Formulation and Commercialization of Rhizobia: Asian Scenario

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Rajendran Vijayabharathi, Arumugam Sathya, and Subramaniam Gopalakrishnan

Abstract

The symbiotic agreement of rhizobia with leguminous plants is making a valuable contribution to agriculture primarily as nitrogen fixers and secondarily as plant growth promoters by their key role as phosphate solubilizers, growth hormone producers, abiotic and biotic stress relievers, and host-plant resistance enhancer. In the so far identified 14 genera and 105 species of rhizobia, a huge number of research reports were reported in various aspects. Genetically modified rhizobia with desirable traits have also been surfed to a large extent. Besides their potentiality, the commercial success of rhizobia as a bio-inoculant is poor, because most of the inoculants produced worldwide are of poor or suboptimal quality. Though voluminous data and better understanding are available on various formulation technologies, longevity and efficacy of the final product are loosed at the farmer's end. This book chapter is focused to address various types of formulations applicable to rhizobia, quality control for longevity, gaps in knowledge on bringing the native potential of rhizobia during formulation, and critical control points to be considered during its development. The chapter also shares ICRISAT's experience in its rhizobial collection, formulation developments, and efficacy testing.

Keywords

Rhizobia • Legumes • Asia • Formulations • Peat • Legislations

R. Vijayabharathi • A. Sathya • S. Gopalakrishnan (✉)
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502324 Hyderabad, Telangana, India
e-mail: s.gopalakrishnan@cgiar.org

H.B. Singh et al. (eds.), *Agriculturally Important Microorganisms*,
DOI 10.1007/978-981-10-2576-1_3

3.1 Introduction

Approximately 80 % of the human dietary nitrogen needs, i.e., 24 Tg/year in tropics and subtropics, are satisfied by the plants. But with the increasing earth's population at a rate of 1.4 % annually, the present scenario of crop production rates will not be sufficient to maintain the dietary needs (Mannion 1998; Fink et al. 1999). Deterioration of agricultural lands and use of marginal lands for crop production are further complicating the scenario, because soil N management plays a critical role in crop yield (Huang and Rozelle 1995; Bramley et al. 1996; Rozelle et al. 1997; Savant et al. 1997). While considering the past scenario, i.e., between 1950 and 1990, N fertilizers played a major role in increasing the cereal grain yield. They yielded 6–9 mg grain/hand take-up 200–300 kg N ha year^{-1} (Vance 1998). Still the use of N fertilizers at global scale is in increasing trend as per the FAOSTAT data. Though nitrogen fertilizers gave an increase in crop production, there was a great impact in the environment which includes NOx loss, acid rain, higher leaching, change in the global N cycle, and polluted ground water. When developed countries were facing such problems, developing countries were affected by the additional issues of fertilizer cost, availability, and distribution problems (Kinzig and Socolow 1995; Vitousek et al. 1997).

In the context of sustainable N management, symbiotic nitrogen fixation (SNF) plays a vital role. Though it represents systems including either rhizobia, *Azolla*, or *Anabaena* with either leguminous or cereal crops, the system of legume-rhizobia symbiosis is the critical factor as it involved in 80 % (approximately 100–122 Tg year^{-1}) of biologically fixed nitrogen by involving a range of species such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* (Vance 1998; Herridge et al. 2008). The process of nitrogen fixation through SNF was reviewed periodically at various aspects covering biochemical and molecular mechanisms and genetic factors (Jiao et al. 2016; Remigi et al. 2016). When considering the fixed nitrogen effect by fertilizer and SNF, two key factors have to be considered: (i) fixed N by SNF is less susceptible to volatilization, leaching, or denitrification than fertilizer N, and (ii) industrial production of N requires approximately 1.5 Kg oil Kg^{-1} fertilizer in order to reduce N to ammonia along with the requirement of high temperature and pressure. Though SNF is also an energy-demanding process involving 16–24 moles of ATP for reducing 1 mole of dinitrogen, its persistence, stability, and absence of post-fixation effects add positive impact over fertilizer N. As per the review by Herridge et al. (2008), symbiosis by rhizobia is the efficient system for SNF as it contributes 55,140 kg N ha^{-1}, whereas 0.330 kg N ha^{-1} is by other biological systems. The symbiosis by cyanobacteria contributes for 5 Tg N, whereas by free-living, associative, and endophytic bacteria provides 10–20 Tg N. Actinorhizal symbiosis estimates about 4–42 g N tree^{-1} (Dommergues 1995), and cycads contribute 8–19 kg N h^{-1} in a year (Vessey et al. 2004).

Rhizobia, the efficient nitrogen fixer, are a term used for collective bacteria that enters symbiosis with legumes. Initially, till 1982, it was considered that *Rhizobium* is the only bacteria that possess these properties, but today, it was identified that there are 14 genera in two subphyla of Proteobacteria, viz., α-Proteobacteria and

β-Proteobacteria. α-Proteobacteria includes the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Shinella*, and *Sinorhizobium* (syn. *Ensifer*), and β-Proteobacteria includes *Burkholderia*, *Cupriavidus*, and *Herbaspirillum*. The number of genera in the rhizobia list is increasing day by day by various studies. This increasing number of rhizobia isolation led to reclassification and redesignation of some species (Lindström et al. 2010).

