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Efficient native rhizobia isolation: A sustainable approach to symbiotic nitrogen fixation in legumes

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Abstract
This article provides an overview on role of efficient native rhizobia isolation and symbiotic nitrogen fixation by legumes. The protein element in legumes; which is among the richest source of protein worldwide relies mostly on nitrogen (N_2). Symbiotic nitrogen fixation is the base for source of nitrogen in legumes. Legume rhizobium symbiosis proves more effective when the rhizobial species works efficiently under particular or sometimes extreme environmental conditions. Therefore the acclimatisation of native rhizobia to native environment for better legume rhizobia symbiosis, thereby better nitrogen fixation and thereby a more better protein source for our population needs to be isolated. The extreme environmental conditions *viz.* salinity, acidity, high temperature, drought and flood often stands as the major causes for yield reduction in pulses due to reduction in growth and metabolism of rhizobial species. Hence, screening of native rhizobia on their efficiency basis by various physical, biochemical as well as molecular characteristics and application of these efficient native rhizobia to legumes will improve the legume yield as well as the protein concentration in them, thereby leading to a sustainable symbiotic relationship between legume rhizobia.

Keywords: Symbiotic nitrogen fixation, native rhizobia, legumes

Introduction
Dinitrogen which constitutes about 80% of earth's atmosphere and considered as one of the key driver of global agricultural production remains inert due to the stability of its triple bond.Most nitrogen is supplied to the plant from either the soil, rainfall or through the process of nitrogen fixation (Peoples, 1995) [36]. Symbiotic N Fixation, the reduction of atmospheric nitrogen into ammonia is the second most important biological process on earth after photosynthesis (Sylvia, 2005) [55]. The Rhizobium-legume symbiosis is superior to other nitrogen fixing systems due to its high potential (Sanaa and Fawziah, 2005) [43]. The terrestrial flux of N from biological nitrogen fixation has been estimated to range from 139-170 million tons per year (Russeille, 2008) [42]. Thus emphasis should be given for establishment of efficient symbiotic N_2-fixing systems in legumes. Although chemical fertilizers have played a significant role in green revolution, inappropriate and imbalanced uses of chemical fertilizers are affecting gradually the soil fertility, crop quality and to environmental degradation. Therefore, bioinoculation with *Rhizobium* in leguminous plants contributed for crop growth stimulation, a substitute for costly nitrogenous fertilizers (Tairo and Ndakidemi, 2013) [56]. 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) [25].

The utilization of inoculants of native rhizobia promote ecologically sustainable agricultural ecosystems by enhancing legume production due to their growth promoting traits and adaptability to soil and environmental stress (Mwangi *et al*., 2011) [29]. Furthermore, crop production using inoculants could be cheaper and more affordable to the resource-poor smallholder farmers (Singh *et al*., 2016) [48]. The ability of native strains to interact positively with the resident soil micro biota and their adaptability to the local agroecological climatic conditions often elucidates their superior performance over the exotic commercial strains (Meghvansi *et al*., 2010) [28]. In order to achieve maximum legume productivity, screening of native isolates for their nitrogen fixation efficiencies is vital (Anglade *et al*., 2015) [4].

Survival, persistence and competitiveness of the *rhizobial* strains are the major factors determining their successful use as inoculants (Purcino *et al*, 2000) [37]. To determine these properties, the inoculated strains must be screened as per the efficiency and the introduced strain must compete with highly adopted indigenous rhizobia for nodulation under specific soil conditions. Many biotic and abiotic factors affect the persistence of symbiotically effective introduced rhizobial strain in soil (Appunu, 2009) [7]. Moreover, soil acidity is one of the factors which restrict production of pulses by restricting root growth, nodulation, N fixation and limiting *Rhizobium* survival and persistence in soils (Appunu, 2009 and Sylvia, 2005) [7, 55]. Achieving good nodulation of pulses on acid soils is generally much harder. These constraints lead to sub optimal productivity of crops raised in acid soils; consequently it becomes inevitable to inoculate the crop with adequate effective *Rhizobium* (Deka and Azad, 2006) [12]. It is self-evident, that nitrogen fixation will play a central role in future agricultural system. It has been estimated that above each hectare of land there are about 80,000 tons of nitrogen available in atmosphere. But atmospheric nitrogen is not available to plants or animals and they cannot make use of free nitrogen. Since, symbiotically fixed nitrogen is in organic form, there would be little loss and more crop uptake (Lupwayi *et al*., 2004) [27]. This review explores the isolation of native rhizobia by various stages of screening, which contributes towards symbiotic nitrogen fixation.

