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Phenotypic characterization of rhizobia from legumes and its application as a bioinoculant

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Symbiosis between leguminous plants and soil bacteria, commonly referred to as rhizobia, is of considerable economic importance, in low input sustainable agriculture, agroforestry and land reclamation, besides being eco-friendly. In the era of development and commercialization of bioinoculant technology for sustainable agriculture development, rhizobial inoculants have been targetted as potential candidate. A Total ten nodulated plants in replica i.e. five of soybean and five of French beans were collected from organic farm of Dehradun, Uttarakhand. Number of nodules present in sample soybean A, B and C were more than French bean. Soyabean showed medium, prominent, white colonies of YEMA whereas French bean showed large white colonies. All isolated *Rhizobium* strains were found to be Gram negative and these were having granules within the cell. All strains of Rhizobium [SbR, ScR, FaR, FbR, FcR] had peritrichous flagellar arrangement while SaR strain of had sub polar flagella. All strains of Rhizobium gave positive congo red and negative test with alkaline broth and lactose agar test. When *Rhizobium* strain isolated from the soyabean was used as bioinoculant, it increased the growth of inoculated plant and the plant appeared much healthier in comparison to the control.

Key words: *Rhizobium,* soyabean, bioinoculant, nodules

Introduction

Rhizobium species are usually defined as nitrogen-fixing soil bacteria capable of inducing the formation of root or stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia for benefit of the plant. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied. During the last decades, the

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assessment of diversity within rhizobial natural population in various regions of the world has received increased attention .The development of numerous molecular genetic methods has been greatly contributed to these investigations.

The availability of several sensitive and accurate PCR-based genotyping method (Jensen *et al.*, 1993) has enabled the differentiation among closely related bacterial strains and detection of a higher rhizobial diversity than previously considered (Dognon-Bourcier *et al.,* 2000). Consequently, the taxonomy of root- and stem nodulating bacteria has been deeply changed in recent years. The rhizobial species are currently classified in following genera, *Allorhizobium, Azorhizobium, Bradyrhizobium, Devosia Rhizobium, Sinorhizobium, and Methylobacterium* belong to α-proteobacteria (Young *et. al.,* 2000; Rivas *et al.,* 2002) whereas *Burkholderia* and *Ralstonia* belong to β-proteobacteria (Chen *et al.*, 2001: Moulin *et al.*, 2001).

As a valuable source of proteins, oil and bioactive molecules of pharmaceutical importance used both for human and livestock nutrition, soybean (*Glycine max* L. (Merill.) is one of the most important legume crops in the world. Therefore, the nitrogen fixing microsymbionts of soybeans are of great agricultural value. Soybean microsymbiont, previously named as *Rhizobium japonicum*, allocated to new genus *Bradyrhizobium* as Bradyrhizobium *japonicum*. Accordingly, the slow growing, alkali producing strains was separated fast-growing growing, acid producing rhizobia. Since then, other nitrogen-fixing bacteria able to nodulate soybean roots like *Bradyrhizobium elkanii* and *Bradyrhizobium liaoningense* (Xu *et al.*, 1995) and *Mesorhizobium tianshanese* (Chen *et al.*, 1995) have also been described. In 1988, the genus *Sinorhizobium* was proposed for fast-growing soybean rhizobia (Chen *et al.,* 1988), with one species named *Sinorhizobium fredii*, Currently, several species of *Bradyrhizobium* and *Sinorhizobium* of soybean are known to nodulate other legumes (Young *et al*., 2001; Yoa *et al.,* 2002). A gram-positive genus *Bacillus*, as PGPR, has also been reported from nodules of soybean. Hence, it is now expected that nodules may be home of lot many bacteria other than rhizobia with multiple functions including nitrogen fixation.

It has been recognised that an important requirement for agronomically useful *Rhizobium*-soybean associations is the ability of inoculant strains to compete with very diverse indigenous rhizobial strains. Therefore, in order to improve the beneficial effect of soybean inoculation it is important to determine the characteristics of rhizobial field populations.

Materials and method

Collection of samples

Total ten nodulated plants in replica i.e. five of soyabean and five of French beans were collected from organic farm of Dehradun, Uttarakhand. Healthy plants were uprooted carefully and those plants possessing healthy nodules with pink colour were selected and brought to the lab in polythene bags. The samples were processed immediately on the same day.

