hydrolysis test (gelatin liquefaction by using 1.5% (w/v) gelatin in nutrient broth; Sadowsky *et al*., 1983), H_2S test (stabbing the bacterial suspensions in YEMA-stabs containing methionine or cysteine for testing the reduction of sulphur containing amino acids and production of H_2S gas, Zobell and Feltham, 1934), urea hydrolysis (urea broth containing phenol red as a pH indicator to observe transformation of colour from pale yellow to an intense red-violet, Christensen, 1946), standard MRVP (methyl red and Voges-Proskauer) test, String test (Potassium hydroxide solubility checked by mixing fresh loopful bacterial suspension into two drops of 3 % (w/v) $KOH_{(aq)}$ on a glass slide for few seconds and lifting loop up in the air upto 1 to 2 cm high for checking thread formation, Suslow *et al*., 1982), starch hydrolysis (Starch agar plates inoculated with 5-7 days old culture and incubated in dark at 28ºC for 4 days and flooding the incubated growth on plates with iodine solution for checking the zone of amylolytic activity around growth, Oliveira *et al*., 2007), oxidase test (rubbing a fresh growth from solid culture medium and rubbing it on oxidase disk containing N, N-dimethyl-p-phenylenediamine oxalate and α-naphthol to check the blue colour for the presence of cytochrome oxidase enzyme production, Kovaks, 1956) and ammonia production (growing isolates into peptone broth and incubation at 30 ºC for 48 to 72 h and then, Nesselerization is done to check brown to yellow colour formation, Cappuccino and Sherman, 1992) were performed and results were recorded.

RESULTS AND DISCUSSIONS

Thousands of isolates showing majorly two types of colony characteristics were found on Congo red YEMA, out of which red coloured, non-mucoid, rough colonies were ignored considering those of common contaminant *Agrobacterium* sp. and from the hyaline mucoid colonies obtained from 10^{-2} dilution level of natural nodular bacteroidal inoculation, few rapidly growing were selected.

A rigorous study of the hundreds of isolated colonies from Congo red YEMA plates to get and select the standard characteristics of *Bradyrhizobium* in accordance with Bergey's Manual an extensive comparison was done and the isolates that do not fall in the genus *Bradyrhizobium* were discarded. The isolates that showed the exact characteristics to that of standards were selected and finally ten isolates were kept for further studies.

The selected isolates showed the cultural and morphological characteristics of *Bradyrhizobium* species that are charted in Table 1. The cultural traits of these isolates showed circular, whitish pink coloured, translucent, gummy, glistening, smooth colonies with entire, convex elevation, mucoid consistency and size ranging between 2-4mm (Fig 1). The microscopic characteristic of these isolates revealed them as singular, non-spore forming Gram-negative bacilli (rod-shaped), motile with a single subpolar or polar flagellum producing slow growth on yeast extract-mannitol agar, slime producing capsular rods. These were in accordance with the earlier findings of Gachande and Khansole, 2011 and Datta *et al*., 2015.

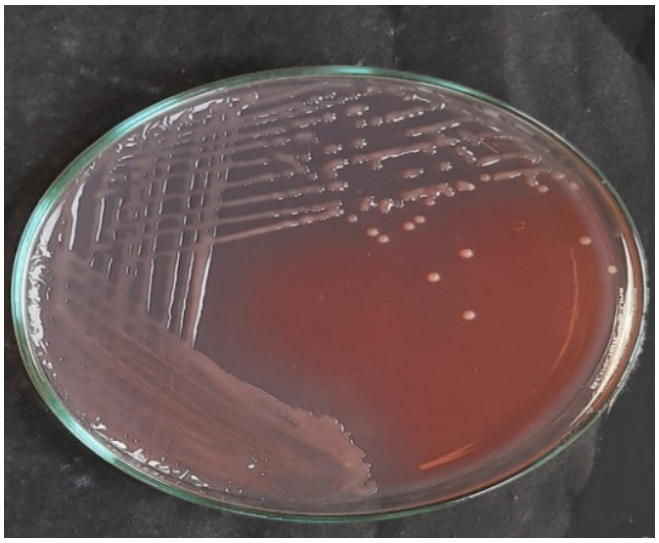

Fig 1: Colonies of *B. japonicum* on CR-YEMA media

Table 1: Cultural and morphological characteristics of *B. japonicum*

Sr. No.	Strains characters	Standards (Jordan, 1982)	Observations
1.	Colony shape	Circular	Circular
2.	Colony size	2-4 mm	2-4 mm
3.	Margin	Entire	Entire
4.	Elevation	Convex	Convex
5.	Colour	Whitish pink	Whitish pink
6.	Opacity	Opaque	Opaque/ Slightly Translucent
7.	Motility	Motile	Motile
8.	Texture	Smooth	Smooth
9.	Spore formation	Non-Spore forming	Non-Spore forming
10.	Gram's nature	Gram negative	Gram negative
11.	Shape of bacteria	Rod shape	Rod shape
12.	Cell Arrangement	Single	Single

From the observations of the growth, it was revealed that the inoculated bacteroids from crushed root nodules utilised the sugar mannitol from the culture media YEMA as a carbon and energy source, used gaseous nitrogen from head space air as a nitrogen requirement and grew in the form of distinct surface growth that was further separated in the form of isolated colonies (Gachande and Khansole, 2011). The rest of the contaminants even if present, probably unable to utilise mannitol as its carbon and energy source as well as gaseous nitrogen from air as its nitrogen source, thus getting retarded for its growth except a common contaminant with non-mucoid rough colonies that could be clearly discriminated due to the dye Congo red added to YEMA. These probably might be the colonies of *Agrobacterium* species (Maruekarajtinplenga *et al*., 2012, Kaur *et al*., 2012) as the colonies of *Bradyrhizobium* do not accumulate the colour of the dye Congo red, while that of the above common contaminant acquires the dye.