Nitrogen as macro or major nutrient

Nitrogen was discovered by Daniel Rutherford in 1772. It was found to be so inert that Antoine Lavoisier named it "azote", meaning "without life". Dinitrogen (N_2) has a triple bond and does not readily accept or donate electrons. As a gas or liquid, nitrogen is colorless and odorless. Even though it is one of the most abundant elements (predominately in the form of nitrogen gas (N_2) in the Earth's atmosphere), plants can only utilize reduced forms of this element. Plants acquire these forms of "combined" nitrogen by:

1. The addition of ammonia and/or nitrate fertilizer (from the Haber-Bosch process) or manure to soil
2. The release of these compounds during organic matter decomposition
3. The conversion of atmospheric nitrogen into the compounds by natural processes, such as lightning
4. Biological nitrogen fixation (Vance, 2001) [59]

N is reported to be the major nutrient required in sufficient amounts to sustain crop yield and quality. Nitrogen is one of the key nutrients necessarily required for the improvement of growth and nutritional contents of plants.

Nitrogen requirement of legume crops

Legumes are vital source of protein for human beings as well as animals but the basic structure of protein is constituted by nitrogen. There is a vital role of microbes in nitrogen stress management. The biological nitrogen fixation (BNF) mediated by microbes contributes 180-106 t of fixed N per year globally, out of which 80% is contributed by symbiotic associations and the rest comes from free-living or associative systems (Graham, 1988). Garg and Geetanjali (2007) [14] reviewed the processes and signaling involved in symbiotic N fixation in legume nodules at a micro scale. This kind of symbiosis exists in many types of legumes, including grain legumes, forage legumes, and some leguminous trees. The symbiosis is typically host-specific and mediated by signaling biomolecules produced by both host plant and bacteria (Oldroyd *et al*., 2011) [34].

Having the ability to carry out symbiotic nitrogen fixation and as an excellent source of high quality protein legume play a special role in human and animal where the diet is poor in protein (Tikhonovich and Provorov, 2011; Rathi *et al*., 2016) [57, 39].

Biological nitrogen fixation in legume crops

Biological nitrogen fixation is the process that changes inert N_2 to biologically useful NH_3. The total BNF is estimated to betwice as much as the total nitrogen fixation by non-biological processes (Bezdicek and Kennedy, 1998) [9]. This process is mediated in nature only by microbes especially by bacteria. Nitrogen fixation by legumes is symbiotic as it is a partnership between a bacterium and a plant (Lindeman and Glower, 2008) [26]. In legumes, the bacteria live in small growths on the roots called nodules. Within these nodules, nitrogen fixation is done by the bacteria and the NH_3 produced is absorbed by the plant.

Recently, there has been a growing level of interest in environmental friendly sustainable agricultural practices and organic farming systems. Increasing and extending the role of biofertilizers such as *Rhizobium* would decrease the need for chemical fertilizers and reduce adverse environmental effects. Thus, in the development and implementation of sustainable agriculture techniques, biofertilization is of big importance in alleviating the deterioration of natural and environmental pollution. Besides, the assessment of rhizobial genetic diversity is contributing both to the worldwide knowledge of the biodiversity of soil microorganisms and to the utility of rhizobial collections. (Adiguzel *et al*., 2010) [1].

Rhizobial efficiency in symbiotic nitrogen fixation

As per Somascgaran and Hoben (1994) [51] the increase of rhizobia numbers in the rhizosphere is a response to the release of nutrients by the host legume. It indicates legume rhizobium association which is a vital source for nitrogen requirement of legumes and source of carbon for bacteria involved is process of symbiotic nitrogen fixation (SNF). Urzua (2005) [58] reported that advantages of symbiotic nitrogen fixation in plants and improved the efficiency of SNF by some methods included strain characterization and laboratory selection, green house studies, N-accumulated, nodulation, C_2H_2reduction. SNF facilitated their management, achieving higher productions and improving the quality of the crops, saving N-fertilizer and at the same time reducing the environmental impacts associated with nitrogen fertilization (Urzua, 2005) [58].