Characterization of nodules by morphological study

Collected samples were washed thoroughly with running tap water. Then number of nodules, colour, size and shape of nodules of each sample were observed keenly. Only healthy and maximum nodulated plants were selected for further study.

Isolation of Rhizobium from selected plants

Healthy, unbroken, firm and pink nodules were selected for the isolation. They were washed with water carefully and isolation of Rhizobium was done on Yeast Extract Mannitol Agar (YEMA). Adhering soil particles were removed by washing root system under running tap water. Then selected nodules were placed in such a manner that piece of root on the side of the nodule was then transferred to 0.1% $HgCl_2$ or 3-5% H_2O_2 for 5 min for surface sterilization. The nodules were repeatedly washed in sterile water for 3-4 min. To get rid of the sterilent. Nodules were then placed in 70% ethyl alcohol for 3 min and were washed with sterile water thoroughly and crushed with sterile glass rod. The resulting suspension was streaked on YEMA medium containing (1% congo-red dye), incubated at 28 +/- 1˚C for 3-10 days (depending upon growth rate). Same protocol was followed for all collected samples and growth on YEMA plate was observed after incubation period.

Maintenance of bacterial isolates

All bacterial strains were streaked in YEMA slants. After the growth of bacterial isolates at 28˚C the slants were preserved at 4˚C in refrigerator and subcultured at monthly intervals in their respective media.

Phenotypic Characterization

Identification of Rhizobium by microscopic method:

Gram staining and carbol fuschin staining were performed for microscopic study of Rhizobium.

Biochemical Test

Following two tests were performed to distinguish Rhizobial strains Growth on NAM plate : Indigenous Rhizobial strain which were isolated from nodulated plants were streaked on Nutrient agar media plates (NAM). Then plates were incubated at 28 +/- 1˚C for 3-4 days and the growth was observed at different intervals of time (Cameron and Sherman, 1935; Graham and Parker, 1964).

Flagellation test: Flagellar arrangement in these indigenous Rhizobial strains were observed by Flagella staining. Smear of Rhizobium culture was prepared. Slide was covered with Flagella mordant "Loffler's mordent" for 10 min. Slide was washed with distilled water. Carbol fuschin was flooded for 5 minutes on the slide and it was washed off with distilled water. Slide was examined under microscope.

Biochemical tests used for differentiation of Rhizobium and Agrobacterium.

Congo Red Test: An aliquot of 2.5 ml of 1% solution of the dye in H_2O was added to a litre of YEMA. On this medium when suspected strains of nodule bacteria were plated, Rhizobia stand out as white, translucent, glistening elevated colonies and comparatively small colonies with entire margins in contrast to stained colonies of Agrobacterium. Isolated bacterial strain were streaked on YEMA plated incubated for 3-5 days at 28 +/- 1˚C.

Hoffer's Alkaline broth Test: This test is based on the fact that Agrobacterium grows at higher pH level whereas Rhizobium unable to do so. A medium i.e. Hoffer's alkaline having high pH of 11.0 was used to screen new isolated nodulated bacteria for this purpose. Bacterial suspension was inoculated in broth and incubated for overnight at 28 +/- 1˚C.

Lactose agar Test: Agrobacteria utilize lactose by the action of enzyme ketolactose whereas Rhizobia cannot utilize the sugar. This can be detected on agar medium containing lactose at 10 g/litre. Lactose agar

was prepared and poured into the sterile plates. Culture of nodulated bacteria was streaked on lactose agar plate and incubated for 4-10 days. After the growth of culture on the plate these were flooded with benedict solution.

Rhizobial strain is used as bioinoculant. The ability of Rhizobium to infect small seeded legume can be tested on nitrogen free media (Jenson modified media). Inoculation of seeds with Rhizobial strain was done to carry out the plant infection test.

Seed sterilization and germination: Healthy seeds of soyaban were taken and sterilized with H_2O_2 for 3 minutes followed by thorough washing with sterilized distilled H_2O at least for 10 times. Then seeds were soaked aseptically in sterile distilled water for overnight at room temperature. Next day seeds were taken out and dried with sterile blotting paper. Then seeds were transferred to the Petri plates which were poured with freshly prepared sterile nutrient agar media and incubated at 28 +/- 1˚C for 24 hours for germination.

