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Editors

Agriculturally Important Microorganisms

Commercialization and Regulatory Requirements in Asia

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Superior Polymeric Formulations and Emerging Innovative Products of Bacterial Inoculants for Sustainable Agriculture and the Environment

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Yoav Bashan, Luz E. de-Bashan, and S.R. Prabhu

Abstract

Plants have been inoculated with plant growth-promoting microorganisms to enhance crop yield and performance over four decades. The two central aspects for success of inoculation are the effectiveness of the bacterial strain and the application technology. This chapter discusses characteristics of ideal carriers for bacterial inoculants [plant growth-promoting bacteria (PGPB) and plant growth-promoting rhizobacteria (PGPR)] and focuses on superior formulations for the future, mainly polymeric and encapsulated formulations and new emerging ideas in the field of inoculation. Future research avenues are highlighted.

Keywords

Inoculants • Plant growth-promoting bacteria • PGPR • PGPB • Rhizobia

2.1 Introduction

Inoculation of plants to enhance yield of numerous crops and growth performance is an old practice. Two main factors control the success of inoculation: effectiveness of the bacterial isolate and application technology.

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In general, inoculant technology, especially with plant growth-promoting bacteria (PGPB and its biocontrol section commonly known as PGPR), has marginal impact on productivity in developing countries. This happens because inoculants are not used, are of poor quality, or are homemade (Bashan 1998; Bashan et al. 2014; Calvo et al. 2014). Probably as a result of the potential for cheaper production of inoculants by small companies, compared to expensive chemical fertilizers and pesticides dominated by the giant agro-industries in developed countries, many practical studies of numerous crops were done successfully in developing countries, showing its potential (1st, 2nd, 3rd, and 4th Asian PGPR conferences, http://www.asianpgpr.com, retrieved December 28, 2015). Most of these studies originated in the Indian subcontinent, Vietnam, with cereals and legumes in Latin America, mainly in Argentina and Mexico, and in Africa (Johri et al. 2003; Roy et al. 2015; Cong et al. 2009; Fuentes-Ramirez and Caballero-Mellado 2005; Diaz-Zorita and Fernandez-Canigia 2009; Hartmann and Bashan 2009; Atieno et al. 2012; Mathu et al. 2012). The inoculant market in developed countries had a market value of US$293 million in 2013, with a projected 9.5 % compounded annual growth rate.

The reason for using formulated inoculants is straightforward. Shortly after suspensions of bacteria are inoculated directly into the soil without a proper formulation, the bacteria population of most species of PGPB/PGPR is decimated. This result, combined with poor production of bacterial biomass, difficulty sustaining bacterial activity in the rhizosphere, and the physiological state of the bacteria at application time, can prevent the buildup of a sufficiently large PGPB population in the rhizosphere. A threshold number of viable cells, which differ with species, are essential to obtain the intended positive plant response.

The inherent heterogeneity of any soil is the key obstacle in inoculation. Introduced bacteria sometimes can find all the niches in the rhizosphere colonized by other microorganisms. These unprotected, introduced bacteria must compete with the often better-adapted native microflora and mostly cannot withstand predation by soil microfauna. As a response, a major role of any formulation is to provide a more suitable microenvironment, combined with physical protection for a prolonged time. Formulations employed in the field should be designed to provide a reliable source of bacteria that can survive in the rhizosphere and become available to crops when needed (Herrmann and Lesueur 2013; Bashan et al. 2014; Calvo et al. 2014). Although this is the main purpose of inoculant formulation, many inoculants failed to do this. A recent review pointed out the shortage of using existing biotechnological techniques for inoculant production and formulation (Vassilev et al. 2015).

In the last decade, several reviews summarized the field of plant inoculation. Most concentrated on specific genera, such as *Rhizobium* and *Azospirillum*, field performance of several PGPB, availability of various PGPBs and their modes of action, reduction in the use of fertilizers by including inoculants, and potential marketing (Stephens and Rask 2000; Catroux et al. 2001; Deaker et al. 2004; Herridge 2007; Bashan et al. 2004; Bashan and de-Bashan 2010, 2015; Pereg et al. 2016; Rizvi et al. 2009; Lodewyckx et al. 2002; Andrews et al. 2003; Vessey 2003; Lucy et al. 2004; Adesemoye and Kloepper 2009; Mathre et al. 1999; Berg 2009; Keswani