Rhizobial efficiency as influenced by climatological and Edaphological factors

Salt stress not only inhibits the process of nodulation and nitrogen fixation but it also induces premature senescence of already formed nodules (Swaraj and Bishnoi, 1999) [54]. Water stress not only affects nitrogen fixation at earlier stage, but also causes a negative impact on already formed nodules. When nodules are subjected to dry conditions, they show retarded growth resulting in a partially developed root cortex embedded organ (Nadeem *et al.*, 2014) [31].

Need for isolation and screening of native strain

The low productivity of this crop is due to many reasons including low soil fertility status and ineffective populations of root nodule bacteria. Inoculation of suitable *Rhizobium* strain causes a great increase in yield and nodulation under field condition (Akhtar *et al.*, 2009) [2]. Therefore, identification of eco-friendly low cost inputs for increasing its sustainable production under different climatic conditions is a paramount important area of research (Singh *et al.*, 2011) [47].

Furthermore, crop production using inoculants could be cheaper and more affordable to the resource-poor smallholder farmers (Singh *et al.*, 2016) [48]. The ability of native strains to interact positively with the resident soil micro biota and their adaptability to the local agroecological climatic conditions often elucidates their superior performance over the exotic commercial strains (Meghvansi *et al.*, 2010) [28]. In order to achieve maximum legume productivity, screening of native isolates for their nitrogen fixation efficiencies is vital (Anglade *et al.*, 2015) [4].

Sobti *et al.* (2015) [50] isolated strains of root nodulating bacteria from the root nodules fodder legume (*Medicago sativa*), the pure isolates showed growth in three days and turned the yeast extract mannitol agar (YEMA) media containing bromothymol blue to yellow color showing that all were fast growers and acid producers. The colonies were large (2-4 mm in diameter) mucilaginous, circular, glistening translucent or white and precipitated calcium glycerophosphate present in YEMA media. Microscopic examination revealed that the isolates were rod shaped and gram negative in nature.

Hung *et al.* (2004) [17] reported that the root-nodulating bacteria were isolated and characterized from 7 native shrubby legumes and measured growth rates in various media, colony morphology and tolerances to extremes of temperature, salt and pH. Among the 83 isolates that were screened, the majority were fast-growing rhizobia, 28 strains tolerated high concentration of salt (45% NaCl) and grew well between temperatures of 37 and 45 °C. The majority of the strains also tolerated extreme pH in their medium from 3.5 to 12. All strains formed nitrogen fixing nodules and the highest activity was detected in the legume.

Koskey *et al.* (2017) [23] reported that four native rhizobia isolates ELM3, ELM4, ELM5 and ELM8 showed higher symbiotic efficiencies compared to the commercial inoculants. There was a significant improvement in nodule dry weight and seed yields of climbing bean upon rhizobia inoculation when compared to the non-inoculated controls. Inoculation with ELM3 isolate resulted to the highest seed yield of 4,397.75 kg ha^{-1}, indicating 89% increase over non-inoculated control (2,334.81 kg ha^{-1}) and 30% increase over commercial inoculant (3,698.79 kg ha^{-1}).

Importance of screening for efficient strain selection

Amarger *et al.* (1994) [3] isolated 287 isolates of *Rhizobium* nodulating *Phaseolus vulgaris* in France from four geographically distinct field conditions. Shamseldin and Warner (2004) also isolated two *Rhizobium* strains, EBRI-2 and EBRI-26, from Egypt and were further tested for nodulation and competitiveness on bean using *Rhizobium tropici*, CIAT 899 G as the competing strain.

