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Studies on cultural, morphological and biochemical characterization of *Bradyrhizobium japonicum* of soybean

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Abstract
Soybean (*Glycine max* L. Merrill), being a leguminous crop, can be fix atmospheric nitrogen through symbiotic association with effective and competitive strains of Rhizobia (*Bradyrhizobium japonicum/Rhizobium japonicum*) and thereby improve soil fertility as well as productivity of subsequent cereal crops. *Bradyrhizobium japonicum* was isolated from root nodules of Soybean (*Glycine max* L.) on Congo-Red Yeast Extract Mannitol Agar (CR-YEMA) medium and its morphological, cultural and biochemical-characteristics were studied. The test bacterium was circular, convex, glistening, whitish pink colonies (0-4 mm dia.) with entire margin. The bacterium was Gram-negative, non-spore forming and motile rods and showed positive reactions to Catalase oxidation, Potassium hydroxide test, and Starch hydrolysis tests.

Keywords: Soybean, *Bradyrhizobium japonicum*

Introduction
Soybean (*Glycine max* (L.) Merrill) is one of the commercially important grain legumes extensively grown in the world, under varying soil types and climatic conditions. Soybean has contributed significantly to "Yellow revolution" in India and as a pulse-cum-oilseed crop forms an integral part of daily diet of Indian peoples. India ranks 5th in soybean production in the world. In India, during 2015-16, the area, production and productivity of soybean were 11.09 million ha, 7.13 million metric tonnes and 6.1 tonnes/ha, respectively (Annonymous, 2015). Soybean producing major states in India are Madhya Pradesh, Maharashtra, Rajasthan, Andhra Pradesh, Uttar Pradesh, Punjab, Tamil Nadu, Uttarakhand, Gujrat, Karnataka, and Chattisgarh (Appunu *et al.*, 2015) [4]. The family Rhizobiaceae, currently includes the genera *viz.*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium* etc., which are collectively referred as Rhizobia (Kumar and Raghuram, 2016) [9]. Soybean, being a leguminous crop, can be fix atmospheric nitrogen through symbiotic association with effective and competitive strains of Rhizobia (*Bradyrhizobium japonicum/Rhizobium japonicum*) and thereby improve soil fertility as well as productivity of subsequently grown cereal crops. However, symbiotic association between *Rhizobium*-legumes, amount of biological nitrogen fixation, plant growth and thereby crop yield potential are generally affected due to adverse environmental conditions such as drought stress, salt stress, acidity/alkalinity, nutrients deficiency, heavy metals and various pesticides (fungicides, insecticides, herbicides) used to combat diseases, insect-pests and weeds.

Materials and Methods
Isolation of *Bradyrhizobium japonicum*
Bradyrhizobium japonicum was isolated on selective synthetic culture medium Congo-Red Yeast Extract Mannitol agar (CR-YEMA), from functional root nodules, obtained from field grown soybean plants and pure culture was maintained on YEMA, for further studies.
For the purpose, functional root nodules (healthy, bold, unbroken, pink colored) from the roots of field growing soybean plants were detached carefully by giving 'V' shaped cut with sharp blade/knife, collected in glass petri plates and surface sterilized with either 75% (v/v) ethyl

These nodules were then washed in 3-4 sequential changes of distilled water, blott dried, 5-10 nodules were dispensed in 5 ml distilled water in test tube and crushed with sterile glass rod. The resultant bacterial suspension was streaked with inoculation needle wire loop on solidified CR-YEMA Petri plates and incubated at 28±2 ˚C. After, 48-72 hrs of incubation, well isolated single colonies of *R. japonicum* developed were picked up, streaked on YEMA plates and incubated. The pure culture obtained was maintained on YEMA test tube slants, for further studies.

Morpho-cultural characters of *Bradyrhizobium japonium*

For the purpose, pure culture of *B. japonicum* was streaked on YEMA plates and incubated at 28±2 ˚C, for 3-5 days. The cultural/colony characterstics *viz*. shape, size, colour, elevation, texture, margin etc. were observed at 72-96 hrs. of incubation. Fast growing Rhizobia generally produced white, semi-transparent/opaque, circular, mucilaginous colonies; while, slow growing strains produced white, opaque, circular, granular colonies microscopically, Gram-staining reaction, shape and size were recorded.

Biochemical characteristics

Four most important biochemical tests *viz*. Gram's staining, Catalase oxidation, Potassium hydroxide (KOH) solubility and Starch hydrolysis of *B. japonicum* were attempted by adopting standard procedures (Aneja, 2003; Vishunavat and Kolte, 2005) [3].

Gram's staining

A loop full of the 24-36 hrs. old culture suspension of the test bacterium was smeared on clean glass slide, air dried and fixed by gentle heating on flame of the spirit lamp. Aqueous Crystal violet solution (0.5%) was spread over fixed smear for 30 to 60 seconds and then washed with gentle flow of running tap water, for a minute. This stained smear was later flooded with Gram's iodine solution (%), for one minute and rinsed gently with tap water. Later decolorized with 95% Ethanol solution, until color of crystal violet runoff, then washed with gentle flow of water. Finally stained with Safranin, as a counter stain for about 10 seconds, washed with gentle flow of water, air/blot dried and the slides observed under research microscope (40X).

Catalase oxidation test

A loop full of 24-28 hrs. old culture of the test bacterium was placed on the clean glass slide, to this a drop of 3% Hydrogen peroxide (H_2O_2) was mixed, allowed to react for few minutes and observed for production of gas bubbles.