The results of growing the isolates on YEMA with BTB revealed their fast growth on solid surface medium. This enabled to discriminate between fast and slow growing Rhizobia. Fast growing isolates were acid producers from mannitol which dropped the pH of medium to acidity almost below 6.0 turning green colour to yellow of the medium and colonies; while slow growers turn the pH of the medium alkaline almost above 7.6 turning the colour of the colonies and surrounding to blue (Fig 2) (Saeki *et al.,* 2005; Sharma *et al*., 2010). Out of the ten isolates, seven were found slow growing and three were found fast growing. The slow growing rhizobia are appreciated as a potential inoculants for the external use in soybean cultivation where the increase in population, metabolic and physiological rates of rhizobia should be slower and in tune with the growth of soybean roots so that those can tolerate the neutral to alkaline soil pH of black cotton to alluvial soils and subsequently approach the roots to infect and nodulate the soybean allorhizic roots. Naturally, 80 % among all rhizobia in soybean root systems are slow growing *Bradyrhizobium* sp., while only 20 % of the strains are fast growing as per the findings of Dinkwar *et al*., 2020. Therefore, this test enabled to discriminate between *Bradyrhizobium* and *Rhizobium* species and select the slow growing maximally occurring *Bradyrhizobium* species isolates.

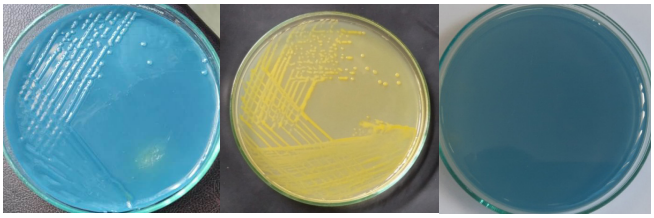

Fig 2: Differentiation of slow growing *Bradyrhizobium* and fast growing *Rhizobium* against control plate.

Table 2: Differentiation of fast and slow growing rhizobia on YEMA (BTB) medium.

Isolates	Colour Reaction on BTB agar	Fast/Slow grower
BJ-1	Blue	Slow
BJ-2	Blue	Slow
BJ-3	Blue	slow
RJ-4	Yellow	Fast
BJ-5	Blue	Slow
RJ-6	Yellow	Fast
BJ-7	Blue	Slow
RJ-8	Yellow	Fast
BJ-9	Blue	Slow
BJ-10	Blue	Slow

The region under study is vulnerable to drastic climate change that leads to the alteration in ambient temperature varying vastly during winter and summer. Therefore, the effect of temperature was checked at various levels by incubating the growth of isolates on agar as well as broth at temperature ranging from 20 to 50 °C with an interval of 5 °C and also at 28 °C. These isolates showed luxuriant growth on plates and in broth in the form of turbidity at 30°C (Table 3). Among these isolates, fast growing showed the tolerance to higher temperatures after 35 °C that was extended to 40 °C, after which fast growers showed a poor vegetative growth, possibly turning to dormant stages (Dinkwar *et al*., 2020). These slow growing isolates showed tolerance merely upto 35 °C after which it showed no growth revealing intolerance to maximal temperatures of mesophilic range. During *kharif* season of soybean cultivation, the ambient soil temperature ranges from 25 to 35 °C that looked favourable for the proliferation of *Bradyrhizobia* in the soil and root system of soybeans. This revealed the possibility of depletion of soil *Bradyrhizobial* population during the subsequent seasons of *rabi* and specifically summer in rainfed areas where the temperature rises beyond 45 °C.

Table 3: Tolerance of *Bradyrhizobium* isolates varying temperatures.

Isolates	Growth in the form of colonies on YEMA at various temperature (°C)								Growth in the form of turbidity in YEMB at various temperature (°C)							
	20	25	28	30	35	40	45	50	20	25	28	30	35	40	45	50
BJ-1	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-
BJ-2	-	+	++	++	+	+	-	-	-	+	++	++	+	+	-	-

BJ-3	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-
RJ-4	-	+	+	+	++	+	+	-	-	+	+	+	++	+	+	-
BJ-5	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-
RJ-6	-	+	+	++	++	+	-	-	-	+	+	++	++	+	-	-
BJ-7	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-
RJ-8	-	+	+	+	++	+	-	-	-	+	+	+	++	+	-	-
BJ-9	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-
BJ-10	-	+	++	++	+	-	-	-	-	+	++	++	+	-	-	-

(++): High growth, (+): Moderate growth, (-): No growth.