Strain efficiency reflects the ability for survival and multiplication in the carrier and soil, growth rate, tolerance to environmental stress, symbiotic properties such as nitrogen fixation, growth stimulant production etc. and competition with native flora existing in soil (Subba Rao *et al*., 1993) [52]. In the case of *Rhizobium* inoculants, the presence of native (soil) rhizobia poses a problem in the form of competition. Since the native rhizobia are well adapted to the soil conditions, they are more competitive and are able to occupy more nodules of the host plant. Often the nodules formed by native rhizobia are low.

The use of efficient strains of *Rhizobium* as seed inoculant has proved to be the cheapest and most effective way to increase the yield of pulse crops (Kumar and Shrivastava, 1994) [24]. According to them the efficiency of local isolates/strains showed better performance (GKP3 and GKP5) and was comparable with those of recommended ones (RCR 3824, VR 169, VR 441 and VR 420). In addition, standard strains SB75 and SB102 (originated from India and USA, respectively) were used to produce bio-fertilizer in powder form to study the symbiotic nitrogen fixing efficiency of different rhizobial strains on soybean. Among rhizobial strains, the strains SB83 and SB177 produced significantly higher number of nodules per plant, nodule dry weight per plant and bean yield than using other strains. It was also possible to select effective and competitive spontaneous mutants for soybean from parental rhizobial strains recommended for commercial inoculants (Carvalho *et al.*, 2005) [10]. Hungria *et al.* (2005) [18], the success of the biological nitrogen fixation process with the soybean crop in South America results from breeding programs searching for both plant and *Bradyrhizobium* genotypes with higher capacity of N_2 fixation, resulting today higher yields are obtained in the total absence of N fertilizers.

A series of trials were conducted during 1996-2002 at IG Agril. University, Raipur to select location specific effective *Rhizobium* isolates for soybean through systematic screening of local *Rhizobium* germplasm against national and international checks. Eight acid tolerant strains of *Bradyrhizobium* isolated from soybean plants grown on acid soils in Madhya Pradesh, India, were examined for their ability to survive in soil and YEMB at low pH levels. All the tested isolates survived in acidic (pH 4-6) conditions and their survival capacity was higher in soil than in nutrient medium at same levels of low pH. Variation among different strains showed that there is potential to improve strain performance under stress conditions. Further, symbiotic effectiveness of these strains was determined under the poly house conditions in sterilized soil (pH 4.5). The strain found to be more tolerant to stress were more effective N_2 fixers in symbiosis with soybean in acid-soil conditions (Appunu *et al*., 2006) [6]. Further, symbiotic efficiency of different *B. Japonicum* strains was tested as well as their compatibility with different soybean cultivars. The application of different *B. japonicum* strains caused significant differences in nodule number and

nodule dry weight.

Katiyar *et al*. (2009) [21] soybean rhizobial isolates were collected from 92 different locations in the hills of Uttarakhand and plains of Uttar Pradesh. After preliminary screening, eleven isolates were tested for effectiveness against soybean varieties based on increase in N content in plants over the uninoculated control. Efficient *Rhizobium* isolates recorded better nodulations and higher grain yield of different soybean varieties. In the current study by Sharma *et al*. (2010) [45], 22 rhizobial isolates (recovered from 12 different soybean growing sites) and 8 reference strains were selected for biochemical and metabolic characterization. Of 22 isolates, 18 were identified as fast growing isolates while the rest were slow growing indigenous and reference strains resulted also in seed yield differences. The results obtained in this study, highlight the importance of rhizobial strain selection as mentioned by Sikora *et al.* (2008) [46].

Characterization of rhizobacteria for efficient screening morphological and biochemical characterization

Geetha *et al.* (2014) isolated a total of 140 bacteria from plant rhizosphere soils during 2010-2012. Among them 30 potential bacterial strains showing antagonistic and PGP activities were selected for characterization. Out of which 6 strains were selected for further studies based on various morphological, biochemical and physiological screening methods. Among those six, 2 isolates belonged to Gram positive and 4 isolates belonged to Gram negative. All isolates exhibited production of indole acetic acid whereas 2 isolates produced HCN and solubilised inorganic phosphate.