Development of such rhizobia as inoculants for legume crops is the most valuable contributions ever made by science to agriculture since it is evident to reduce N fertilizer use. Initial studies of inoculation were performed at a very basic level and laborious moving of soil from fields of well-nodulated legumes to legume-free fields (Fred et al. 1932). European countries initiated the inoculums development by advising their farmers to treat legume seeds with glue and sieved air-dried soil from well-nodulated plants (Walley et al. 2004). The work of Hellriegel and Beijerinck in the 1880s has brought a record on using pure cultures of rhizobia on inoculation of legume seeds. Within a couple of years, rhizobia were available in the European market for a range of species, and still it is getting developed involving new technologies (Guthrie 1896; Perret et al. 2000). But in the context of Asian countries, still the legume inoculant technology is underdeveloped due to a range of factors. Hence, this book chapter is focused to discuss the factors affecting rhizobia inoculant development in Asia.

3.2 Beneficial Traits of Rhizobia

Rhizobia are primarily considered for nitrogen fixation. Still the research on SNF in relation to rhizobia is ongoing including genetically modified rhizobia (Lindström and Mousavi 2010; Okazaki et al. 2016). After the concept of plant growth-promoting rhizobacteria by Kloepper, rhizobia have also been surfed to a large extent for its plant growth-promoting (PGP) properties (Kloepper and Schroth 1978). Hence, a developed rhizobial inoculum will provide additional plant and soil health benefits besides fixing nitrogen. PGP properties of rhizobia have been reviewed previously by Gopalakrishnan et al. (2014) and Naveed et al. (2015). The representatives of rhizobia with PGP traits have been given here.

3.2.1 Rhizobia as Phosphate Solubilizers

Rhizobia including *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Mesorhizobium mediterraneum*, *Bradyrhizobium* sp., and *Bradyrhizobium japonicum* (Vessey 2003; Afzal and Bano 2008) are the potential P solubilizers. The solubilization was aided by low molecular organic acids produced by them, for instance, 2-ketogluconic acid production by *R. leguminosarum* (Halder et al. 1990) and *R. meliloti* (Halder and Chakrabarty 1993). Enhanced growth in chickpea and barley plants by P-solubilizing rhizobia *M. mediterraneum* has been demonstrated by Peix et al. (2001).

3.2.2 Rhizobia as Iron Mobilizers

Iron exists as insoluble hydroxides and oxyhydroxides which cannot be accessed by both plant and microbes. Some bacteria synthesize low molecular weight compounds termed as siderophores which are capable of sequestering Fe^{3+}. Many rhizobia species including *R. meliloti*, *Rhizobium tropici*, *R. leguminosarum*, *Sinorhizobium meliloti*, and *Bradyrhizobium* sp. are reported to be potent siderophore producers (Arora et al. 2001; Carson et al. 2000).

3.2.3 Phytohormone Production of Rhizobia

Phytohormones are the essential substances for plant growth stimulation. They include indole-3-acetic acid (IAA), cytokinin, and gibberellins. IAA is the foremost phytohormone and plays a role in cell division and differentiation and also in nodule formation. Rhizobia strains are also reported to produce IAA via indole-3-pyruvic acid and indole-3-acetaldehyde pathway (Camerini et al. 2008). Similarly rhizobia have been reported to produce cytokinins which are involved in root development and root hair formation (Senthilkumar et al. 2009). Gibberellins which are responsible for stem elongation and leaf expansion are also reported in *Rhizobium* (Boiero et al. 2007). Some reports are there for production of abscisic acid which stimulates stomatal closure, induces seeds to store proteins, and induces gene transcription for protease inhibitors (Dobbelaere et al. 2003).

3.2.4 Rhizobia as Biocontrol Agents

Biocontrol properties have been demonstrated in several rhizobia strains through the mechanisms like competition for nutrients (Arora et al. 2001), production inhibitory substances including antibiotics (Chandra et al. 2007), production of hydrolytic enzymes (Ozkoc and Deliveli 2001), siderophores (Carson et al. 2000; Deshwal et al. 2003), and low molecular weight metabolites (Bhattacharyya and Jha 2012). Phytopathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Fusarium solani* were found to be controlled by rhizobia.

3.2.5 Rhizobia as Abiotic Stress Relievers

The stress of the plant depends on host-plant reaction which can be influenced by rhizobia and the symbiosis (Yang et al. 2009). Several reviews periodically documented the stress tolerance of *Rhizobium* and *Bradyrhizobium* against soil salinity, acidity, alkalinity, osmotic stress, and temperature fluctuations (Graham 1992; Kulkarni and Nautiyal 2000; Grover et al 2010).

3.3 Development of Rhizobia Formulations

Development of an inoculant technology for microbes is a time-consuming and cumbersome process as it faces various issues because many of the microbes produce fruitful laboratory results but fail to reflect similar effects under field conditions. So the success of an inoculant depends on its optimal results in situ and sophisticated use including cost-benefit ratio by end user (Xavier et al. 2004). In the context of inoculant development, carrier, a vehicle which transfers the microbes from laboratory to field, plays a crucial role. An ideal carrier should provide a beneficial microenvironment for the inoculated microbes against a range of biotic and abiotic stress factors including contaminants, soil antagonists, soil health deterioration, temperature, dryness, UV light, and mechanical stress. It should include the features such as (1) sustained availability, (2) low cost, (3) high moisture absorption capacity, (4) easy to process, (5) easy to sterilize, and (6) buffering capacity (Keyser et al. 1993). An overview on the available carrier materials and different types of inoculants is given in Fig. 3.1. Each carrier and formulation technology has its own pros and cons; and several reviews summarizing the same are available (Jung et al. 1982; Van Elsas and Heijnen 1990; Daza et al. 2000; Catroux et al. 2001; Amarger et al. 2001; Deaker et al. 2004; Bashan et al. 2014; Nehra and Choudhary 2015; Gopalakrishnan et al. 2016). Different rhizobial formulations tested on various crops are summarized in Table 3.1.