et al. 2014). Recently, in a whirl of announcements, several large agrochemical industries and microbiology-based companies declared their overlapping interests in developing microbial-based products to improve plant performance without genetic manipulations of the plants. This showed the confidence that major agribusiness and chemical companies have in the potential growth of this industry. Since 2012, multiple acquisitions, licensing agreements, and partnerships with face values worth of hundreds of millions of US dollars show the depth and breadth of investment that large companies are making in microbial product development (Fox 2015; Olson 2015). Even with all the renewed interest and investments in microbial products, the challenges of formulations, quality, and field performance are yet to be tackled (Herrmann and Lesueur 2013; Bashan et al. 2014).

Because of the common unclear literature mix between inoculants, biofertilizers, biopesticides, carriers, and formulations, in this review, "bacterial isolates" refer to specific bacterial strain of PGPB/PGPR that can promote plant growth after inoculation. "Carrier" refers to the abiotic substrate (solid, liquid, or gel) that is employed in the formulation process. "Formulation" refers to the laboratory or industrial process of unifying the carrier with the bacterial strain. "Inoculant" refers to the final product of formulation containing a carrier and bacterial agent or consortium of microorganisms.

2.2 The Ideal Inoculants

When designing an inoculant, the farmers' and the manufacturers' requirements must be considered, which are mostly complementary but not the same. In practice, and above all, farmers always seek maximum yield of their crops. The main practical features of inoculants expected by farmers are that the inoculant has to be compatible with routine field practices, such as seed disinfection and the common use of pesticides. Other important features of inoculants are (1) ease of use, (2) compatibility with the seeding equipment at the time of seeding, (3) tolerance of unintentional abuse during storage, (4) ability to work under different field conditions and types of soil, and (5) ability to help prolong survival of the inoculated bacteria for the time needed by the plant. The additional requirements of manufacturers from the same inoculant are (6) shelf life that lasts more than one growing season, (7) reproducible yield results in the field, and (8) human, animal, and plant safety required by laws. The last item is achieved by eliminating hazardous materials.

The marketplace for inoculants does not have any international standards for quality. In practice, inoculant quality can be under governmental regulations, as in the Netherlands, India, China, Thailand, Russia, Canada, France, Australia (voluntarily), and Argentina (since 2016), or leave product quality to the discretion of the manufacturer, as is common in the USA, Mexico, and the UK, claiming that the market will decide if a product survives. This ambiguity in the 1980s–1990s led to inadequate performance of commercial PGPB inoculants and subsequent abandonment of their use on a global scale (Stephens and Rask 2000; Catroux et al. 2001). Inoculants made a strong comeback since 2000, concentrated in Latin America

(Fuentes-Ramirez and Caballero-Mellado 2005; Diaz-Zorita and Fernandez-Canigia 2009; Hartmann and Bashan 2009) and Southeast Asia, mainly in India (Nguyen et al. 2003; Selvamukilan et al. 2006; Reddy and Saravanan 2013; Roy et al. 2015).

The three fundamental and essential characteristics for inoculants are to (1) support the growth of the PGPB/PGPR, (2) support the necessary number of viable microbial cells in good physiological condition for an acceptable time (Stephens and Rask 2000), and (3) deliver enough microorganisms at the time of inoculation to reach a threshold number that is usually required to obtain a response, i.e., the inoculant must contain enough viable bacteria after the formulation process (Date 2001).

In practice, the formulated carrier (inoculant) is the sole delivery vehicle of live microorganisms from the factory to the plants in the field. The carrier is the major portion (by volume and weight) of the inoculant. Carriers of inoculants can be divided into five categories: (1) peat, coal, biochar, clays, and inorganic soil; (2) waste plant materials of diverse industrial and agriculture origins; (3) inert materials, polymers, and treated rock fragments, such as vermiculite and perlite; (4) plain lyophilized microbial cultures and a mix of oil and dried bacteria (these preparations can later be incorporated into a solid carrier or used as they are); and (5) liquid inoculants, where some chemical is added to the liquid medium containing the PGPB to improve stickiness, stability, surface tension, function, and dispersal (Bashan et al. 2014; Bashan 1998).

