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A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture

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Abstract: Plant growth promoting rhizobacteria are the soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere. There has been much research interest in PGPB and there is now an increasing number of PGPB being commercialized for various crops. Today a lot of efforts have been made for searching and investigating the PGPB and their mode of action, so that they can be exploited commercially as biofertilizers. Because of the various challenges faced in screening, formulation, and application, PGPB have yet to fulfill their promise and potential as commercial inoculants. Recent progress in our understanding of their diversity, colonization ability, mechanisms of action, formulation, and application should facilitate their development as reliable components in the management of sustainable agricultural systems. Several reviews have discussed specific aspects of PGPB as bioinoculants. We have tried to critically evaluate the current status of bacterial inoculants for contemporary agriculture in developed and developing countries. This review focuses on some important information regarding the biofertilizing potential of some important group of microbes, their formulations, their application for the development of sustainable technology, scope of improvement by genetic engineering, steps to be undertaken for their commercialization and their future prospects.

Keywords: Beneficial bacteria, Bioinoculants, PGPB, Carrier, Formulation, Sustainable agriculture

INTRODUCTION

Different bacterial genera are involved in various biotic activities of the soil ecosystem making it dynamic for nutrient turn over and sustainable for crop production (Ahemad and Khan, 2010a). They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (like pesticides) (Ahemad and Malik, 2011; Ahemad, 2012). Biofertilizer are defined as the preparation that contains live or latent cells of efficient strains of nitrogen fixing, phosphate solublising or cellulolytic microorganisms. On their application on seeds, soil or composting areas. the number of beneficial microorganisms increases and also enhance the rate of those microbial processes which augment the availability of nutrients that can be easily assimilated by plants. They are also called as the 'microbial inoculants'. Inoculant is the means to transport living bacteria from the factory and introduce them onto living plants, so they may produce the desired effects on plant growth (Tittabutr *et al*., 2007) which includes nitrogen fixation in legumes, biocontrol of soil-borne diseases, the enhancement of mineral uptake, weathering of soil minerals, and nutritional or hormonal effects. They also help in stimulating the plant growth hormones providing better nutrient uptake and increased tolerance towards drought and moisture stress. They don't have any ill effect on soil health and environment. A small dose of biofertilizer is sufficient to produce desirable results because each gram of carrier of biofertilizers contains at least 10 million viable cells of a specific strain (Anandaraj and Delapierre, 2010).

The most established use of bacterial inoculants is the practice of inoculating legumes with cultures of *Rhizobium* spp. During late 1970s, *Pseudomonas fluorescens* and *P. putida* groups began to be intensively investigated (Glick, 1995; Glick and Bashan, 1997) and *Azospirillum* was found to enhance the growth in the nonlegume plants (Döbereiner and Day, 1976), by directly affecting plant metabolism. In recent, years, evaluation of various other bacterial genera, such as *Bacillus*, *Flavobacterium*, *Acetobacter*, and several *Azospirillum*- related microorganisms has been done (Tang and Yang, 1997).

In the near history the first commercial preparation of PGPB entered the market but it has been a century that *Rhizobium* inoculants have been in the market place (Fages, 1992; Tang and Yang, 1997). Nobbe and Hiltner

launched 'Nitragin' in 1896 using a laboratory culture of Rhizobia, from there the commercialization of biofertilizers started followed by the discovery of *Azotobacter* and then the blue green algae and a host of other micro-organisms. *Azospirillum* and *Vesicular - Arbuscular Micorrhizae* (VAM) are fairly recent discoveries. In India the first study on legume *Rhizobium* symbiosis was conducted by Joshi (1920) and the first commercial production started as early as 1956. However, the Ministry of Agriculture under the Ninth Plan initiated the real effort to popularize and promote the input with the setting up of the National Project on Development and Use of Biofertilizers (NPDB).

ORGANISMS ACTING AS BIOINOCULANTS

The term 'rhizobacteria' implies a group of rhizosphere bacteria competent in colonizing the root environment (Kloepper *et al*., 1991). Therefore rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with roots hairs and plant-produced materials (Ahemad and Kibret, 2014). In accordance with Vessey (2003), soil bacterial species burgeoning in plant rhizosphere which grow in, on, or around plant tissues stimulate plant growth by a plethora of mechanisms are collectively known as plant growth promoting rhizobacteria (PGPR). The rhizobacteria are the dominant deriving forces in recycling the soil nutrients and consequently, they are crucial for soil fertility (Glick, 2012) they are commonly used as inoculants for improving the growth and yield of agricultural crops (Ghevariya and Desai, 2014). A number of bacterial species belonging to various genera are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth (Kumar *et al*., 2012). Nitrogen (N) is the most vital nutrient for plant growth and productivity. The atmospheric N_2 is converted into plant-utilizable forms by biological N_2 fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms (Biswas and Gresshoff, 2014). Nitrogen fixing organisms are generally categorized as (a) symbiotic N_2 fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. rhizobia) (Ahemad and Khan, 2012) and non-leguminous trees (e.g. *Frankia*) and (b) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (*Anabaena*, *Nostoc*), *Azospirillum*, *Azotobacter*, *Gluconoacetobacter diazotrophicus* and *Azocarus etc*. (Bhattacharyya and Jha, 2012). Rhizobia (including *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*) are generally regarded as microbial symbiotic partners of legumes and are mainly known for their role in the formation of nitrogen -fixing nodules (Antoun and Pre´ vost, 2005). However, non-symbiotic nitrogen fixing bacteria provide only a small amount of the fixed nitrogen that the bacterially -associated host plant requires (Glick, 2012). There are many legumes like sesbania which have a very high capacity to fix atmospheric nitrogen and support the growth of the plant (Ladha and Reddy, 1995). *Agrobacterium* which is a rhizobia related bacteria has also been found to act as a biofertilizer and help in the plant growth (Mia and Shamsuddin, 2010). Phosphate solubilizing microorganisms (PSM), are those micro-organisms that provide the available forms of P to the plants and hence are the viable substitute to chemical phosphatic fertilizers (Khan *et al*., 2009). Bacterial genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012). Vesicular Arbuscular Mycorrhiza (VAM) fungi enhances the availability of phosphorus and nitrogen to host plants and help the plant to survive under a variety of salinity stress conditions (Bargali, 2011).