America, Europe, and Australia have potential market for rhizobia and have well-developed inoculant technologies. It is estimated that, in Australia, legumes

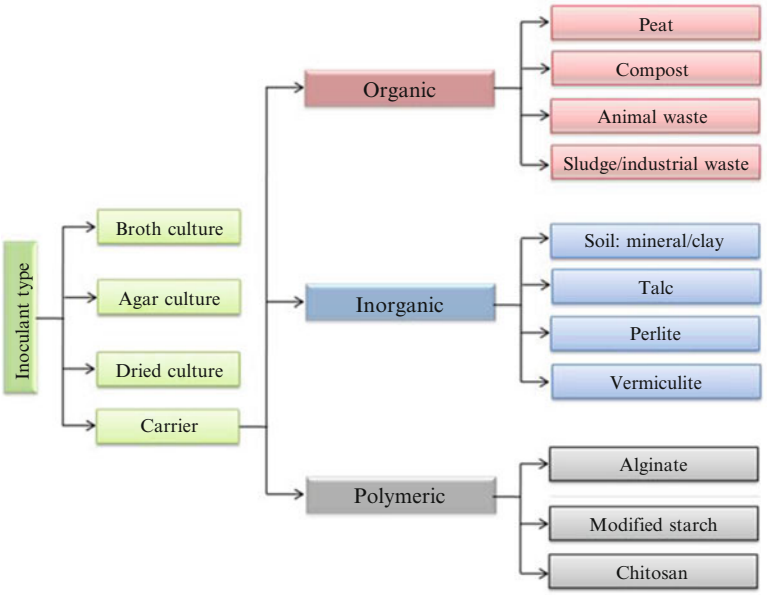

Fig. 3.1 Overview of inoculant types

Table 3.1 Different formulations of rhizobia tested at field levels

Formulation types	Additive/ treatment	Rhizobia	Crop tested	References
Liquid (culture media or water)	Glycerol, PVP, trehalose, FeEDTA	*B. japonicum*	Soybean	Singleton et al. (2002)
	PVP; FeEDTA	Several rhizobia; *B. japonicum*	Soybean	Albareda et al. (2008)
	Unknown (commercial)	*B. japonicum*	Soybean	Maurice et al. (2001)
	Gum Arabic	*Bradyrhizobium* sp., *Rhizobium* sp.	*Acacia mangium*, green gram, *Leucaena leucocephala*	Diouf et al. (2003); Wani et al. (2007)
Lyophilized cells	Soybean oil/ peanut oil	*Rhizobium* sp.	Bean, cowpea, peanut	Kremer and Peterson (1983)
Organic carrier				
Peat	None or with undisclosed additives	*B. japonicum*; *Rhizobium* sp., *R. leguminosarum* bv. *viciae*	Chickpea; faba beans; maize; pea; soybean; wheat	Clayton et al. (2004a, b), Hamaoui et al. (2001), Hungria et al. (2010), Hynes et al. (2001), Khalid et al. (2004), and Revellin et al. (2000)
	Gum Arabic	*Rhizobium*, *Bradyrhizobium*	Bean, *Lupinus*, *Hedysarum* Soybean	Albareda et al. (2009) and Temprano et al. (2002)
Coir dust/coco peat	None	*Azorhizobium caulinodans*	Rice	Van Nieuwenhove et al. (2000)
Vermicompost/ earthworm compost	Lignite	*R. leguminosarum*	Not tested	Raja Sekar and Karmegam (2010)
Sawdust	Composted by inoculation with *Cephalosporium* sp. and *Azospirillum brasilense*	*B. japonicum*, *R. meliloti*	Groundnuts, lucerne, and grass mixture of bird's foot trefoil and ryegrass; soybean	Kostov and Lynch (1998)
Sawdust	None	*R. leguminosarum*	*Trifolium repens*	Arora et al. (2008)

(continued)

Table 3.1 (continued)

Formulation types	Additive/ treatment	Rhizobia	Crop tested	References
Grape bagasse, cork compost	Gum Arabic, CMC	Several rhizobia; *B. japonicum*	Soybean	Albareda et al. (2008)
Wastewater sludge	Acid, alkaline, and oxidative pretreatments	*S. meliloti*, *R. leguminosarum* bv. *viciae*, *B. japonicum*, *B. elkanii*	Not tested	Ben Rebah et al. (2002a, b)
Inorganic carrier				
Clay minerals, perlite	Gum Arabic, CMC	Several rhizobia, *B. japonicum*	Soybean	Albareda et al. (2008)
Coal	None	*R. leguminosarum* bv. *phaseoli*	Pinto bean	Crawford and Berryhill (1983)
Vermiculite	None	*B. japonicum*, *S. meliloti*, *R. leguminosarum* bv. *phaseoli*	Navy beans	Graham-Weiss et al. (1987) and Sparrow and Ham (1983)
Perlite	Gum Arabic	*Rhizobium*, *Bradyrhizobium*	Bean; *Lupinus*, *Hedysarum*; soybean	Temprano et al. (2002)
	Sucrose	*R. leguminosarum* bv. *phaseoli*, *R. tropici*, *B. japonicum*	Bean, soybean	Daza et al. (2000)
Polymeric carrier				
Alginate	None	*Rhizobium* spp.	*Leucaena leucocephala*	Forestier et al. (2001)

Modified from Bashan et al. (2014)

growing on 25 M ha of land fix US$3–4 billion worth of N annually (Bullard et al. 2005). Report of Vessey (2004) states the benefits of rhizobial inoculants in the Northern Great Plains of the USA and Canada on soybean, lentil, pea, and faba bean with an overall response of 45 % yield increase. In the context of Asia, the situation is typically different though it contributes for maximum production of pulses than the other regions/continents. The statistical data of FAO (FAOSTAT 2016) on top seed producers and fertilizer users clearly indicates that the major seed producers are India and China; also the two Asian countries are the relatively top consumers of fertilizers (Fig. 3.2). All these together give a clear indication that the Asian countries depend more toward N fertilizers than the biofertilizers contributing nitrogen