Two different formulation types of inoculants exist, i.e., sterile or non-sterile. A sterile carrier has significant advantages of delivering the right microorganism at the precise concentration, avoiding the unpredictable potential for the indigenous organisms to suppress cell counts. Therefore, there is more control over inoculum potential; however, the sterilization process renders the inoculant far less cost-effective, especially in developing countries. Sterile and more pricy inoculants have been successfully marketed in the USA, Australia, Canada, Mexico, and Argentina.

Formulation is the crucial issue for commercial inoculants. This industrial process can determine the commercial success or failure of a biological agent that has outstanding performance under research conditions. Formulation is the industrial "witchcraft" of converting a promising laboratory-proven microorganism, cultivated by skilled specialists in carefully designed and supervised greenhouse experiments into a commercial product used by farmers under diverse and uncontrolled field conditions.

Chemical formulations in agro-products have high standards for long shelf life, ease of use, and resistance to abuse by farmers. PGPB/PGPR inoculants are expected to match them. Yet inoculants must overcome two major problems common to all living microorganisms: (1) loss of viability during storage in the farmer's warehouse and (2) long shelf life and stability at temperatures ranging from −5 to 30 °C.

Regardless of the formulation, the consistency of the inoculants can be liquid, slurry, granular, or powder (Bashan et al. 2014). The raw material of the carrier and the type of formulation vary greatly. The raw material carriers for most commercial inoculants are cheap and naturally abundant. Beside the most common material,

peat, other materials have been proposed, including bagasse, animal manure, alfalfa powder, coir dust (coco peat), biochar, perlite, rock phosphate, charcoal, and a range of coals, lignite, talc, and inorganic soil fractions, mainly clays (Bashan 1998; Stephens and Rask 2000; Bashan et al. 2014).

The five categories of desirable general characteristics for a good formulation are listed here (Bashan et al. 2014):

(1) *Chemical and physical characteristics*: The carrier of a contaminant-free inoculant should be nearly sterile or cheaply sterilized, as chemically and physically uniform as possible, of consistent batch quality, of high water-holding capacity (for wet carriers), and suitable for many bacteria species and strains of PGPB/PGPR. Consistency and availability of raw material are an absolute requirement for all carriers. Because the carrier is a major ingredient of the inoculant production, when varied, established quality control process of such inoculants cannot be adjusted for every batch of raw material during industrial production of an inoculant.
(2) *Manufacturing qualities*: Inoculants must be easily manufactured and mixed by the microbial fermentation industry. It should allow addition of nutrients, have an easily adjustable pH, and be made of a reasonably low-priced raw material with adequate supply and availability.
(3) *Farm-handling qualities*: A major concern is ease of handling, providing rapid and controlled release of bacteria into the soil, and application with standard seeding equipment. This is fundamentally important because farm practices seldom change to accommodate a new technology that delivers a high-quality inoculant with specialized machinery, especially in conservative farm areas (Date 2001).
(4) *Environmentally friendly characteristic*: Complying with contemporary environmental laws over substances that may change soil characteristics, the inoculant should be nontoxic and biodegradable, leave no carbon footprint, and be nonpolluting. Application should minimize environmental risks, such as the dispersal of cells of PGPB/PGPB to the air or groundwater.
(5) *Long-storage quality*: The inoculant must have a sufficient shelf life. One or 2 years at room temperature are often necessary for successful integration into the agricultural distribution system in developed countries (Catroux et al. 2001; Deaker et al. 2004).

Naturally, no single inoculant can have all these capacities at top-end quality. However, a good inoculant should have as many of these characteristics at a reasonably good quality. Synthesizing "super-inoculants" or finding a polymer used in more expensive industries, such as pharmaceuticals, nanotechnology, or cosmetics to accommodate all the desired features is theoretically feasible (John et al. 2011; Schoebitz et al. 2013b). So far, these formulations are produced in laboratories and have not employed in commercial products. So far, no effort to synthesize a carrier with defined superior characteristics for agricultural and environmental purposes has been reported, presumably because of high cost.

2.3 Inoculant Formulations

The performance of an inoculant is often the Achilles heel for commercialization. A microbial strain may function optimally under skillful personnel and precise laboratory conditions; yet, formulating this microorganism into an affordable product used by farmers, where similar results under real field condition are expected, is a difficult task and failure is common (Stephens and Rask 2000; Bashan et al. 2014). The literature describes many tested inoculants (Bashan 1998; Bashan et al. 2014; Calvo et al. 2014), but commercial inoculants appear in only a very few variations.