Sugiyama *et al.* (2014) [53] used both culture-dependent physiological profiling and culture independent DNA-based approaches to characterize the bacterial communities of the soybean rhizosphere during growth in the field and found highly diverse communities of bacteria inhabiting soybean rhizospheres. Pyrosequencing analysis revealed that differences between the bacterial communities of rhizosphere and bulk soils at the phylum level; i.e., *Proteobacteria* were increased, while *Acidobacteria* and *Firmicutes* were decreased in rhizosphere soil during growth.

Niste *et al.* (2015) isolated and biochemically characterized twenty rhizobacteria strains from red clover (*Rhizobium trifolii*) and alfalfa (*Sinorhizobium meliloti*). From which they reported that phenotypically all isolated strains had the similar colony morphology, conical with a smooth margin, the color and texture was watery to translucent. The isolates were fast-growing and failed to absorb Congo red. API 20NE and API 20E showed a negative reaction for the reduction of nitrate in RtS1, RtR2, and positive for SmM1, SmM2. The reaction was positive for β-galactosidase, β-glucosidase and negative for urease, arginine dihydrolase, for all rhizobial isolates. Carbon sources: glucose, arabinose, mannose, mannitol, maltose, were also positive for all strains. Rhizobial strains utilized a wider range of carbohydrates.

Parthiban and Mahesh (2015) [35] isolated rhizobia from root nodules of *Vigna mungo* and characterized morphological and biochemically to ascertain in its physiology under the normal condition and they reported that all samples were streaked on Bromothymol blue (BTB) added Yeast Extract Mannitol (YEM) selective media confirmation. The positive samples from all target areas showed hazy appearance in the motility media and also were positive for catalase, motility and O-nitro phenyl-D-Galactopyranoside (ONPG) Tests. The samples found negative for methyl red (MR), voges-proskauer (VP), indole, citrate utilization test, hydrogen sulphide production, urea hydrolysis test and gel liquefaction test

Lalitha and Emamnuel (2013) [25] conducted an isolation study of *Rhizobium* from rhizosphere soil of leguminous plants grown in various parts of Coimbatore and isolated four isolates (AhV01, VmV01, VmG02 and VrV01). All the four isolates were positive for catalase, amylase, oxidase and nitrate reductase tests. These isolates variably showed resistance to various antibiotics in antibiotic susceptibility testing.

Devi *et al.* (2015) [13] isolated a novel *Burkholderia* strain AU4i (B-AU4i) from pea rhizosphere and they have conferred it to have phosphate solubilization, indole-3-acetic acid production, N_2 fixation, ammonia production, siderophore production, HCN production.

Significance of molecular and NIF gene characterization

Malleswari and Bagyanaryana (2013) [11] isolated 219 bacterial strains and initially screened them for their PGP activities. From the 219 isolates four bacterial strains were selected and tested for *in vitro* specific plant growth promotion activities such as ammonia production, IAA production, phosphate solubilization, HCN production and antifungal activity. These PGPR isolates were characterized through 16S rRNA gene sequencing which led to their identification as Pantoea sp. (Cf 7), Bacillus sp. (Cf 60) and Pseudomonas sp. (Te1, Av 30) respectively.

Naveed *et al.* (2014) [32] identified bacterial isolates by 16S rRNA gene sequence analysis and the bacterial strains belonged to 5 genera *i.e. Ensifer*, *Bacillus*, *Pseudomona*, *Leclercia* and *Rhizobium*. The bacterial strains were characterized for morphological, physiological, biochemical tests and glucose dehydrogenase (*gdh*) gene that involved in the phosphate solublization using cofactor pyrrolo quinolone quinone (PQQ). Seven rhizosphere and 3 root nodulating stains are positive *for gdh* gene.

Ji *et al.* (2014) [19] have isolated 576 endophytic bacteria from the leaves, stems, and roots of 10 rice cultivars and identified 12 of them as diazotrophic bacteria using a specific primer set of Nif gene. Through 16S rDNA sequence analysis, nifH genes were confirmed in the two species of Penibacillus, three species of *Microbacterium*, three *Bacillus* species, and four species of *Klebsiella*. Among 12 isolates tested, 10 strains have shown higher auxin producing activity, 6 isolates were confirmed as strains with high siderophore producing activity while 4 isolates turned out to have high phosphate-solubilizing activity.