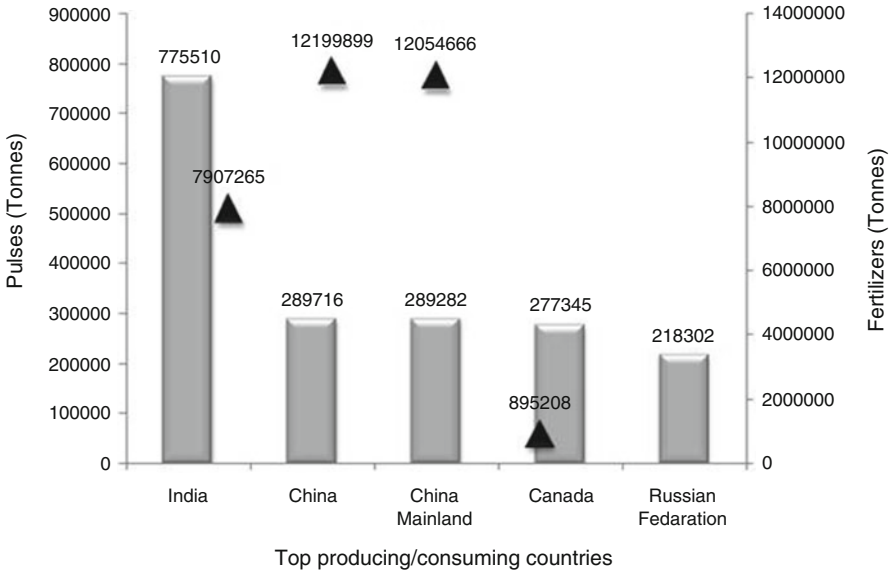

Fig. 3.2 Top pulse producers and fertilizer consumers of the world (Note: ■ Top seed producer (based on average data of 1993–2013) on the left axis ▲ Top fertilizer consumers (based on average data of 2006–2009) on the right axis)

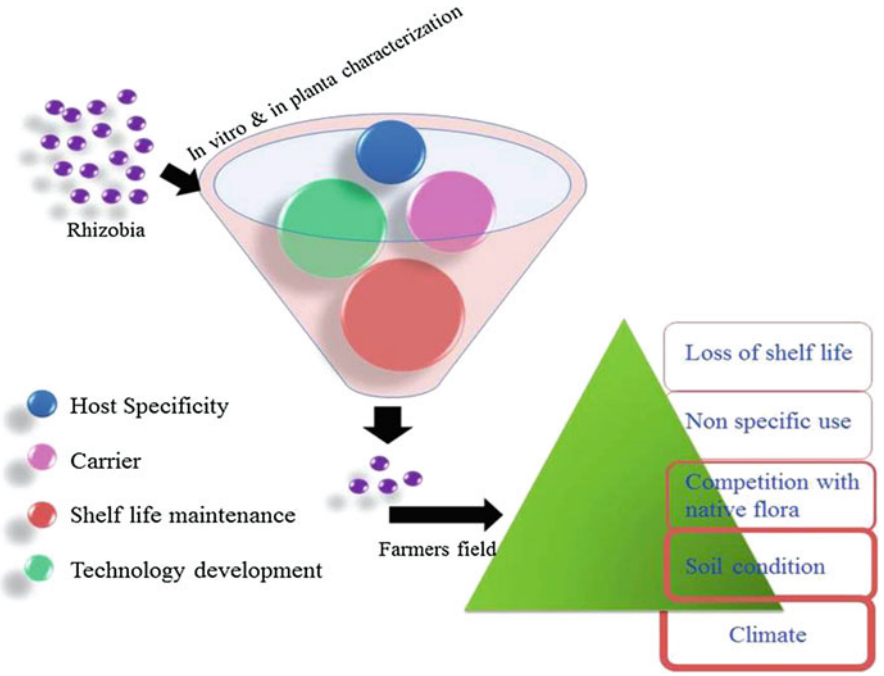

Fig. 3.3 Overview of barriers in inoculant development

including *Rhizobium* inoculants. The problem in the context of Asian scenario in *Rhizobium* inoculant technology is described here in various aspects, and an overview is given in Fig. 3.3.

3.3.1 Inoculant Strain Selection

Effective rhizobial strain is the central core for developing an inoculant which is necessitated in order to provide rhizobia for new legume cultivars and species and extend and optimize the legume cultivation under fluctuating environmental conditions. Brockwell et al. (1995) have listed a set of essential and desirable characters for inoculant strains including host specificity, competence with native rhizobia population and also with agrochemicals, genetic stability, etc. Asian countries including India (Ansari et al. 2014), China (Jiao et al. 2015), Nepal (Adhikari et al. 2012), and Myanmar (Htwe et al. 2015) have been reported with vast diversity of nodulating rhizobia. Recent reports on diversity analysis of rhizobia under hostile environments such as soils with acidity (Mishra et al. 2014), alkalinity (Singh et al. 2016), and micronutrient deficiency (Unno et al. 2015) indicate the research initiatives on the exploration of Asian rhizobial strains. The large genetic diversity noticed on soybean native rhizobia of Asian countries further supports the phenomenon (Biate et al. 2014). Reeve et al. (2015) captured the phylogenetic and biogeographic diversity of root nodule bacteria across the world through two genome sequencing reports, which has only 7 entries for rhizobia from Asian origin among the 107 selected strains. However, these 7 entries include 3 among the total of 13 type strains and 1 among the total of 14 elite strains with commercial significance, indicating that the complete characterization and exploration of rhizobial biodiversity of Asian countries will pave way for inoculant development.