2.3.1 "Primitive" Inoculants: Raw Culture Media with No Additional Formulation

The old method of inoculating seeds and plants with bacterial culture suspension, as done since the pioneering times of plant inoculation four decades ago, still prevails today. It is a common practice among researchers because it is the least laborious and most described method in the literature.

2.3.2 Liquid Inoculants

Liquid inoculants are an improvement of "no-formulation" inoculants to address some of the limitations listed above. Basically, they are simple microbial cultures or suspensions amended with substances that may improve stickiness, stability, and dispersal abilities (Singleton et al. 2002; Bashan et al. 2014). The main advantage of these inoculants over solid inoculants is that they are easy to handle by farmers. These inoculants are very common and the preferred commercial inoculants specifically for the PGPB *Azospirillum* in the developed countries.

2.3.3 Inoculants Using Organic Carriers

Without doubt, peat is the main carrier for rhizobia in North and South America, Europe, and Australia and the main ingredient of inoculants that is sold in large volumes. It is also suitable for most other PGPB/PGPR. Yet, peat is rarely available and expensive in most of Asia and Africa. All other carriers proposed for PGPB/PGPR–rhizobia are compared with the standard peat carrier. Performance of peat-based inoculants and its shortcomings were intensively and continuously reviewed, as well as details of production of variants. Currently, technical details of the basic peat-based inoculant such as grain size, pH, optimal moisture, other amendments, quality of inoculants, quality control standards, and occupational health and safety are common knowledge (Stephens and Rask 2000; Catroux et al. 2001; Date 2001; Deaker et al. 2004, 2011; Xavier et al. 2004).

Alternatives to peat-based inoculants, popular in the 1980s–1990s, were lignite, charcoal, coir dust, composts of various origins and compositions, sugarcane filter mud, bagasse, soils mixed with various organic amendments, and vermiculite. Most were considered inferior to peat as a carrier (Bashan 1998; Singleton et al. 2002). Some organic inoculants made of some waste materials were tested with success in recent years, mainly in developing counties (Ben Rebah et al. 2007). Some were tested on a large scale and others only had inoculant production reported but not evaluation in situ (Hale et al. 2014). Examples for these formulations were described by Bashan et al. (2014). Although some organic wastes can perform equally well or better than peat as a carrier, the main limitation is the availability of sufficient raw material for an industry. Compost made from cork, bagasse, sawdust, brewery waste, or banana leaves can sustain a small, local inoculant industry where the materials are available. They cannot form the base for a large industry, especially when the raw material batch is variable.

2.3.4 Inorganic and Partly Organic Inoculants

Inorganic inoculants can be made from natural inorganic materials, natural polymers, or synthetic materials. Apart from polymeric inoculants, inorganic inoculants are the oldest version of inoculants (Bashan 1998), and a few such as clay and biochar (300 °C burnt carbon) are experimentally used for reforestation in semiarid zones and as a potential carrier for PGPB/PGPR and carrier for processing polluted water with bacteria (Hale et al. 2014; Schoebitz et al. 2014; Stelting et al. 2014). While most of these inoculants are used on a small scale for crop production, all polymeric inoculants, as far as we know, are experimental. Yet, because they open a new approach to formulation with endless industrial variations, these inoculants are described and discussed in detail in this chapter.

2.3.5 Polymeric Inoculants

Synthetic formulations based on a large variety of polymers have been continuously tested for decades because they offer substantial advantages over peat and better options for industrial production (Table 2.1). These include far longer shelf life, appropriate survival in the field, sufficient cell density, ease of manufacturing, and improved performance of plants in general (Bashan 1998; John et al. 2011; Bashan et al. 2014). So far, for agricultural and environmental uses, these polymers include alginate, agar, kappa carrageenan, pectin, chitosan, bean gum, and several proprietary polymers.