The PGPR belonging to various bacterial genera are known to participate in many important biological activities (Table 1), such as the biological control of plant pathogens, nutrient cycling and seedling/plant growth (Zahir *et al*., 2004; Ahemad and Khan, 2010b) through the production of various substances. Among PGPR, *Pseudomonas* and *Bacillus* are the most commonly described genera possessing plant growth promoting activities but many other taxa are also included in PGPR group. Selected strains of PGPR are being used as seed inoculant **(**Sahin *et al*., 2004; Zahir *et al*., 2004; Rani *et al*., 2009; Ahemad and Khan, 2009; Ahemad and Khan, 2010c).

The use of PGPR to augment crop productivity has been limited largely due to the variability and inconsistency of results observed under laboratory, greenhouse and field trials. Soil is an unpredictable environment and an intended result is sometimes difficult to achieve. Climatic variations has also a large impact on the effectiveness of PGPR but sometimes unfavorable growth conditions in the field are to be expected as a normal functioning of agriculture (Zaidi *et al*., 2009). Despite all these factors, increase in crop yields following PGPR applications in the growth chambers and field trials have also been observed. Plant growth promoting traits do not work independently of each other but additively as it was suggested in the ''additive hypothesis,'' that multiple mechanisms, such as phosphate solubilization, dinitrogen fixation, ACC deaminase and antifungal activity, IAA and siderophore biosynthesis *etc*. are responsible for the plant growth promotion and increased yield (Bashan and Holguin,1997).

FORMULATIONS OF INOCULANT

In inoculant industry producing a formulation containing an effective bacterial strain is a crucial aspect and can determine the success or failure of a biological agent (Bashan, 1998). A microorganism which is functioning optimally under laboratory conditions might not be

able to produce equivalent results under field conditions after formulation production. Once an inoculant formulation which works *in situ* has been developed, it must be refined to allow for the sophistication of the end-user (Stephens and Rask, 2000). It is imperative that the formulation remain stable during production, distribution, storage, and transportation, irrespective of whether product is new or improved. The formulation produced should also be easy to handle and apply by the end users, it should be delivered to the target site in the most appropriate manner and form, it should be able to protect the agent from various harmful environmental factors, and should be able to maintain or enhance activity of the organism in the field (Jones and Burges, 1998). Another important consideration is the cost-effectiveness of the formulation it should not put much pressure on the end users financially (Xavier *et al*., 2004).

Optimal characteristics of a carrier for inoculants: The delivery vehicle of live microorganisms from the factory to the field is called carrier (Trevors *et al*., 1992). The carrier is the major portion (by volume or weight) of the inoculant that helps to deliver a suitable amount of PGPM in good physiological condition (Smith, 1992). The carrier should be designed to provide a suitable microenvironment for the PGPM and should assure a sufficient shelf life of the product (at least 2-3 months for commercial purposes, possibly at room temperature). The formulation should allow an easy dispersion or dissolution in the volume of soil near the root system. A good carrier should therefore posses as much as the following properties: good moisture absorption capacity, easy to process and free of lump-forming materials, near-sterile or easy to sterilize by autoclaving or by other methods (*e.g*., gamma-irradiation), low cost and availability in adequate amounts, and good pH buffering capacity (Keyser *et al.*, 1993). For carriers that shall be used for seed coating, a good adhesion to seeds is also important. Other characteristics that are affecting the carrier appropriateness are a standardized composition ensuring chemical and physical stability, suitability for as many PGPM species and strains as possible, the possibility of mixing with other compounds (*i.e*., nutrients or adjuvants), and being composed of biodegradable and nonpolluting compounds (Smith, 1992). In case the inoculant is used as seed coating, the carrier shall assure the survival of the PGPM on the seed since normally seeds are not immediately sown after seed coating (Muresu *et al*., 2003). A good carrier should have one essential characteristic: the capacity to deliver the right number of viable cells in good physiological condition at the right time (Smith, 1992; Trevors *et al*., 1992).

CARRIERS

Various organic carriers and inorganic substances have been used as carriers. The organic carriers suffer from the disadvantage that due to their high nutrient content, they support the growth of a large number of contaminants as well. Also, during heat sterilization of the formulations, organic carriers may release some compounds, which are toxic to bacteria resulting in low bacterial counts (Weiss *et al*., 1987). While selecting the inorganic carriers, we consider their bulk density and local availability. Any carrier-based formulation with low bulk density, such as vermiculite, will require larger packing volume per kilogram of the formulation (Saharan *et al.*, 2010).

Talc formulation: Talc, chemically referred as magnesium silicate ($Mg_3Si_4O_{10}(OH)_2$, is used as a carrier for formulation development due to its inert nature and easy availability as raw material from soapstone industries. The potential of talc to be used as a carrier was demonstrated by Kloepper and Schroth (1981). Rhizobacteria could survive in talc for 2 months. The *Fluorescent Pseudomonads* after storage for two months in talc mixture with 20% xanthum gum at 4°C did not decline in no. While *P. fluorescens* isolate Pf1could survived up to 240 days in storage (Vidhyasekaran and Muthamilan, 1995). *P. putida* strain 30 and 180 survived up to 6 months (Bora *et al*., 2004) and *B. Subtilis* survived up to 45 days in talc based formulations (Amer and Utkhede, 2000). Saravanakumar *et al*. (2007a) demonstrated that application of talc-based bioformulation of *P. fluorescens* Pf1 consistently reduced the blister blight disease and increased the yield on tea plants. The same research group has demonstrated that *P. fluorescens* Pf1 effectively controlled the dry root rot disease on mung bean plants (Saravanakumar *et al*., 2007b). Further, seed treatment, soil application and seedling dip of talc-based bioformulation of Pf1 effectively reduced the sheath rot disease on rice plants under glasshouse and field conditions (Manikandan *et al*., 2010).