3.3.2 Genetically Modified Rhizobia

Besides the native flora, genetic modification has also been done in rhizobia, mainly to compete with the indigenous strains and to improve its efficacy to form nodules and to fix nitrogen.

3.3.2.1 Modification in Nodulation

To increase the nodulation efficiency, two approaches were carried out. One is by introducing genes encoding for trifoliotoxin, an antibiotic to which indigenous flora is sensitive. Robleto et al. (1998) used this construct in *Rhizobium elti*, the common bean microsymbiont. They differ with the indigenous strain only in the production of nodules. Over 2 years, the genetically modified strains had occupied 20 % of the nodules in comparison to non-trifoliotoxin-producing strains. Another approach is to modify the expression of metabolite *putA* gene which is responsible for root surface colonization. Dillewijn et al. (2001) followed this approach in alfalfa field with *S. meliloti* strains overexpressing *putA* gene. On 1 month of inoculation, a large

number of strains occupied the nodules than the control strains. It appears to be an efficient method of nodulation, but on the yield of crop after 3 years of experimentation, they were all equal in inoculated and un-inoculated plants. This informs that inoculant strains will improve in nodulation only when indigenous competing population is less efficient which might not be frequent.

3.3.2.2 Modification in Nitrogen Fixation

To improve the nitrogen fixation, two approaches were followed. One is involving modification in *nifA* gene which regulates the expression of genes necessary for enzymes involved in nitrogen fixation. The other is by modulating dicarboxylate transport (*dct*) genes which supplies the carbon and energy required for nitrogen fixation. A construct with extra copy of *nifA* and/or *dct* genes was inoculated in *S. meliloti* and released in four fields (Bosworth et al. 1994). There was an increase by 13 and 18 % of alfalfa biomass in wild-type strains and non-inoculated control, respectively. But they were shown only at the sites with very low population of indigenous flora and low nitrogen content. Further, these were not found after 3 years of exploitation (Scupham et al. 1996). A study on soybean cultivation with release of *B. japonicum* with or without extra copy of *nifA* gene did not neither increase the yield nor the nitrogen fixation (Ronson et al. 1990). Summarily, the success of genetic modification has the potential to bring out a success in poor agricultural conditions.

3.3.2.3 Interaction Between Indigenous and Genetically Modified Rhizobia

In response to the introduction of genetically modified rhizobia, there was a change in number, composition, and activities of indigenous microflora and most importantly exchange genetic material with indigenous microflora. There were very less differences observed in rhizospheres of different hosts (Hirsch and Spokes 1994; Amarger et al. 2001) which informs only less changes happen on introduction. Similarly, vice versa transfer, i.e., plasmids from native flora to the introduced flora, was also not detected on re-isolating the genetically marked rhizobia after 1–2 years of introduction (Hirsch 1997). Data predicts that plasmid acquisition takes place at a frequency of 8×10^6/recipient cell in one site after 7 years of release which is not a stable conjugant. Studies have reported that there is no transfer of Tn-7 plasmid that occurs at any stage. If occurred also, the frequency is less than 10^7 events/gram of soil (Drahos et al. 1986). Lilley and Bailey (1997) had reported that transfer from indigenous to genetically marked rhizobia takes place with a frequency from 5×10^7 to 1 per recipient which varies with the year of experiment. However, the generated transconjugant is not stably maintained in the cell.

3.3.3 Nutritional Attributes for Rhizobia

After the selection of effective rhizobia, nutritional attributes have to be considered in order to evaluate whether the given carrier material will be enough to hold the viability or it requires any additional supplements for rhizobial maintenance.

Knowledge of nutritional requirement is a key factor when selecting complex material like agricultural, industrial, and sewage sludge wastes for inoculant production. Broadly rhizobia are divided into two categories depending on nutritional requirement and growth rate. They are fast-growing and slow-growing rhizobia which are placed in the genus *Rhizobium* and *Bradyrhizobium*, respectively (Jordan 1984). Fast growers are acid producers with 2–4 h as generation time. Slow growers are alkaline producers with 6–8 h as generation time (Jordan 1984). Fast growers can grow on various carbon sources such as hexoses, pentoses, disaccharides, trisaccharides, and organic acids (Allen and Allen 1950), whereas the other type can grow only in the presence of pentose but can utilize many aromatic substrates (Parke and Ornston 1984). In the context of nitrogen, some fast growers are potent in utilizing nitrate, ammonia, and amino acids (Quispel 1974). Amino acid glycine, alanine, and certain D-forms of amino acid might create a negative impact in nitrogen fixation (Burton 1979). Vitamin requirements vary between the genera, for example, *R. leguminosarum* (bv. *trifolii* and bv. *phaseoli*) requires biotin, thiamine, or calcium pantothenate separately or in combination, whereas *S. meliloti*, *B. japonicum*, etc. need only biotin (Graham 1963). In case of minerals, deficiency of Ca^{2+} and Mg^{2+} affects the growth and results in abnormal cells (Vincent 1962).

3.3.4 Inoculant Development

Among the inoculants are the primitive types such as broth culture, agar culture, and dried/lyophilized cells. These types of inoculants could not be promoted to practical technology, though it is least laborious and has proved records at research centers, because of impractical application at large scales and its failure to meet economic and commercial needs (Bashan et al. 2014). Hence, a carrier is necessary for the development of a successful inoculant.