Table 2.1 A sample of polymeric formulations used for producing inoculants of plant growth-promoting bacteria for plants from 1998 to 2015

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	None	*Azospirillum brasilense; Azospirillum* combined with *Methylobacterium* sp.	Tomato	Bashan et al. (2002), Yabur et al. (2007), and Joe et al. (2014)
Alginate	None	*Azospirillum brasilense*	Several desert trees	Bashan et al. (2009a, b, 2012)
Alginate	Organic olive residue	*Azospirillum brasilense* and *Pantoea dispersa*	*Pinus halepensis*	Mengual et al. (2014)
Alginate	None	*Azospirillum brasilense; A. lipoferum; Pseudomonas fluorescens; Bacillus megaterium; Serratia marcescens; Enterobacter* sp.	Wheat; maize	Bashan and Gonzalez (1999), Bacilio et al. (2004), El-Komy (2005), Bashan et al. (2006), Schoebitz et al. (2013a, c), Ben Farhat et al. (2014), and El-Gamal et al. (2015)
Alginate	None	*Agaricus bisporus* (champignon)	*Agaricus bisporus*	Friel and McLoughlin (1999)
Alginate	None	*Chlorella vulgaris, C. sorokiniana* together with *Azospirillum brasilense, Bacillus pumilus*, or *Phyllobacterium myrsinacearum; Synechococcus elongatus* together with *A. brasilense*	Tertiary wastewater treatment	Gonzalez and Bashan (2000), Gonzalez-Bashan et al. (2000), Lebsky et al. (2001), de-Bashan et al. (2002, 2004), (2005), (2008), de-Bashan and Bashan (2004, 2008), Hernandez et al. (2009), Perez-Garcia et al. (2010), Covarrubias et al. (2012), Cruz et al. (2013), and Ruiz-Güereca and Sánchez-Saavedra (2016)

(continued)

Table 2.1 (continued)

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	None	*Pseudomonas fluorescens*	Sugar beet	Russo et al. (2001)
Alginate	None	*Pseudomonas striata; Bacillus polymyxa (PSB); Azospirillum brasilense*	None	Viveganandan and Jauhri (2000) and Cortés-Patiño and Bonilla (2015)
Alginate	None	*Glomus deserticola (AM mycorrhizae); Yarrowia lipolytica (PS-yeast)*	Tomato; faba bean	Vassilev et al. (2001) and Morsy (2015)
Alginate	None	*Pseudomonas putida*	Corn; velvet leaf	Gurley and Zdor (2005)
Alginate	None	*Rhizobium* spp.	*Leucaena leucocephala*	Forestier et al. (2001)
Alginate	Pea protein	*Bacillus subtilis*	*Brachypodium distachyon* and *Phleum pratense*	Gagné-Bourque et al. (2015)
Alginate	Kaolin, starch, talc	*Streptomyces* sp.	Tomato	Sabaratnam and Traquair (2002)
Alginate	Bentonite	*Raoultella planticola*	None	He et al. (2015)
Alginate	Attapulgite	*Pseudomonas* sp.	None	Wang et al. (2014)
Alginate	Starch	*Raoultella terrigena, Azospirillum brasilense*	None	Schoebitz et al. (2012)
Alginate	Gelatin	*Bacillus subtilis*	None	Tu et al. (2015)
Alginate	Starch	*Streptomyces* sp.	Shrub *Rhamnus lycioides*	Mengual et al. (2016)
Alginate	Humic acid	*Pseudomonas putida, Bacillus subtilis, B. megaterium, Azospirillum lipoferum*	Lettuce; rice	Rekha et al. (2007), Reetha et al. (2014), and Sivakumar et al. (2014)
Alginate	Maltodextrin	Diverse nitrogen-fixing bacteria	None	Campos et al. (2014)
Alginate	Peanut oil	*Beauveria bassiana*	Red fire ants	Bextine and Thorvilson (2002)
Alginate	Skim milk	*Bacillus subtilis* and *Pseudomonas corrugata*	Maize	Trivedi et al. (2005)

(continued)

Table 2.1 (continued)