Seed inoculation with pure culture of Rhizobium of soyaban: Four slants of modified Jensen media were made. Single seed producing radical in down position was aseptically transferred into slants. So that radicle should be in direct contact of gelled medium.

Then 2-5 drops of log phase culture of Rhizobium isolated from soyaban, were added and kept as uninoculated (control). The base of the tubes were covered with black paper so as to create darkness required for growth of root system. Test tubes were plugged with non-absorbent cotton plug. The germinated seed were exposed to sunlight periodically. Tubes were incubated for 20-30 days and effect on growth in inoculated and uninoculated tubes was observed.

Result

Nodules of each plant viz., soyaban and French bean were morphologically studied shown in Table1. All selected samples streaked on YEMA plates were found white, gummy, translucent, colonies at different incubation time period. Results obtained are given in the table 2. The Rhizobia isolated from Soyabean and French bean were found to be slow grower. And the large colonies were observed in case of Soyabean but medium sized colonies were found in French bean.

Table 1. Morphological study of nodules

S.No.	Sample	No. of nodules (more than 50)	Size and Shape	Colour of nodules
1.	French bean	Less than 50	Round in shape and large size	Black and Brownish
2.	Soyaban	Above 50	Oval and medium size	Pink

Table 2. Colony characteristics of Rhizobia on YEMA

S.No.	Sample	Size of colonies	Colour	Rate of growth
1.	French bean	Medium sized mucoid colonies	White	Slow
2.	Soyaban	Large sized mucoid colonies	White	Slow

Morphotypic characteristics

All selected Rhizobia were stained with Gram staining and carbol fuschin and results obtained by observing the slide under microscopic are shown in table 3. Sa R designated for Rhizobium strain of Soyabean [a], SbR strain of Soyabean [b], ScR strain of Soyabean [c] and French beans were Gram negative, spherical, pleomorphic in shape and had granules within the cell when stained with carbol fuschin.

Table 3. Identification of Rhizobia by Gram's staining and Carbol fuschin stain

Sr.No	Sample	Isolate name	Gram's staining	Carbol fuschin staining
1.	Soyaban A	SaR	Gram-ve, pleomorphic shape	Presence of granules
2.	Soyaban B	SbR	Gram -ve, small rod shape	Presence of granules
3.	Soyaban C	ScR	Gram -ve, small rod shape	Presence of granules
4.	French bean A	FaR	Gram -ve, small rod shape	Presence of granules
5.	French bean B	FbR	Gram -ve, small rod shape	Presence of granules
6.	French bean C	FcR	Gram -ve, small rod shape	Presence of granules

Biochemical tests

All selected rhizobia were characterzed by biochemical test which growth on NAM plate and Flagellation tests are shown in table 4. Rhizobial strain from French bean and soyabean were slow growers. The rhizobial strains [SbR, ScR, FaR, FbR, FcR] had peritrichous flagella while SaR had subpolar flagella.

Table 4. Growth behaviour of Rhizobial strain on Nutreint agar medium and Flagellar arrangement

Sr. No.	Rhizobial strain	Growth Rate	Flagellar arrangement
1.	SaR	Slow	Subpolar
2.	SbR	Slow	Peritrichous
3.	ScR	Slow	Peritrichous
4.	FaR	Slow	Peritrichous
5.	FbR	Slow	Peritrichous
6.	FcR	Slow	Peritrichous

tests for differentiation of Rhizobium and Agrobacterium : results are given in the table 5 . All the isolated strains were positive for Congo-red. No organism showed any growth in Hoffer alkaline broth and lactose agar. On the various morphological and biochemical tests the isolated strains are possibly identified as : SaR-*R.leguminosarium,* Sb-*R.leguminosarium*, ScR -*R.leguminosarium*, FaR -*R.leguminosarium,*FbR – Bradyrhizobium FcR – Bradyrhizobium.