The major markets, such as Europe and Australia, supply the inoculants in solid carriers, most commonly peat, for seed application (Catroux et al. 2001; Singleton et al. 2002). However, in North and South America, the inoculants supplied are clay- and peat-based granular and liquid inoculants (Singleton et al. 2002; Xavier et al. 2004). The Asian market also depends on peat for its inoculants because of its potential in holding high numbers of rhizobia (greater than 10^8 cells/g) during the storage. Unlikely, they do not have enough peatlands due to the lack of harmonized policies related to the management of peatlands besides their presence in Indonesia, India, Malaysia, Myanmar, the Philippines, Singapore, Thailand, and Vietnam. In the last few years, a forward look for its sustainable management has arisen. The projects ASEAN Peat l and Forests Project (APFP) and SEApeat were aimed in reducing deforestation and degradation of peatland forests and to strengthen the policies for its management. On the other end, a large area of peatlands in Vietnam has been designated as protected area and national parks (http://www.aseanpeat.net/).

It should also be considered that whether the peat belongs to these regions is original peat. Thomas et al. (1974) have evaluated the physicochemical characters of peat obtained from Nilgiri reserves of India and concluded that the material was

not an original peat as it lacks the main traits like water-holding capacity and organic carbon content. It is noticed that the Indian peat has 20–50 % organic carbon, whereas Australian and American peat has 65 and 86 % organic carbon content, respectively (NIIR 2004). Conservation policies for peatland management by Europe, Australia, and America have become stringent as they have key roles in biodiversity, carbon sequestration, and fuel-related application. This indirectly leads to the unavailability and high export cost for other countries (Joosten 2015).

As an alternative to peat, other organic carriers such as lignite and charcoal can be used which have also proved to be efficient in carrying rhizobia with the shelf life of 4–6 months (Argal et al. 2015; Gao et al. 2015). Research on alternate carrier was started more than four decades ago on carriers such as lignite and coal, clays and mineral soils, compost, farmyard manure, pressmud, agricultural waste, and inorganic materials like vermiculite, perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels, and alginate (Kandasamy and Prasad 1971; Dube et al. 1980; Chao and Alexander 1984; Iswaran et al. 1972; Philip and Jauhri 1984; Sadasivam et al. 1986; Sparrow and Ham 1983; Dommergues et al. 1979; Jung et al. 1982).

There are numerous reports on research and development of successful rhizobial formulations which were tested in fields of various research stations. However, there are very few number of coordinated network projects on large-scale evaluation in Asian countries. On the contrary, the International Network of Legume Inoculation Trials (INLIT) funded by the US Agency for International Development (USAID) in the University of Hawaii's NifTAL project assessed the need for inoculation in tropical agricultural systems by conducting 228 trials on various legumes such as green gram, soybean, black gram, groundnut, cowpea, chickpea, lentil, pigeon pea, and common bean. Worldwide Rhizobial Ecology Network (WREN), the follow-up program of NifTAL, evaluated the factors contributing to variations in inoculation response including a number of infective rhizobia, edaphic characteristics, crop fixed-N demand, and soil fixed-N supply (Singleton et al. 1992).

Effective regulatory quality control (QC) program has key role in the successful production of rhizobial inoculants. This may be supported by appropriate legislation as in Canada, Uruguay, and France or may be voluntary as in Australia, Thailand, New Zealand, and South Africa. Contrarily, in the USA, regulatory control and independent testing are considered unnecessary, with manufacturers conducting their own internal QC. Irrespective of the QC nature, all QC programs should monitor the numbers and quality of the strains in the inoculants along with the contaminating microorganisms. In Asia, 90 % of inoculants sampled had $<10^8$ rhizobia g^{-1} carrier and most of samples were contaminated (Thompson 1992).

Besides these barriers, many Asian countries commercialized rhizobia inoculants. This includes the following: (1) Pakistan, Fasloon Ka Jarasimi Teeka (AARI), BioPower (NIBGE), Biozote (NARC), and Rhizogold (ISES, UAF), consists of *Rhizobium* sp. (Naveed et al. 2015), and (2) Japan, the Tokachi Federation of Agricultural Cooperative (TFAC), produces Mamezo (rhizobia are mixed with peat and the natural organic matters), R-processing seeds (leguminous seeds inoculated with rhizobia), and hyper-coating seeds (leguminous grass seed coated by rhizobia within the capsule of calcium carbonate) (Yokoyama and Ohyama 2007).

Table 3.2 Viability and longevity of five different formulations of six different rhizobia