Press mud formulation: Press mud is a by-product of sugar industries. Its usefulness as fertilizer is based on the nutrient content of the mud. It is rich in micronutrients and can reduce the requirement of chemical fertilizers. It also provides suitable conditions to bacteria for carrying out nitrogen fixation and phosphate solubilisation that keeps the soil healthy and develops the self-reclamation cycle. The fertilizer produced is free from all pathogens, harmful bacteria, weeds and seeds due to the high temperature produced during biocomposting. Fertilizer is easy to handle, to pack and transport. The biocompost contains 25-30% organic carbon, 1.2-2.0% nitrogen, 1.5-2.0% phosphorous and 2.5-3.0% potash (Partha and Sivasubramanian, 2006). This carrier maximizes the survival of *Azospirillum* spp. by providing favourable conditions in comparison to lignite, which is predominantly used as a carrier material in India (Muthukumarasamy *et al*., 1999).

Vermiculite formulation: Vermiculite is a naturally occurring layer silicate mineral [$(Si_3Al)Mg_3(OH)_2O_{10}.Mg_{0.5}.nH_2O$] (Alexandre-Franco *et al*., 2011; Bozzolo and Evans, 2013) and could also be considered as possible carriers, especially when the process of

their production involves the use of specific selected strains. For example, increased amount of N and P availability in the final product can be achieved by adding N-fixing and P-solubilizing bacteria to a vermicompost (Vassileva *et al*., 2010). It can be sterilized easily due to its inorganic and pre expanded nature by the common sterilization processes without the risk of producing toxic by-products or causing further structural changes. It provides enough space for microbial proliferation and also provides superior aeration due to its multilamellate structure. It is widely available and is relatively less expensive (Meisinger, 1984). So vermiculite has become a very attractive material for the inoculant production due to the various properties exhibited by it like anticrusting (Hemphill Jr., 1982), moisture-holding, and plant growth promoting (Lima *et al*., 1984; Reid *et al*., 1983) abilities. Vidhyasekaran and Muthamilan (1995) reported *P. fluorescens* (Pf1) to survive for 8 months and *B. Subtilis* for 45 days (Amer and Utkhede, 2000) in vermiculite formulation.

Peat formulations: Peat formulations have been the carriers of choice, and are the most commonly used in the *rhizobia* inoculation industry (Kaljeet *et al*., 2011). Peat is widely available and has a long history of field trials, therefore commonly used as a carrier for PGPR, particularly for rhizobia inoculants. Peat inoculant applied to the seed as slurry is the most commonly -used method to inoculate grain legumes with rhizobia (e.g. *Bradyrhizobium* spp., *Mesorhizobium* spp., *Rhizobium* spp. *etc*.). Peat slurry inoculants are made using finely -milled peat that have been sterilised by gamma irradiation and these sterilised inoculants can support high concentrations of rhizobia, generally 10^9 to 10^{10} cellsg^{-1} peat at manufacture (Hartley *et al.*, 2005). Further, the use of a sterile peat carrier significantly reduces threats to quality resulting from the presence of contaminants. Sterile peat also lowers costs of culture production by extending the broth, through culture dilution, while still achieving a higher final population density (Stephens and Rask, 2000). Further to ensure contact between the rhizobia and the legume seed coat, and to reduce rhizobial desiccation inoculation is improved by the use of adhesives and polymers (Deaker *et al*., 2004). The peat inoculant is not difficult to produce, is easy to apply and usually maintains a high concentration of viable bacteria. In this form, the bacteria are metabolically active, and bacterial multiplication continues during the storage period also as long as sufficient nutrients, moisture, and the correct temperature are maintained in some inoculants. Since peat was adopted decades ago, farmers are by now quite comfortable using it, and governmental agencies are also very familiar with how to monitor its quality.

The principle drawbacks originate from the different batches of peat and peat collected from various sources differ greatly in composition, structure, pH and microbial populations (Graham-Weiss *et al.*, 1987). Due to this the final product is greatly affected and may cause difficulties in deciding the inoculant dosage, storage conditions (Van Elsas and Heijnen, 1990), and inoculant variation in effectiveness between different manufacturers and between different batches from the same manufacturer (Bashan *et al*., 1992). Some peat has been known to contain inhibitors to *Rhizobium* strains (Brockwell, 1985). Due to organic nature of peat, it cannot be completely sterilized by steam or by gamma irradiation because toxic by-products are produced due to high temperatures and high dosage of irradiation (Mulligen and Cooper, 1985) and it also undergoes structural and compositional changes which are unfavourable for subsequent growth and survival of the bacterial spp. (Strijdom and van Rensburg, 1981). From the delivery standpoint, peat powder is easily blown away from the seeds by the commonly used seed air-delivery system used by the planter. Peat interferes with the seed monitoring mechanism of the planters which has been rectified by the addition of adhesives to peat formulations, which has also ensured enhanced seed coverage.