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	Glycerol, chitin	*Pantoea agglomerans*	None	Zohar-Perez et al. (2002)
Alginate	Chitin, bran	None	Cabbage, basil, radish, wheat	Sarrocco et al. (2004)
Alginate	None	*Raoultella planticola*	Cotton	Wu et al. (2014)
Chitosan	None	Several PGPB	Tomato	Murphy et al. (2003)
Carrageenan	None	*Azospirillum brasilense*	None	Cortés-Patiño and Bonilla (2015)
Liquid culture	Alginate	*Sinorhizobium meliloti*	Alfalfa	Rouissi et al. (2014)
CMC/corn starch	MgO	Rhizobia; *Gluconacetobacter diazotrophicus; Herbaspirillum seropedicae; H. rubrisubalbicans; Azospirillum amazonense;* and *Burkholderia tropica*	Cowpea, sugarcane	Júnior et al. (2009) and da Silva et al. (2012)
HPMC	None	*Azotobacter chroococcum*	None	Rivera et al. (2014) and Rojas-Tapias et al. (2015)
Ethyl cellulose; modified starch	Silica	*Pseudomonas fluorescens*	None	Amiet Charpentier et al. (1998)

For earlier studies, see Bashan (1998). *CMC* carboxymethyl cellulose, *HPMC* hydroxypropyl methylcellulose

All these polymers share several basic requirements:

(1) Nontoxic in nature and free of harmful preservatives that affect bacteria within the inoculant and later the inoculated plants
(2) Slowly degradable in the soil by soil microorganisms, thereby gradually releasing the bacteria in needed quantities, usually at the time of seed germination and emergence of seedlings, leaving no secondary pollution
(3) Provide significant physical protection for the bacteria from soil competitors and many environmental stresses (Zohar-Perez et al. 2003; Covarrubias et al. 2012; Cruz et al. 2013)
(4) Hold sufficient moisture for survival of the bacteria
(5) Dispersible in water to allow movement of the bacteria from the polymer to the plants, when necessary

More beneficial features are that these polymeric inoculants (1) can be stored dried at various ambient temperatures for extended time without refrigeration, (2) offer a consistent batch quality for manufacturing and at the same time a better environment for the bacteria, (3) can be industrially manipulated to fit the needs of specific PGPB/PGPR, and (4) can be further amended with nutrients to improve short-term survival of the bacteria upon inoculation, which enhances the success of the inoculation process. This last feature is especially essential for associative PGPB competing in the rhizosphere with native microbes.

The major drawback of polymeric inoculants is that the raw materials for most polymers are relatively expensive, compared to peat, soil, and organic inoculants, and require further expensive handling by the industry at costs similar to those in the fermentation industry. As a direct result, so far no commercial polymeric inoculants are currently available. Yet, these inoculants may represent the future technology. A positive aspect of polymeric inoculants is that they are still the domain of research laboratories and does not have proprietary protection of private companies; hence, relatively more information is available in the scientific literature.

2.3.6 Encapsulated Formulations

The encapsulation of live microorganisms in polymers (also known as immobilization when one microorganism is used and co-immobilization when more than one organism is used) is currently experimental in the fields of agricultural and environmental bacteria inoculation technology. The fundamental industrial concept underlying immobilizing microbial cells is to entrap live microorganisms into a polymeric matrix while maintaining their viability and capacities. The encapsulated final product (bacteria–polymer) is then fermented in a bacterial growth medium for different industrial products concentrating in the production of organic acids, amino acids, enzymes, vitamins, and environmental applications, including bioremediation of toxic materials. The desired bacterial products are taken from the fermenter while fermentation continues. Primarily, immobilized microbial cells are easy to produce, store, and handle during industrial production. The main goal of industrial immobilizations is to maintain the immobilized cells in an active form, at high concentrations, for as long as possible. Any premature release of the microorganisms from these immobilized substrates is undesirable. These industrial formulations are not the topic of this chapter and can be reviewed elsewhere (Gibbs et al. 1999; Kourkoutas et al. 2004).

Immobilized PGPB/PGPR formulations for agricultural and environmental applications have at least two very distinctly different purposes from those of the fermentation industry: (a) They have to provide temporary physical protection for the immobilized PGPB/PGPR in the soil from stressful environmental conditions, microbial competitors, and mini-predators, all hostile to any change in the biological makeup of the soil, and (b) for successful root colonization, they have to release the PGPB/PGPR strain gradually. Liberation of the immobilized bacteria from

the beads occurs when the polymer is slowly degraded by the native soil microorganisms and in the process releasing the PGPB–rhizobia to the soil where plants that need inoculation are growing.