Table 5. Biochemical tests for differentiation of Rhizobiumand Agrobacterium

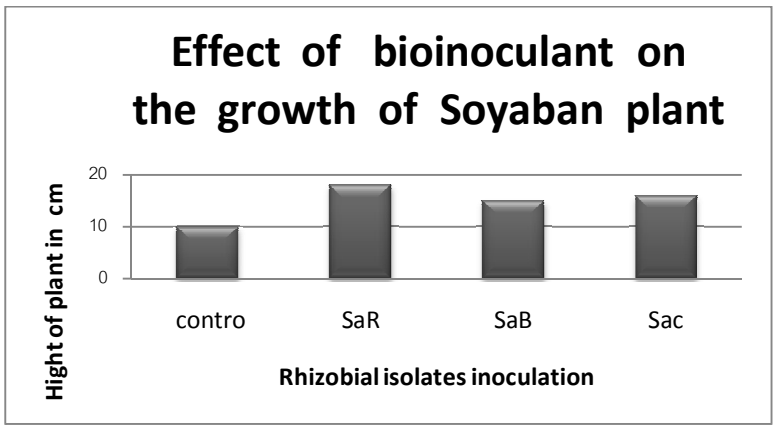
Sr.No.	Isolates names	Congo-red	Hoffer's alkaline broth	Lactose agar	Possible organism
1.	SaR	+ve	No growth	No growth	*R. Leguminosarum*
2.	SbR	+ve	No growth	No growth	*R. Leguminosarum*
3.	ScR	+ve	No growth	No growth	*R. Leguminosarum*
4.	FaR	+ve	No growth	No growth	*R. Leguminosarum*
5.	FbR	+ve	No growth	No growth	Bradyrhizobium
6.	FcR	+ve	No growth	No growth	Bradyrhizobium

Application of strain of rhizobium as bioinoculant for its specific plant

Use of rhizobial strain as bioinoculant for its specific plant was done by plant infection test. The test was performed by using rhizobial strain of Soyaban as bioinoculant for its plant growth. Growth rate was different in test tube a and other three tubes. The effect of bioinoculant was studied on Soyaban plant in a test tube by using specific isolation of that particular plant. As evident from the result shown in table, the inoculated plants showed faster growth as compared to control. The inoculated plants were looking much healthier than uninoculated one.

Number of nodules present in sample Soyabean A, B and C were more than French bean. Soyabean showed medium, prominent, white colonies

of YEMA whereas French bean showed large white colonies. All isolated Rhizobium strains were found to be Gram negative and these were having granules within the cell. All strains of Rhizobium [SbR,ScR,FaR,FbR,FcR] had peritrichous flagellar arrangement while SaR strain of had sub polar flagella. All strains of Rhizobium gave positive congo red and -ve test with alkaline broth and lactose agar test. When Rhizobium strain isolated from the Soyaban was used as bioinoculant, it increased the growth of inoculated plant and the plant appeared much healthier in comparison to the control Fig. 6.



Discussion

Legume *Rhizobium* symbiosis is one of the biological N2-fixing systems and is the most cost effective means of N addition to terrestrial ecosystem. But, to certain extent the survival of these organisms in nature largely depends on the genetic and physiological traits of the organisms in addition to the environmental conditions brought about by cultivation practices.

Before the introduction of molecular taxonomy for identification of bacteria, bacteria which induced morphologically distinct outgrowths called nodules on the root surface of leguminous plants (except *Parasponia*) were collectively called as rhizobia. Accordingly, all bacteria that nodulates leguminous plants are at present collectively called as legume nodulating bacteria. Variability in the environment and management practices of legumes is likely to be associated with diversity in legume nodulating bacteria, an aspect that has received only marginal attention in India (Manvika Sahgal and Johri, 2003). However, very little work is carried out in India on the population dynamics and diversity of LNB in different agro-climatic conditions in different soils.

Kumar *et al*. (1997) reported that cultivation of legumes led to an increase in the population of rhizobia in soil. They suggested that the nodules of leguminous plants decay after maturity and as a result of that, rhizobial cells were released in the soil atmosphere. Hegde (1983) determined the red gram (*Cajanus cajan*) rhizobial population in 14 soil samples from different places in Karnataka using siratro and red gram as trap plants. The population ranged from 1.8 x 10^2 cfu g^{-1} to 2.6 x 10^5 cfu g^{-1} of dry soil. Population changed as with soil depth, soil types and cropping patterns.