Other alternatives of peat: Due to increase in demand and cost rise of peat as a substrate in horticulture have led to the search of an alternative substrate which possess high quality and low cost (Gil *et al*., 2008; Moral *et al*., 2009). A number of studies have shown that organic residues such as urban solid wastes, sewage sludge, animal manure and dung, paper waste, pruning waste, spent mushroom and even green wastes, after proper composting, can be used with very good results as container growth substrates instead of peat (Bustamante *et al*., 2008; Moral *et al.,* 2009). Cattle manure compost (CMC) (Ko *et al*., 2008), freeze-dried cells (McInnes and Date, 1999) and lyophilized rhizobial cells (Caesar and Burr, 1991) can also be used as an alternatives to peat.

All of the above mentioned carriers rely on the absorption of the microorganisms by the substance/matrix of the carrier but this method has some drawbacks, particularly in context with the survival of the microorganisms and their protection during transport, storage, and handling. Nevertheless, some processes with different carriers using such approach have been patented: the patent no. 521.850 of Belgian for *Rhizobium* which uses diatomaceous earth and colloidal silica; the British patent no. 1.777.077 for the use of bentonite for *Rhizobium*; French Patent no. 1.180.000 for the *Azotobacter* group using a must juice, to which substances such as cellulose, bone meal, kaolin, or silica gel are added which has an adsorbing action; United States Patent no. 4956295 for the stabilization of dried bacteria extended in particulate carriers, where dried viable bacteria are mixed in a particulate carrier composed primarily of an inorganic salt such as sodium or calcium carbonates, bicarbonates, sulfates, or phosphates of low moisture absorbing capacity together with a minor proportion of a silica gel absorbent. Different types of carriers used for inoculant development are mentioned in table 2.

Table 1. Beneficial Interactions between plant growth promoting rhizobacteria.

PGPR	Hosts	Colonization	Plants Response	Reference(s)
Rhizobium meliloti Pseudomonas	*Medicago sativa, Medicago polymorph, Melilotus* sp., *Trigonella foenum-graecum Trigonella* sp., *Trifolium* spp	Roots	Biological nitrogen fixation	(Maheshwari *et al*., 2010 Zhao *et al*., 2012)
Pseudomonas Pseudomonas sp Strain 267 and R., *Azopirillum lipoferum Trifolium* spp	*Pisum* spp., *Lathyrus* spp. *Vicia faba*, *Vicia lentils, Lens* spp.,	Roots	BNF	(Tchebotar *et al*., 1998; (Mishra *et al*., 2011)
Pseudomonas sp *and Bacillus* sp *Rhizobium Phaseoli*	*Phaseolus vulgaris, Phaseolus coccineus, Vicia faba*	Roots	Increases nodulation in bean	(Stajkovic *et al*., 2011)
Rhizobium A. brasilene Pseudomonas, B. megaterium	*Cicer arietinum, Dactylis glomerata, Phaseolus vulgaris*	Roots	BNF and Phosphate solubilization	(Gunasekaran *et al*., 2004; (Dardanelli *et al*., 2008)
Bradyrhizobium sp., *Bacillus* sp., *Serratia marcescens*	*Vigna radiata, Arachis hypogaea, Glycine max*	Roots	BNF	(Badawi *et al*., 2011; Black *et al*., 2012)
B. japonicum Azospirillum brasilense, Pseudomonas, Bacillus subtilis, B. thuringiensis Aeromonas sp, *Serratia* sp	*Glycine max, Zea mays* L.	Rhizosphere	BNF, promote seed germination and early seedling growth .	(Bai *et al*., 2003)
AM fungi free living N_2 fixing bacteria like *Azospirillum brasilense* or *Azotobacter Pseudomomas sp*	*Pinus sabiniana, Solanum lycopersicum, Lactuca sativa, Triticum aestivum and Zea mays*	Roots	stimulates root colonization, BNF, Increases biomass, limits soil salinity stress, and affects plant yield	(Kohler *et al*., 2010)
Pseudomonas Bacillus Mycobacterium	Zea mays	Roots	stimulates plant growth , N, P & K uptake in nutrient deficient soil	(Egamberdiyeva 2007)
Bacillus substilis strain 101 *Azospirillum Brasilemse* sp.245	*Lycopersicon esculentum*	Rhizosphere	coinoculation shows more Plant height,node number total biomass	(Felici *et al*., 2008)
Pseudomonas putida.Azo -spirilium, Azotobacter	Artichoke (*cynara Scolymus)*	Rhizosphere	Phosphate solublizing bacteria along with nitrogen fixing bacteria led to significant increase in radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling vigority index, and significant decrease in mean time of germination	(Jahanian *et al*., 2012)
Pseudomonas sp.A3R3	*Allysum serpyllifolium Brassica juncea*	Rhizosphere	Increased significantly the biomass (B. juncea) and Ni content (A. serpyllifolium) in plants grown in Ni-stressed soil	(Ma *et al*., 2011)
Pseudomonas sp. SRI2 *Psychrobacter* sp. SRS8 *Bacillus* sp. SN9	*Brassica juncea, Brassica oxyrrhina*	Rhizosphere	Increased the biomass of the test plants and enhanced Ni accumulation in plant tissues	(Ma *et al*., 2009a)
Psychrobacter sp.SRA1 *Bacillus cereus* SRA10	*Brassica juncea, Brassica oxyrrhina*	Rhizosphere	Enhance the metal accumulation in plant tissue by facilitating the release of Ni from non soluble phase in the soil.	(Ma *et al*., 2009b)
Pseudomonas aeruginosa Pseudomonas fluorescens Ralstonia metallidurans	Maize	Rhizosphere	Promoted plant growth, facilitated soil metal mobilization, enhanced Cr and Pb uptake	(Braud *et al*., 2009)

Conti.