Potassium hydroxide (KOH) test

A drop of 3% Potassium hydroxide was placed on clean glass slide, to this 48 hrs old test bacterial culture was mixed with clean inoculation loop, stirred for 10 second and observed for appearance of slime threads.

Starch hydrolysis test

The autoclaved and cooled Starch agar medium was poured in sterile glass petri plates. On solidification of the medium, pure culture of the test bacterium was streaked on it and incubated for 96 hrs at 28±2^{0}C. Then these plates were flooded with Lugol's iodine and allowed to react for few minutes.

The bacterium *B. japonicum* was isolated from the root nodules of healthy growing soybean plants collected from various fields, on basal culture medium YEMA with Congo-red, by applying streak plate method. After 3-4 days of incubation, whitish translucent colonies of the test bacterium were developed (Figure 1). Through frequent sub-culture, the test bacterium was purified and its pure culture maintained on YEMA test tube slants for further studies.

Characterization of *Bradyrhizobium japonicum*

Morpho-cultural characteristics

The bacterium**,** *Rhizobium japonicum* (Krichner and Buchanan) Syn. *Bradyrhizobium japonicum* grew better on YEMA plates. The colonies developed were circular, convex, glistening, whitish pink, with entire margin and measured about 2-4 mm (Table 1, Figure 1). The bacterium was non-spore forming and motile rod shaped.

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

Sr. No.	Shape	Size (mm)	Gram reaction	Margin	Elevation	Colour
1.	Rod	2-4	Negative	Regular	Convex	Milky white and glistening

Biochemical characters

Different biochemical tests *viz*., Grams staining, catalase oxidation test, KOH (Potassium hydroxide), Starch hydrolysis, etc. were attempted of *B. japonicum* and the results obtained (Figure 2) revealed that the bacterium *B. japonicum* as Gram-negative (Figure 2 a); whereas, it showed positive reactions in respect of Catalase oxidation (Figure 2 b), Potassium hydroxide test (Figure 2 c) and Starch hydrolysis test (Figure 2 d).

These results of present study obtained on morpho-cultural and biochemical characteristics of *B. japonicum* are in conformity with earlier reports of several workers. Singh *et al*. (2008) [6, 12] characterized the *B. japonicum*, isolated from soybean root nodules and reported its colonies as milky white, translucent, circular (2-4 mm diam.), shiny, raised (convex) and sticky with musty odor. The bacterium was Gram-negative rods. Deora and Singhal (2010) [6] reported that the bacterium *Rhizobium* on YEMA medium produced sticky and mucoid colonies, Gram-negative rods and showed positive reactions to starch hydrolysis and negative reaction to methylene blue, lactose and gelatin test. Gachande and Khansole (2011) [8] reported that *Rhizobium/Bradyrhizobium japonicum* produced circular, light pink, convex, opaque colonies with entire margin. The bacterium was Gram-negative rod, aerobic, non-spore forming and motile. It showed positive reactions for citrate utilization, catalase and ammonia production and negative to methyl red and hydrolysis of gelatin test. Rajpoot and Panwar (2013) [11] reported that the bacterium *Rhizobium* spp., isolated from root nodules of *Vigna radiata*, produced white colonies (2-4 mm in diameter), Gram-negative rods, showed positive reactions to Catalase and Urea hydrolysis, and negative reactions to Citrate, Gelatin and Starch hydrolysis.

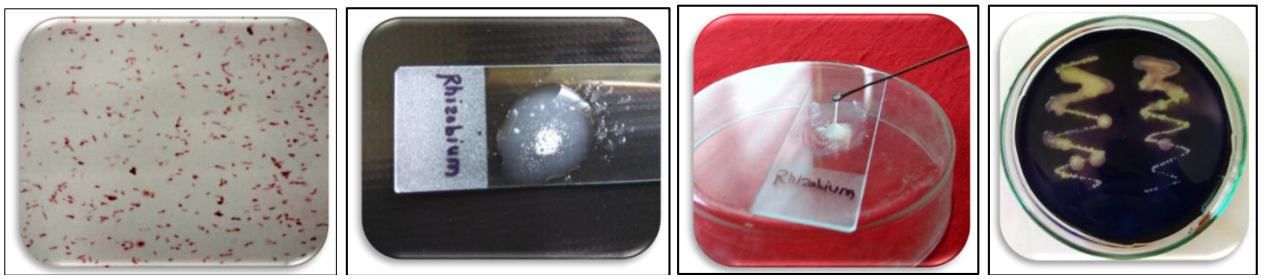
Similar morpho-cultural and biochemical characteristics of *Rhizobium/Bradyrhizobium japonicum* and other *Rhizobium* spp. were reported earlier by many worker. (Amin, 2014; Deshwal and Chaubey, 2014; Deb *et al*., 2015; Nushair 2017) [2, 5, 7, 10].

Results and Discussion

Isolation of *Bradyrhizobium japonicum*

a) Colonies on CR-YEMA b) Slant Culture c) Subculture of *B. japonicum* on YEMA d) Pure culture slant tubes

Fig 1: The bacterium was non-spore forming and motile rod shaped



a) Gram's Staining (40X) b) Catalase oxidation test c) Potassium hydroxide test d) Starch hydrolysis test

Fig 2: These results of present study obtained on morpho-cultural

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