	Colony-forming unit (CFU/ml) of different carrier materials in months												
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th
	Chickpea													
IC-59														
T1	4.5×10^{9}	3.0×10^{9}	2.4×10^{9}	2.2×10^{9}	2.0×10^{8}	1.5×10^{8}	1.3×10^{8}	1.1×10^{8}	9.1×10^{7}	5.7×10^{7}	2.3×10^{7}	1.2×10^{7}	8.6×10^{6}	3.0×10^{6}
T2	2.0×10^{9}	1.8×10^{9}	1.4×10^{9}	1.2×10^{9}	8.9×10^{8}	7.5×10^{8}	5.4×10^{8}	4.3×10^{8}	1.2×10^{8}	9.0×10^{6}	4.2×10^{6}	1.2×10^{6}	7.5×10^{5}	4.5×10^{6}
T3	2.9×10^{9}	2.0×10^{9}	2.1×10^{9}	1.7×10^{8}	1.2×10^{8}	8.9×10^{7}	6.3×10^{7}	4.8×10^{7}	2.3×10^{7}	1.1×10^{7}	9.7×10^{6}	5.6×10^{6}	2.3×10^{6}	1.1×10^{6}
T4	7.5×10^{8}	4.7×10^{8}	5.5×10^{8}	4.2×10^{8}	1.0×10^{8}	9.3×10^{7}	7.1×10^{7}	5.6×10^{7}	2.8×10^{7}	9.0×10^{6}	7.0×10^{6}	1.1×10^{6}	7.3×10^{5}	2.6×10^{5}
T5	1.9×10^{9}	1.1×10^{9}	1.4×10^{9}	1.1×10^{9}	8.4×10^{8}	8.5×10^{7}	6.7×10^{7}	4.5×10^{7}	2.7×10^{7}	1.7×10^{7}	9.3×10^{6}	4.8×10^{6}	2.5×10^{6}	1.2×10^{6}
IC-76														
T1	3.9×10^{9}	4.3×10^{9}	2.15×10^{9}	1.75×10^{9}	1.3×10^{9}	1.1×10^{9}	1×10^{9}	9.6×10^{8}	5.1×10^{8}	4.3×10^{8}	1.2×10^{8}	1.0×10^{8}	9.3×10^{7}	2.0×10^{7}
T2	3.3×10^{9}	1.8×10^{9}	9.5×10^{8}	5.5×10^{8}	1.1×10^{8}	9.9×10^{7}	7.1×10^{7}	5.0×10^{7}	3.4×10^{7}	2.1×10^{7}	1.1×10^{7}	7.6×10^{6}	2.5×10^{6}	1.0×10^{6}
T3	5.0×10^{9}	2.0×10^{9}	1.1×10^{9}	1.1×10^{9}	6.6×10^{8}	1.1×10^{8}	8.9×10^{7}	6.1×10^{7}	4.9×10^{7}	3.1×10^{7}	1.2×10^{7}	5.6×10^{6}	3.1×10^{6}	1.2×10^{6}
T4	7.0×10^{8}	4.0×10^{8}	2.5×10^{8}	1.5×10^{8}	1.1×10^{8}	7.6×10^{7}	4.3×10^{7}	2.1×10^{7}	8.1×10^{6}	6.1×10^{6}	3.2×10^{6}	1.1×10^{6}	5.5×10^{5}	2.1×10^{5}
T5	2.3×10^{9}	1.1×10^{9}	5.5×10^{8}	4.0×10^{8}	1.0×10^{8}	6.6×10^{7}	3.3×10^{7}	1.1×10^{7}	9.3×10^{6}	3.7×10^{6}	2.3×10^{6}	1.1×10^{6}	7.5×10^{5}	3.5×10^{5}
	Pigeon pea													
IC-3195														
T1	7.6×10^{9}	5.9×10^{9}	3.2×10^{9}	1.7×10^{9}	1.0×10^{9}	9.0×10^{8}	8.3×10^{8}	8.1×10^{8}	7.6×10^{8}	6.3×10^{8}	2.1×10^{8}	1.2×10^{8}	4.1×10^{7}	1.4×10^{7}
T2	4.7×10^{9}	2.6×10^{9}	1.4×10^{9}	7.0×10^{8}	5.9×10^{8}	2.5×10^{8}	1.3×10^{8}	8.7×10^{7}	7.0×10^{7}	5.6×10^{7}	1.1×10^{7}	8.3×10^{6}	3.7×10^{6}	1.3×10^{6}
T3	6.8×10^{9}	1.6×10^{9}	9.0×10^{8}	5.0×10^{8}	4.0×10^{8}	1.7×10^{8}	9.7×10^{7}	5.3×10^{7}	2.1×10^{7}	1.8×10^{7}	9.3×10^{6}	7.6×10^{6}	5.0×10^{6}	1.5×10^{6}
T4	9.5×10^{9}	4.0×10^{9}	2.1×10^{9}	1.1×10^{9}	7.0×10^{8}	2.3×10^{8}	8.9×10^{7}	7.1×10^{7}	6.5×10^{7}	4.0×10^{7}	1.3×10^{7}	8.9×10^{6}	4.2×10^{6}	1.7×10^{6}
T5	8.7×10^{9}	3.4×10^{9}	1.9×10^{9}	1.0×10^{9}	8.0×10^{8}	5.6×10^{8}	2.3×10^{8}	1.0×10^{8}	9.6×10^{7}	7.0×10^{7}	3.0×10^{7}	9.1×10^{6}	5.0×10^{6}	2.1×10^{6}
IC-4062														
T1	9.9×10^{9}	8.5×10^{9}	6.3×10^{9}	2.85×10^{9}	2.3×10^{9}	1.6×10^{9}	8.6×10^{8}	4.7×10^{8}	2.5×10^{8}	1.8×10^{9}	1.1×10^{9}	6.6×10^{7}	4.6×10^{7}	2.1×10^{7}
T2	3.8×10^{9}	2.1×10^{9}	1.6×10^{9}	9.0×10^{8}	4.2×10^{8}	3.2×10^{8}	1.3×10^{8}	1.0×10^{8}	9.1×10^{7}	7.0×10^{7}	3.0×10^{7}	8.6×10^{6}	4.3×10^{6}	1.2×10^{6}
T3	2.1×10^{9}	7.9×10^{9}	6.3×10^{9}	3.6×10^{9}	1.6×10^{9}	1.1×10^{9}	8.8×10^{8}	4.7×10^{8}	3.1×10^{8}	2.4×10^{8}	1.1×10^{8}	7.8×10^{7}	5.2×10^{7}	1.6×10^{7}

(continued)

Table 3.2 (continued)