***B**radyhizobium* sp. 750 *Pseudomonas* sp., *Ochrobactrum cytisi*	*Lupinus luteus*	Rhizosphere	Increased both biomass and nitrogen content,accumulation of metals (phytostabilisation potential)	(Dary *et al*., 2010)
Pseudomonas putida CC-R2-4, Bacillus subtilis CC-pg 104	*Lectuca sativa* L.	Rhizosphere	Significant increase in shoot length and root length achieved through encapsulated inoculant	(Rekha *et al*., 2007)
Pseudomnas putida strain R-168, *Pseudomonas fluores cens* strain R-93,*Pseudomonas fluorescens* DSM 50090, *Pseudomonas putida* DSM291 *Azospirillum lipoferum* DSM 1691,*Azospirillum brasilense* DSM 1690	Maize (*Zea mays* L.)	Rhizosphere	Plant height, seed weight.no. of seeds per ear and leaf area, shoot dry weight significantly increased.	(Gholami *et al*., 2009)
Bacillus subtilis, Pseudomonas aeruginosa	*Solanum lycopersicum L(.tomato),Abelmoschus esculentus(*okra*)Amaranthus p.*(African spinach	*Rhizosphere*	*Dry biomass increased 31% for tomato, 36% for okra* 83% for African spinach	*(*Adesemoye *et al., 2008)*
Pseudomonas tolaasii ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes* sp. ZN4, *Mycobacterium* sp. ACC14	*Brassica napus*	*Rhizosphere*	Protected Canola plants against the inhibitory effects of Cadmium	(Dell' Amico *et al*., 2008)
Azotobacter chroococum Azospirillum lipoferum	Cotton (*Gossypium hirsutum* L.)	Rhizosphere	Seed yield (21%), plant height (5%) and microbial population in soil (41%) increased over their respective controls while boll weight and staple length remained statistically unaffected	(Anjum *et al*., 2007)
Bacillus subtilis, Bacillus pumis, Rhizobium sp. IC3 123	*Cajanus cajan*	Rhizosphere	Increase in plant fresh weight, chlorophyll content, nodule.	(Rajendran *et al*., 2008)
Bacillus sp. *Paenibacillus* sp.	Rice	Roots	Promote significantly root and shoot growth	(Beneduzi *et al*., 2008)
Bacillus cereus RS18 *Bacillus licheniformis* RC08	Wheat, Spinach	Roots	All bacterial strains were Effective in IAA production and significantly incresaed growth of wheat and spinach	(Cakmakci *et al*., 2007)
Xanthomonas sp. RJ3, *Azomonas* sp. RJ4 *Pseudomonas* sp. RJ10, *Bacillus* RJ31	*Brassica napus*	Rhizosphere	Stimulated plant growth and Increased cadmium accumulation	(Sheng and Xia, 2006)
Pseudomonas sp., *Bacillus* sp.	Mustard	Rhizosphere	Stimulated plant growtjh and Decreased CR (VI) Content	(Rajkumar *et al*., 2006)
Ochrobactrum Bacillus cereus	Mungbean	Rhizosphere	Lower the toxicity of cadmium to seedlings by reducing Cr (VI) to Cr (III)	(Faisal and Hasnain, 2005)
Azospirillum brasilense, Bacillus pantothenticus, Pseudomonas pieketti	Rice (Oryza sativa)	Rhizosphere	Increased rice grain yield maximum upto 76.9%	(Thakuria *et al., 2004)*
Pseudomonas fluorescens PGPR1, PGPR2, PGPR4	*Peanut (Archis hypogoea L.)*	Rhizosphere	*Significantly enhanced pod* yield,haulm yield and nodule dry weight over the control	*(Day et al*., 2004)

POLYMER- BASED POLYMERS

Alginate formulations: Alginate is the most commonly used substance for microbial cell encapsulation. It is a natural polymeric compound made up of D -mannuronic acid and L-glucuronic acid. It is derived mainly from brown macroalgae such as *Macrocystis pyrifera* (kelp), but recently it has been found that another macroalga (*Sargassum sinicola*) produce alginate of similar physical characteristics (Yabur *et al*., 2007). It is also available from several bacteria (*Pseudomonas* and *Azotobacter*) (Hay *et al*., 2010). Alginate beads generally have a diameter of 2-3mm, but microbeads with a size of 50 to 200 μm that can entrap up to 10^8 to 10^9 $CFUg^{-1}$ have also been proposed (Bashan *et al*., 2002). Different AMF structures have also been entrapped into alginate matrixes

Table 2. Carriers materials used for biofertilizers.

Carrier material	Inoculant Bacterium	Characteristics	Viability of cells per g or mL or per seed	Reference(s)
Sterilized oxalic acid industrial waste	*B. japonicum*	- seed inoculation - multiplication up to 90 days in carrier -Enhancement in Grain yield, nodule number and nitrogen content	10^9 at room Temp. 90 days	(Ajay *et al*., 1996; Rebah *et al*., 2007)
Perlite	*R. leguminosarum* bv.*phaseoli, R. tropici* and *B. Japonicum*	- soil inoculation - Can be stored in dry state without losing viability	- 6 months at 4°C and 28 °C	(Daza *et al*., 2000)
Composted sawdust	*B. japonicum, S. Meliloti M. Loti,*	- seed inoculatin -Good growth and survival of the inoculant strains	-13.9 × 10^8 at 6–9 °C, 9 months -15 × 10^9 at 6–9 °C, 9 months -7 × 10^9 at 6–9 °C, 9 months	(Kostov and Lynch, 1998)
Alginate beads	B.subtilis P.corrugata	-seed inoculants -maximal viability of bacterial inoculant -maximum no. of inoculated bacteria recovered from rhizosphere	180 days of storage at 4°C	(Pankaj *et al*., 2005)
Vermiculite (nutrient supplemented)	*B. japonicum, S. meliloti* and *R. Leguminosarum* bv. *Phaseoli*	-Seed inoculant - good survival of Temp., - can be heat sterilized -can be prepared in various sizes -can be directly released into the soil	10^8–10^9 at ambient 4 weeks	(Graham- Weiss *et al*.,1987; Sparrow and Ham, 1983 a, b)
Vermiculite	*A. lipoferum* *Chroococcum* *Megaterium* *P. fluorescens*	-Seed inoculant -good growth and survival in consortium -can be successfully employed for large scale preparation of commercial inoculant	4.32 × 10^8 cfu g^{-1} 6 months at 25-30°C 1.98 × 10^8 cfu g^{-1} - 6months at 25-30°C 1.14×10^8 cfu g^{-1} 6 months at 25-30°C 3.32×10^8 cfu g^{-1} 6 months at 25-30°C	(Sangeetha and Stella, 2012)

Conti..