Biofertilizers may be defined as "the microbial inoculants which are biologically active products containing active strains of specific bacteria, algae, fungi alone or in combination which may help in increasing crop productivity by helping in the biological N2 fixation, stimulate plant growth or help in decomposition of plant residues by cellulolytic organism. Biofertilizer, an alternative source of N-fertilizer, especially rhizobia in legume symbiosis is an established technology. Use of the biofertilizers can also prevent the depletion of the soil organic matter (Jeyabal and Kuppuswamy, 2001). Inoculation with bacterial biofertilizer may reduce the applica tion of fertilizer-N by increasing N uptake by plants (Kennedy *et al*., 2004). But most of this technique mainly limited between legume and *Rhizobium* in symbiotic process, which can fix atmospheric N2. However, biological N2 fixation (BNF) technology can play a vital role in substituting for commercially available N-fertilizer in cereal production thereby reducing the environmental problem to some extent. BNF and its transfer of NH4 activate the growth promotion of associated plants. Nitrogen fixation and plant growth enhancement by rhizosphere bacteria might be important factors for achieving a sustainable agriculture in the future. This is associated with roots and grasses have been recognized as an important component of the nitrogen cycle in a range of ecosystems (Chalk, 1991). In recent years use of Rhizobial species as a source of biofertilisers has become a hope for most of the countries as far as economical and environmental view point are concerned (Mia and Shamsuddin, 2010). Higher production of cereals brings higher production cost and pollutes the soil environment due to excessive use of chemical fertilizers. Therefore, biofertilizers were used which are cost effective and environment friendly. In the biofertilizer technology, *Rhizobium*-legume is most common and widely used in different countries. Recently, it is also found that rhizobia can make an association with graminaceous plants such as rice, wheat, maize, barley millets and other cereals some time as endophytic without forming any nodule-like structure or causing any disease symptoms. Increasing the ability of rhizobia in biofertilizer, crop enhancing activity in nonlegumes especially cereal grains would be a useful

technology for increased crop yields among resource-poor farmers. Recent findings showed both more crop enhancing and biofertilizer attributes in cereal crops due to rhizobial inoculation. In addition, plant nutrients like P, K, Ca, Mg and even Fe accumulation were also observed (Mia and Shamsuddin, 2010).

Nitrogen fixation and plant growth promotion by rhizobacteria are important criteria for an effective biofertilizer. One of the most important factors in the generation of high yields from modern rice cultivars is nitrogen fertilizer. Farmers are applying high amounts of the fertilizers which is very costly and make the environment hazardous especially when use discriminately. In addition, more than 50% of the applied N-fertilizers are some how lost through different processes which not only represent a cash loss to the farmers and consequently polluted the environmental (Ladha *et al*., 1998). Crop scientists all over the world are facing this alarming situation and they are trying to overcome this condition by exploring alternative sources which is cost effective and save the environment.

The agriculturally important microorganisms have their own unique place and scope in overall context of agro biodiversity. They play an important role in nutrient acquisition for plants and other micro elements. Indirect influence on crop production can also be acquired by inoculation with bio control agents. The use of *Rhizobium* as inoculants has been found to increase nodule formation , high yield of N2 fixation and increase in growth of many legume crop plants.

In the present study, an attempt was made to explore the possibility of use of atmospheric N2 as nitrogen fertilizer of Biological nitrogen fixation with indigenous Rhizobial strain of legume crops. Biological nitrogen fixation offers a better alternative over chemical fertilizers as the process, besides supplying nitrogen to crop, enriches soil nitrogen content and maintains soil health and productivity (Reddy and Reddy, 2004). The symbiosis between the root nodule bacteria of the genus *Rhizobium* and legumes is of special significance to legume husbandry as seed inoculation with effective strains of *Rhizobium* could meet the partial nitrogen requirement of legumes and hence reduction in dependence of external nitrogen inputs. The results obtained in the present study are conclued as Number of nodules present in sample Soyabean were maximum than French bean. Soyabean showed small prominent, white colonies of YEMA whereas French beans showed large white colonies. All isolated Rhizobium strains were found to be gram negative, and these were having granules within the cell. All strains of Rhizobium having peritrichous flagellar arrangement while SaR strain had subpolar flagella. All strains of Rhizobium gave positive congo red test and -ve test with alkaline broth and lactose agar test. When

Rhizobium strain, isolated from the Soyaban, was used as bioinoculant, it improve plant growth.

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