	Colony-forming unit (CFU/ml) of different carrier materials in months													
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th
T4	2.0×10^{9}	1.7×10^{9}	1.3×10^{9}	8.0×10^{8}	4.0×10^{8}	9.8×10^{7}	7.9×10^{7}	5.5×10^{7}	3.2×10^{7}	1.0×10^{7}	6.9×10^{6}	3.3×10^{6}	1.1×10^{6}	6.8×10^{5}
T5	2.0×10^{9}	2.3×10^{9}	1.8×10^{9}	1.0×10^{9}	6.6×10^{8}	9.1×10^{7}	6.3×10^{7}	4.3×10^{7}	2.7×10^{7}	1.9×10^{7}	9.4×10^{6}	7.8×10^{6}	3.3×10^{6}	1.1×10^{6}
	Groundnut													
IC-7001														
T1	9.4×10^{9}	8.0×10^{9}	7.0×10^{9}	6.3×10^{9}	1.9×10^{9}	1.0×10^{9}	9.2×10^{8}	8.3×10^{8}	6.0×10^{8}	5.6×10^{8}	2.4×10^{8}	1.1×10^{8}	7.9×10^{7}	3.4×10^{7}
T2	8.4×10^{9}	5.8×10^{9}	4.9×10^{9}	3.6×10^{9}	1.5×10^{9}	8.8×10^{8}	5.4×10^{8}	4.8×10^{8}	3.1×10^{8}	2.2×10^{8}	1.1×10^{7}	7.7×10^{7}	3.5×10^{7}	9.1×10^{6}
T3	7.3×10^{9}	4.0×10^{9}	3.6×10^{9}	3.1×10^{9}	1.8×10^{9}	5.2×10^{8}	4.2×10^{8}	3.7×10^{8}	2.5×10^{8}	2.5×10^{8}	2.1×10^{7}	1.1×10^{8}	6.7×10^{7}	3.0×10^{7}
T4	8.1×10^{9}	5.6×10^{9}	4.8×10^{9}	3.9×10^{9}	1.3×10^{9}	1.1×10^{9}	7.3×10^{8}	4.2×10^{8}	3.1×10^{8}	1.4×10^{8}	9.0×10^{7}	3.1×10^{7}	5.6×10^{6}	3.1×10^{6}
T5	10×10^{9}	9.1×10^{9}	8.1×10^{9}	5.8×10^{9}	2.8×10^{9}	9.3×10^{8}	5.2×10^{8}	3.1×10^{8}	1.5×10^{8}	8.0×10^{7}	4.8×10^{7}	1.6×10^{7}	7.9×10^{6}	2.9×10^{6}
IC-7113														
T1	8.3×10^{9}	7.9×10^{9}	4.7×10^{9}	2.65×10^{9}	2.1×10^{9}	8.8×10^{8}	5×10^{8}	4.5×10^{8}	3.7×10^{8}	2.2×10^{8}	1.2×10^{8}	8.6×10^{7}	3.9×10^{7}	1.8×10^{7}
T2	5.1×10^{9}	1.4×10^{9}	9.1×10^{8}	4.2×10^{8}	1.1×10^{8}	6.1×10^{7}	4.8×10^{7}	3.4×10^{7}	2.5×10^{7}	1.3×10^{7}	1.1×10^{7}	9.0×10^{6}	4.8×10^{6}	1.2×10^{6}
T3	4.2×10^{9}	1.4×10^{9}	5.0×10^{8}	1.2×10^{8}	9.0×10^{7}	4.0×10^{7}	2.9×10^{7}	2.3×10^{7}	2.1×10^{7}	1.6×10^{7}	8.8×10^{6}	4.3×10^{6}	1.2×10^{6}	6.1×10^{5}
T4	1.8×10^{9}	9.9×10^{8}	4.0×10^{8}	1.5×10^{8}	1.0×10^{8}	9.8×10^{7}	6.6×10^{7}	4.8×10^{7}	2.3×10^{7}	1.5×10^{7}	8.9×10^{6}	5.8×10^{6}	2.1×10^{6}	4.5×10^{5}
T5	2.8×10^{9}	1.4×10^{9}	8.8×10^{9}	4.6×10^{9}	2.2×10^{9}	6.3×10^{7}	5.4×10^{7}	3.3×10^{7}	2.5×10^{7}	1.9×10^{7}	7.6×10^{6}	3.7×10^{6}	1.2×10^{6}	5.3×10^{5}

TI, peat; T2, talc; T3, talc + starch; T4, charcoal; and T5, charcoal + sugarcane powder

3.4 Work at ICRISAT

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), based at Patancheru, Hyderabad, India, has been using peat-based rhizobial formulation for its mandate crops chickpea, pigeon pea, and groundnut. In order to find an efficient alternative carrier material, a total of six rhizobia (two specific for chickpea, IC-59, IC-76; two specific for pigeon pea, IC-3195, IC-4062; two specific for groundnut, IC-7001, IC-7113) were formulated as five different inoculants using peat, talc, talc amended with starch, charcoal, and charcoal amended with sugarcane powder, and shelf life was evaluated for a period of 13 months (Table 3.2). Among the carrier materials, peat was found to be the best as it holds 10^7 rhizobia for IC-76, IC-3195, IC-4062, IC-7001, and IC-7113 and 10^6 for IC-59 even after 13 months of storage. On the whole, the shelf life maintenance was observed in the order of peat > talc amended with starch > talc > charcoal amended with sugar cane powder > charcoal. The results also suggest that the use of proper additives to the inoculants can tremendously enhance the shelf life of the product.

3.5 Conclusions

From the literature survey, it is observed that legume inoculants gained more attention in developed countries with successful stories like soybean in Brazil, pea and lentil in Canada, and subterranean clover in Australia. In Asia, though there is a considerable interest in rhizobial inoculant development, still many factors such as undisturbed supply of good-quality carrier material, well-developed technology, quality control legislations, well-defined good manufacturing practices, training programs, well-planned field demonstrations, and governmental support for small-scale industries are creating constraints for further development. Unification of all these sectors can lead to the development of a low cost, high shelf life, and highly effective rhizobial inoculants.

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