Cheese whey grown cells in peat	*S. meliloti*	- seed inoculation - Better survival of temperature during storage	4.7×10^9, survived freezing at −18 °C	(Bissonnette *et al*., 1986, Bissonnette and Lalande, 1988)
Mineral Soils	*R. leguminosarum* bv.*phaseoli*	- seed inoculant -*Rhizobium* survived better at 4^0C	10^6 at 4 °C and 10^5 at 25°C,105 days viability on coated seeds: mineral soil > peat	(Chao and Alexander, 1984)
Coal (8 types)	*R. leguminosarum* bv.*phaseoli*	- seed inoculant - supports growth and survival of *R. phaseoli* strains. - 10^7 rhizobia per g after 12months	10^7 to 10^{10} at 20– 22 °C, 4 wks Viability on coated seeds: coal 10^4 < peat 10^5	(Crawford and Berryhill, 1983)
Soybean oil or peanut oil added with lyophilized cells	*Rhizobium*	- seed inoculant - Provide more protection from draught and high temperature	10^5 viable rhizobia after 56 days of incubation at 60°C	(Kremer and Peterson, 1983)
Perlite	*R. leguminosarum bv.phaseoli*, *R. Tropici* and *B. Japonicum*	- seed inoculant - Combination with sucrose adhesive gave better results - similar to peat based - inoculants in action	-6months at 4 °C -Better than peat at 28 °C, 6 mo	(Daza *et al*., 2000)
Wastewater Sludge	*Sinorhizobium meliloti*	- seed inoculant - high potential to support survival of *S. Meliloti*	1.12×10^9 (primary sludge), 2.20×109 (Secondary Sludge), 0.85×109 (Mixed Sludge)	(Rebah *et al*., 2001;Rebah *et al*., 2002a, 2002b)
Wheat bran, sugarcane Baggas	*Rhizobium*/ *Bradyrhizobium* and rock- phosphate -solubilizing fungus *Aspergillus niger*	- soil inoculant - The number of codoultured microorganisms was the highest with peat, followed by bran and sugarcane baggas.	10^5–10^6 at 30 °C, 90 d	(Muniruzzaman and Khan, 1992)
Nutrient-supplemented Pumice	*Bradyrhizobium* sp. (*Lupinus*)	- seed inoculant - good storage and handling properties and could be mixed directly with the seeds during the sowing process	10^8–10^9 at 22 °C, 35 weaks	(Einarsson *et al*., 1993; Rebah *et al*., 2007)
K-carrageenan	Yeast, *E. coli*, *Seratia marcescens*, and*Acetobacter suboxydans*	- gives higher cell density in the beads	-contains ten times more cells than the free cell -drop in the viable cells can be delayed with inclusion of 5% (w/v) tri calcium phosphate	(Pooet *et al*., 1986; Keppeler *et al*., 2009)

Conti.

Xanthan -carob Gum	*Rhizobium, Agrobacterium* and *Arthrobacter*	Provides good protection for Bacteria	viable cells of *B. japonicum* reduced from $\log_{10}$7.8–8.0 to $\log_{10}$6.0 –7.3 $ml^{-1}$of broth; after 100d at 28°C	(Mugnier and Jung, 1985; Deaker *et al*., 2004
Bacterial Flocs	*Azospirillum* and *Rhizobium*	Not studied	Not studied	(Neyra *et al*., 1995)
Polyacrylamide	*Rhizobium, Enterobacter aerogenes*	Readily available	-entrapped rhizobia survive better than peat and liquid Cultures after storage at 30 °C	(Deaker *et al*., 2004)
Alginate	*A. lipoferum* *A. chroococcum* *P.fluorescens* *B. megaterium*	-seed inoculant -good growth rate and survival in consortium	64.61×10^8 cfu g^{-1} 6 months at 25-30°C 56.81×10^8 cfu g^{-1} 6months at 25-30°C 47.83×10^8 cfu g^{-1} 6months at 25-30°C 63.89×10^8 cfu g^{-1} 6months at 25-30°C	(Sangeetha and Stella, 2012)
Alginate-based aggregate inoculant	*M. oryzae* CBMB20 *M. suomiense* CBMB120 *A. brasilense* CW903	-co-aggregated bact. inoculant of methylo bacterium sp. and A.brasilense conferred better shelf life and stress abatement in inoculated tomato plant.	Biofilm formation efficiency of CBMB20 & CBMB120 increased by 15% & 34% on co-cultivation with CW903.Co-aggregation with CW903 Enhance survivability of CBMB20	(Joe *et al*., 2014)

(Strullu and Plenchette, 1991) or in beads formed with different polymers (Vassilev *et al.*, 2005). Encapsulation of living cells in polymeric gel is a well-established technology in a wide range of different applications (Park and Chang, 2000). The gel-like matrix with its catalytic ability allows the cells to remain viable for longer duration. Moreover, alginate beads entraps sufficient number of bacteria (Zohar-Perez *et al*., 2002) which shows several advantages over free cell formulations like, it protects the bacteria from biotic stresses (Smit *et al*., 1996) and abiotic stresses such as the inhibitory effect of toxic compounds (Cassidy *et al*., 1997), enhanced survival and improved physiological activity (Weir *et al*., 1995), supply of encapsulated nutritional additives (Trevors *et al*., 1993), increased cell densities and preferential cell growth in various internal aerobic and anaerobic zones of encapsulating gel. This technology was firstly used to encapsulate the plant-beneficial bacteria like *A. brasilense* and *P. fluorescens* (Bashan, 1986), which were later successfully used to inoculate wheat plants under field conditions. The bacteria survived in the field long enough and their populations were comparable to the survival of bacteria originating from peat-based inoculants (Bashan *et al*., 1987). Inclusion of filamentous fungi such as *Aspergillus* (Jain *et al*., 2010) has been also proved possible.