Two novel, Gram-negative, motile, rod shaped, aerobic bacterial strains, MH17T and RD15, were isolated from the sterilized root and rhizosphere soil of rice, respectively. Phylogenetic analysis based on 16S rRNA gene sequences showed that the similarity between strains MH17T and RD15 was 100%. The isolates exhibit high sequence similarities to *Rhizobium oryzae* CGMCC 1.7048T (98.7%) and *Rhizobium petrolearium* SL-1T (97.0% and 97.1%), which supports that they belong to a novel species in the genus *Rhizobium*. The major cellular fatty acids were identified as summed feature 8 (C18:1 x7c and/or C18:1 x6c). Type strain MH17T had 87.5% DNA-DNA relatedness with RD15 by using the initial renaturation rate method. Based on draft genome sequences, strain MH17T showed 30.1% DNA-DNA hybridization values to R. oryzae CGMCC 1.7048T, the closely related strain, which supported that MH17T represents a novel species in the genus *Rhizobium*. Average nucleotide Identity

(ANI) between strains MH17T and RD15 were 97.8%, and strain MH17T showed 82.2% ANI value with R. oryzae CGMCC 1.7048T. The DNA G+C content was 60.4 mol% (Tm) (Zhao *et al*., 2017) [62].

The *nif* genes (nitrogenase genes) are N_2 fixation genes and are present in both symbiotic and free-living systems (Kim *et al*, 1994) [22]. These genes include structural genes, genes involved in the activation of iron proteins, iron molybdenum co-factor biosynthesis, and electron donation, and regulatory genes required for the synthesis and function of enzymes. In diazotrophs, the *nif* genes are typically found in a cluster of around 20-24 kb with seven operons encoding 20 different proteins (Glick, 2012).Nitrogen fixation is mainly governed by nitrogen fixation (nif) genes whose expressions are strictly regulated by environmental oxygen and ammonium. The nifD and nifK genes specify α and β subunits, respectively, of the molybdenum iron protein (dinitrogenase) and the iron protein (dinitrogenase reductase) is encoded by nifH (Rubio and Ludden 2005) [41]. *Bacillus* and *Paenibacillus* are Gram-positive, spore-forming bacteria and can survive even in strict environments. The members of nitrogen-fixing Bacillus and *Paenibacillus* have great potential for use as a bacterial fertilizer in agriculture. Presently, there are only a few reports about nitrogen fixation in these bacteria. For example, *Paenibacillus azotofixans* contains three copies of nifH (Choo *et al*. 2003) [38] and *Paenibacillus massiliensis* T7 contains a nif BHDKENX cluster (Zhao *et al*. 2006) [16].

The *Paenibacillus sabinae* T27 is a gram-positive, spore-forming diazotroph which is a novel species isolated and named by our lab and it has high nitrogenase activities (Ma *et al*. 2007) [60]. Currently, nothing is known about the nitrogen fixation of this bacterium. In this study, three nifH genes are cloned, and their activities and putative promoter regions are characterized.

The current understanding of nitrogenase diversity has been based largely on Phylogenetic analyses of nifH and nifD, the nitrogenase structural genes (Zehr *et al*., 2003) [61]. Recently, Raymond *et al*. (2004) [40] performed genomic analyses of nif genes encoding the core components of nitrogenase, including the NifH, NifD, NifK, NifE and NifN proteins.

Conclusion

The biological nitrogen fixation is an important biochemical process through which atmospheric di-nitrogen can fix through symbiotic or asymbiotic process. Rhizobium is a member of class alpha proteobacteria can fix N_2 efficiently. The above mentioned point indicates that, the efficient local strain is needed for the better survival in indigenous environment. Their identification and efficiency is needed. Different authors in their previous studies reported that colony of rhizobia are convex, translucent, circular margin, gram –ve and gummy in nature. The biochemical characterization indicates that rhizobia have the plant growth promoting rhizobacteria (PGPR) activity, which facilitate the growth of plant as well as the growth of other rhizobacteria present in rhizosphere. The Nif gene characterization is essential for conformation of the symbiotic ability of isolates.

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