2.4 Macro- and Micro-formulations of Alginate

Through 2015, alginate derivatives are the preferred polymer for immobilization of microorganisms. The alginate formulations are used for various purposes: application of biocontrol agents and mycoherbicides, immobilization of cellular organisms and enzymes, increase of stability of recombinant plasmids in host cells, bacterial chemotaxis research, and primary polymer in formulations of drugs. Alginate is a naturally occurring polymer available worldwide, mainly from different marine macroalgae in large and sustainable quantities (Draget et al. 2002; Yabur et al. 2007), as well as from several bacteria (Sabra et al. 2001; Trujillo-Roldan et al. 2003). The preparation of beads containing PGPB/PGPR is fairly easy and straightforward and involves a multistep procedure at room temperature with minimal amounts of additional chemicals and equipment; thus, it is very popular in research. Procedures to formulate PGPB/PGPR in alginate are available (Bashan et al. 2002; de-Bashan et al. 2004, 2015; de-Bashan and Bashan 2010; Bashan and de-Bashan 2015). Occasionally, biomass of the immobilized PGPB/PGPR strain is low for the specific application; thus, a second multiplication of the immobilized PGPB/PGPR in the already-formed beads is necessary. This step is fairly simple (Bashan 1986). The advantages of alginate formulations are their nontoxic nature, biodegradability, availability at low costs (in 2015, US$ 2 per kg: Chinese product), slow release of the entrapped microorganisms into the soil that can be designed and controlled by variation in the polymeric structure, and approval for human use by the US Federal Food and Drug Administration (Bashan et al. 2002; Zohar-Perez et al. 2002).

2.4.1 Macro-alginate Beads

The technology of macro-alginate beads (1–4 mm diameter) was used to immobilize several PGPB/PGPR and mycorrhizal fungi. For example, *Streptomyces* sp. was formulated in an alginate–kaolin (aluminum silicate) carrier. Initially, the bacteria were mixed with the kaolin, then mixed with alginate, and formed into beads. Finally, the formulation was freeze-dried. This dry form was further formulated as wettable powder by adding starch, talcum, and more kaolin to improve survival of *Streptomyces* sp. in the inoculant for up to 14 weeks. These formulations yielded large variations in biocontrol efficacy of the fungal pathogen *Rhizoctonia solani* in tomato (Sabaratnam and Traquair 2002).

Encapsulation of the PGPB/PGPR *Bacillus subtilis* in alginate beads supplemented with humic acid yielded high viability of immobilized bacteria, with excellent survival after storage for 5 months. Slow release of bacteria from the bead was shown for 1 week at various levels of pH. Successful promotion of lettuce by the

encapsulated bacteria was demonstrated (Young et al. 2006). The success of this particular immobilization technique was attributed to the dual benefits of humic acid for the microbes and plant and the chemical properties of the humic acid, including easy mixing with alginate without interfering in the formation of the alginate gel beads. One benefit of humic acid in the structure of alginate bead is that it serves as a carbon source for encapsulated *Pseudomonas putida* and *B. subtilis*, which promotes survival of the encapsulated microorganisms during storage (Rekha et al. 2007). Immobilization of an encapsulated phosphate-solubilizing bacteria *Serratia* sp. was superior to a non-immobilized inoculant of the same strain on wheat plants (Schoebitz et al. 2013c). The effectiveness of free and encapsulated PGPR *Raoultella planticola* in promoting cotton growth under saline stress demonstrates that encapsulated inoculants have more positive effects on cotton seedlings than free cells (Wu et al. 2014). The efficacy of wet macrobeads of an alginate/starch mix was demonstrated when several *Streptomyces* spp. were inoculated onto a perennial shrub under revegetation conditions in the field in semiarid environment (Mengual et al. 2016).

An extended survival time in dry alginate bead was demonstrated when two PGPB, *Azospirillum brasilense* and *Pseudomonas fluorescens*, immobilized in two types of alginate bead inoculants recovered after being dried and stored at ambient temperature for 14 years. Although the populations in the beads had decreased, significant numbers survived (10^5–10^6 CFU g^{-1} beads). After inoculating wheat plants in a growth chamber, both species colonized and enhanced growth equal to those that had not been stored (Bashan and Gonzalez 1999). Vassilev et al. (2001) demonstrated that tomato plants inoculated with an AM fungus (*Glomus desert-icola*) and phosphate-solubilizing yeast (*Yarrowia lipolytica*) that were co-immobilized in alginate are a useful technique for establishing plants in nutrient-deficient soils. Several plant growth parameters were equal in treatments whether the tomatoes were inoculated with the free AM fungus or alginate-entrapped fungus, but inoculation with the fungus and the yeast produced better results.