Alginate beads can maintain a sufficient amount of live cells to assure inoculation up to several months (van Veen *et al*., 1997). The viability of inocula can be improved by adding some nutrients (*e.g*., skimmed milk) to the inoculum (Hernandez *et al*., 2006) or freeze-drying gel beads in presence of glycerol (Tal *et al*., 1997) .However, freeze-drying of alginate beads can result in some collapse of the matrix (Rassis *et al*., 2002) therefore, while planning this technological process some materials can be added to the mixture which can reduce the cost and/or improve the mechanical properties. Adding chitin to the beads (Zohar-Perez *et al.*, 2005) helped preserve their porous cellular structure resulting in significantly higher porosity values when compared to starch filled beads (Tal *et al.*, 1999) and resulted in higher bacterial efficacy when evaluating their effect on plants. Addition of 0.5% kaolin to freeze

Table 3: Comparison of vermiculite, peat and alginate related to production of bacterial inoculants.

Properties	Vermiculite	Peat	Alginate	Reference(s)
pH	Approximately neutral	Usually require neutralization before use as carrier	Used at low pH for cell immobilization and encapsulation	(Graham-Weiss *et al*., 1987; Pandey and Khuller, 2005)
Buffering capacity	Good	Little	Good	(Orlando *et al*., 1994; Bashan *et al*., 2002)
Toxicity	Inorganic; will not produce organic toxic products or undergo structural changes sterilization	Organic; known to occasionally contain inhibitors to bacterial strains; upon may produce toxic substances and undergo compositional and structural changes upon sterilization	Produces no environment pollution, non -toxic and biodegradable	(Bashan, 1998; Daza *et al*., 2000)
Contamination	Exfoliated at extremely high temperature which kills microorganisms; its mineral nature does not support microbial growth	Usually contains unknown microbial contaminants able to grow on organic compounds	Cannot be contaminated after Production	(Bashan, 1998; Young *et al*., 2006)
Physical properties	Multilamellate; provides good aeration, quick temp equilibration and space for microbial growth during fermentation	Not layered; structure may change at high temperature or upon exposure to strong gamma radiations acid	Physically uniform, Composed of f1-1, 4-linked D-mannuronic and L-glucuronic acid, provides good aeration, mechanical strength and protects from abiotic stress	(Klein *et al*., 1983; Hartley *et al*., 2005)
Seed sticking ability	Flaky; good sticking properties for seed coating	Powder or granular; often requires sticker to adhere to seeds	In the form of macro or microbeads; requires adhesives for the attachment to the seeds	(Graham-Weiss *et al*., 1987; Bashan *et al*., 2002)
Industrial application	Extensively used in industries for various purposes	Various successful industrial processes exist	No inexpensive industrial technology exists	(Alexandre-Franco *et al*., 2011)
Consumer friendly	Requires larger packing volume per kilogram of the formulation so a bit clumsy for the farmer	Simple to use for the farmer	Simple to use for the farmer	(Kalra *et al*., 2010; Siddiqui and Kataoka, 2011)
Storage space	Bulk	Bulk	Storage requires little space	(Saharan *et al*., 2010)

Survival Time	Long and stable survival by sustaining 86% of viable bacteria cells after 6 months of storage. Found to be one of the best carriers for *A. radiobacter* K84, *Burkholderia* sp. and *Pseudomonas* sp.	Survival of rhizobia is just long enough to soil incur root colonization	Long term survival in under water field capacity	(Deaker *et al*., 2004; Bazilah *et al*., 2011)
Mosture Retention	Good moisture retention capacity	Susceptible to moisture fluctuations	Resistant to moisture fluctuations	(Bazilah *et al*., 2011)
Nutritional supplementation	Can be supplemented with a nutrient source after sterilization	Nutritional supplements in sterile preparations only	Possible to add nutrients for auxotrophic bacteria or to accommodate special nutritional requirements of some bacteria. Nutrition also increases survival time	(Graham-Weiss *et al*., 1987)
CFU /inoculant	Formulations are found to contain at least 10^8 cfu/ cfu/ inoculant	Inoculants normally do not exceed 10^8 cfu/ inoculant	Can be loaded with a to 10^{11} cfu/ Inoculant	(Graham-Weiss *et al*., 1987)

-dried alginate-glycerol beads significantly increased bacterial survival also under UV light radiation (Zohar -Perez *et al*., 2003). Intraradical structures of *G. intraradices* embedded in alginate beads were still infective after up to 62 months after storage in plastic vials at 4°C (Plenchette and Strullu, 2003).