The *Varhad* region is maximally and typically rainfed and the soils are affected by anthropogenic and indiscriminate application of chemical fertilizers that resulted into variation in soil pH ranging from slightly alkaline to neutral. Soybean favours neutral soil pH, and thus, the beneficial rhizospheric microflora also flourishes well into neutral soils. For deriving appropriate pH for maximal growth of isolates, pH tolerance and favourability was checked by growing these isolates on solid and liquid culture media of adjusted pH to 2, 3, 4, 5, 6, 7, 8 and 9 (Table 4). All the isolates preferred maximal growth at pH 7. After the pH 8, tolerance was observed and at pH 9, growth was restricted. At pH 5, tolerance was observed and at pH 4, minimal growth was observed. No growth was seen at pH 2. This was in accordance with the findings of Dinkwar *et al*. (2020) and Sing *et al*. (2011).

Table 4: Tolerance of *Bradyrhizobium* isolates varying pH.

Isolates	Growth in the form of colonies on YEMA adjusted with various pH							Growth in the form of turbidity in YEMB adjusted with various pH						
	2	4	5	6	7	8	9	2	4	5	6	7	8	9
BJ-1	-	-	-	++	++	+	-	-	-	-	++	++	+	-
BJ-2	-	-	-	++	++	+	-	-	-	-	++	++	+	-
BJ-3	-	-	-	++	++	+	-	-	-	-	++	++	+	-
RJ-4	-	-	+	++	++	-	-	-	-	+	++	++	-	-
BJ-5	-	-	+	++	++	+	-	-	-	+	++	++	+	-
RJ-6	-	-	+	++	++	-	-	-	-	+	++	++	-	-
BJ-7	-	-	-	++	++	+	-	-	-	-	++	++	+	-
RJ-8	-	-	+	++	++	-	-	-	-	+	++	++	-	-
BJ-9	-	-	-	++	++	-	-	-	-	-	++	++	-	-
BJ-10	-	-	-	++	++	-	-	-	-	-	++	++	-	-

(++): High growth, (+): Moderate growth, (-): No growth.

These isolates showed negative methyl red test and hence, were unable to perform mixed acid fermentation and could not produce stable acid end products that can change the colour to red of the dye methyl red. These isolates also showed negative *Voges Proskauer* test revealing that those were unable to produce acetyl methyl carbinol from glucose fermentation. The gelatin medium remained solidified after inoculation of these isolates confirming that those were unable to produce extracellular gelatinase enzyme. These isolates were unable to split amino acid tryptophan to form the compound indole as it was evident from negative indole test. This (Table 5) was in accordance with previous findings of Graham and Parker (1964) and Dinkwar *et al*. (2020).

Table 5: Biochemical Characterization of *Bradyrhizobium japonicum*

Test	Strain of Bradyrhizobia									
	BJ-1	BJ-2	BJ-3	RJ-4	BJ-5	RJ-6	BJ-7	RJ-8	BJ-9	BJ-10
Catalase	+	+	+	+	+	+	+	+	+	+
Citrate Utilisation	+	+	+	+	+	+	+	+	+	+
Gelatine Hydrolysis	-	-	-	-	-	-	-	-	-	-
H2S test	+	+	+	+	+	+	+	+	+	+

Urease test	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-
KOH solubility test	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-	-	-
Ammonia production	+	+	+	+	+	+	+	+	+	+

(+): Positive test, (-): Negative test.

These isolates tentatively soybean rhizobia showed positive results for oxidase (confirming the ability of these isolates to produce enzyme oxidase, thus further revealing that these isolates are aerobic in nature), urease (confirming the ability of these isolates to produce enzyme urease, thus further revealing that these isolates can split urea into carbon dioxide and ammonia), citrate utilisation (confirming that these bacteria were able to utilize citrate as its sole carbon and energy source), catalase (confirming the ability of these isolates to produce enzyme catalase, thus further revealing these isolates as aerobic in nature), H_2S production, starch hydrolysis (confirming that these bacteria were able to produce extracellular amylases) and ammonia production from peptone (confirming that these bacteria can utilize peptone and can produce ammonia). These findings (Table 5) were matched with Gachande and Khansole (2011), Kaur *et al*., (2012), as reported for soybean Rhizobia.

CONCLUSIONS

The isolates were screened from soybean root nodules and were slow-growing, non-acid producing, Gram negative, aerobic, non-sporulating and short rods 0.5 to 0.9 µm x 1.2 to 3.0 µm in size, motile by singular, polar or sub-polar flagellum. Colonies did not exceed 2mm in diameter within 5 to 7 days at the optimal temperature of 25 to 30 °C. The colonies were circular, opaque to rarely translucent, white and convex. Growth on carbohydrate media was usually accompanied by extracellular slime. These characteristics concluded that the isolates were of *Bradyrhizobium japonicum* from root nodules of soybean [*Glycine max* (L.) Merrill] from the western *Vidarbha* region and have a potential of serving as a bioresource for its exploitation as nitrogen fixing biofertilizers.

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