Recently, a process using starch industry wastewater as a carbon source for the production of *Sinorhizobium meliloti* with simultaneous formulation using alginate and soy oil as emulsifier has been proposed, showing a cell viability of more than 10^9 CFUmL^{-1} after 9 weeks of storage (Rouissi *et al*., 2010). Two patents have also been registered: French Patent application no. 77.10254 (Corresponding to U.S. Patent no. 4.155.737) which makes use of a polymer gel based on polyacrylamide gel or a silica gel for different microorganisms; the US patent 5021350 on the process for inclusion of mycorrhizae and actinorhizae in a polymer gel matrix based on at least one polymer from the polysaccharide group, with at least partial crosslinking of the polymer (Malusa *et al*., 2012)

It appears that alginate is the most promising of the encapsulating materials tested so far but it is still very premature to predict whether it will be able to displace peat in the inoculation technology due to its possible deficiencies, especially their higher price than peat. Although commercial alginate preparations are not yet available for bacterial plant inoculation, several other materials, which are used in industrial and environmental microbiology, may be considered as substitutes when the microorganism fails to adapt to alginate preparations (Table 3). To the best of our knowledge, almost none have been tested in soil or in the field.

PROMISING NEW TECHNOLOGIES

One of the methods for storing and delivering microorganisms through liquid formulations is water-in-oil emulsions (Vandergheynst *et al*., 2006). This formulation slows down water evaporation as the oil traps the water around the organism which is particularly beneficial for organisms that are sensitive to desiccation. Water-in-oil emulsions allow the addition of substances to the oil and/or aqueous phases which could improve both cell viability and release kinetics. However, one of the major issues of concern is cell sedimentation during storage. Thickening the oil phase using hydrophobic silica nanoparticles significantly reduced cell sedimentation and improved cell viability during storage (Vandergheynst *et al*., 2007).

Recently, a new process named PGSS (Particles from Gas Saturated Solutions), based on the application of supercritical fluid properties is used which is carried out at low temperatures and uses carbon dioxide as a supercritical fluid. The final product of the process is almost spherical particles that form a free-flowing powder which can be suspended in water. The possibilities of the PGSS process have already successfully been demonstrated for several solids and liquids (Cocero *et al*., 2009).

Another interesting new technology is proposing the exploitation of the natural production of bacterial biofilms as a possible carrier. Two types of biofilms are employed in that case: biofilms growing onto inert

supports (charcoal, resin, concrete, clay brick, sand particles) in which biofilms grow all around the particles, and the size of the biofilm particles grows with time usually to several mm in diameter and biofilms that are formed as a result of aggregate formation also called granular biofilm which may take from several weeks to several months (Qureshi *et al*., 2005). Application of a biofilmed inoculant containing a fungalrhizobia consortium significantly increased N_2 fixation in soybean compared to a traditional rhizobium inoculant (Jayasinghearachchi and Seneviratne, 2004). Wheat seedlings inoculated with biofilm-producing bacteria exhibited an increased yield in moderate saline soils (Ashraf *et al*., 2004). Inocula made with biofilms were shown to allow their rhizobia survive at high salinity (400 mM NaCl) by 105-fold compared to rhizobial monocultures (Seneviratne *et al*., 2008). Interestingly, beneficial endophytes were observed to produce higher acidity and plant growth-promoting hormones than their mono- or mixed cultures with no biofilm formation (Bandara *et al*., 2006).

Bionanotechnology applications which employ nanoparticles made of inorganic or organic materials could also provide new avenues for the development of carrier-based microbial inocula (Malusa *et al*., 2012). The physical stability and the high surface area of nanotubes, together with the ease and cost-effective fabrication of nanotube membranes may thus expand their use in the production of biofertilizer. The use of nanoformulations may enhance the stability of biofertilizers and biostimulators with respect to dessication, heat, and UV inactivation.

Conclusion

Microbial inoculants have long been incorporated into field practices worldwide, with satisfactory results, especially for rhizobia. The recent area of interest is the use of plant-growth promoting rhizobacteria (PGPR) as inoculants. The use of PGPRs on wheat and other cereal crops are being taken as an opportunity to be pursued for similar purposes. Yet, there is a little knowledge about the methods which are used for identifying the best bacteria for the task, and even less is known about their rhizocompetence. We need to work on the other characteristics which are required of potentially beneficial bacteria to function, and survive in their new environment. An additional challenge which remains with us is developing improved carriers that can consistently provide higher number of bacteria under field conditions and extend the shelf life of the bacteria.

During the last century, peat formulations have been developed into effective and accepted carriers, but their development has almost reached its limits. Synthetic carriers offer greater potential and flexibility for the inoculation industry but they still have to be transferred from experimental concepts into commercial inoculants. It is quite early to declare these carriers as potentially universal due to the lack of information about new developments from inoculant companies, even though they overcome many of deficiencies of peat-based inoculants. Due to the high cost of development companies are reluctant to develop synthetic inoculants for the target crop, but it might be supported by the bioremediation industry as many types of encapsulated forms of microorganisms has already been developed for bioremedation use.

The demand for natural biofertilizers is rising steadily in all parts of the world. Public awareness about the environment is increasing, while the apprehension about pollution and health hazards due to synthetic chemicals esp. in rich countries is growing (Shukla and Shukla, 2012). Significant advances has been made by some Asian countries in the development and use of biofertilizers but their potential remains largely underutilized due to the difference in efforts and experiences in different countries.

Several policy and technological gaps need to be addressed which includes: Inconsistency in efficacy toxicology and general safety including allergenic risks in inhaling pertinacious materials; the required degree of stringency of regulation; location, characterization and indexing of agents and creation of repositories; characterization of agro-ecological conditions/regions for key traits and raising the thresholds of desired traits; standard and stable products; quality control; matching performance with synthetics; bioprospecting and allied chemical profiling; scientifically sound use packages; well and joint use with the synthetics are some of the technological aspects.

Special attention should be paid to the needs and constraints of developing countries that need easy-to-use and inexpensive formulations as agriculture in developed countries is the major promoter of microbial inoculants that are environmental friendly. For the future, more research should be focused on the development of better and more economical feasible, synthetic inoculant carriers, while sustaining peat-based inoculant production for agriculture. The other options should be considered as long-term goals.

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