White mushroom (*Agaricus bisporus*) cultivation was improved with wet alginate inoculant applied to spawn, providing a shorter adaptation (lag) period and higher growth rate in pasteurized compost, compared to liquid spawn and conventional commercial grain spawn. Superiority of this delivery system is attributed to high biomass loading capacity of the beads, protection of the mycelia in the bead microenvironment, and even spatial distribution of beads in the compost (Friel and McLoughlin 1999).

Protection against high temperatures can be provided by alginates. Alginate formulation supported high populations and survival of the phosphate-solubilizing bacteria *Pseudomonas striata* and *Bacillus polymyxa* at storage temperatures of 40 °C (Viveganandan and Jauhri 2000). Several macrobead alginate formulations of *B. subtilis* and *Pseudomonas corrugata* were found superior over liquid inoculants or charcoal-based inoculants for improving maize growth under low temperatures in the Indian Himalayas (Trivedi et al. 2005). Survival of the rhizobacteria *Raoultella terrigena* and *A. brasilense* during encapsulation can be improved by incorporating

starch in bead composition and using trehalose, a disaccharide, in growth culture medium (Schoebitz et al. 2012).

Another application of macro-alginate bead inoculants is in tertiary wastewater treatment by microalgae (de-Bashan and Bashan 2010; de-Bashan et al. 2015). A combination of microalgae (*Chlorella vulgaris* or *C. sorokiniana*) and microalgae growth-promoting bacterium *A. brasilense* was co-immobilized. This unique system removes phosphorus and nitrogen nutrients from municipal wastewater. Co-immobilization of the microalgae and the bacterium provided superior results in experiments over several years to remove these nutrients than the microalgae used alone (de-Bashan et al. 2002, 2004; Hernandez et al. 2006; Covarrubias et al. 2012; Cruz et al. 2013). Co-immobilization of the cyanobacteria *Synechococcus elongatus* with the bacterium *A. brasilense* removed more phosphorus from aquaculture wastewater than cyanobacteria cells that were immobilized alone (Ruiz-Güereca and Sánchez-Saavedra 2016). Leftover debris from wastewater treatment that contain alginate beads with the two microorganisms were dried and stored for a year and then used to improve growth of sorghum and enhance eroded desert soil fertility (Trejo et al. 2012; Lopez et al. 2013). Similarly, the combination of co-immobilized *A. brasilense* and *C. vulgaris* improved the growth of tomato plants under saline condition (Escalante et al. 2015).

While alginate formulation may have solved difficulties associated with common peat inoculants (Bashan 1998), the application of macro-alginate beads as inoculants has two major disadvantages. (1) An additional treatment during sowing is needed even if the inoculant is planted by the seeding machine. In developed countries, the grower who is already too busy during sowing may be short of time and reluctant to incur additional expense and time. In developing countries, the farmer might not inoculate the seeds at all. The root of this problem is insufficient agricultural education and conservative cultural traditions that make some small-scale farmers suspicious of new technologies, especially those involving live bacteria. (2) The bacteria released from the inoculant needs to migrate through the soil toward the plants. Under typical agricultural practices, when beads are loosely mixed with seeds and sown together by planters, the beads might fall up to a few centimeters from the seeds. The bacteria released from the beads must move through the soil, facing competition and predation by the native microflora, often more aggressive and better adapted to the soil than the added PGPB/PGPR. Sometimes the absence of a continuous film of water, essential for such movement, is a limiting factor. These distances, large on a microbial scale, might prove prohibitive for many added PGPB/PGPR, even *Azospirillum*, with proven motility in soil (Bashan and Levanony 1987; Bashan and Holguin 1994).

2.4.2 Alginate Microbeads

The microbead (50–200 μm in diameter or smaller) was developed to overcome the two fundamental difficulties of macrobeads. The idea considers that, if the beads are small enough but capable of encapsulating a sufficient number of bacteria, it would

be possible to produce a "powder-like" formulation similar to powdered peat inoculants. The seeds are coated with bead powder at the seed-handling facility and sold to the farmer as "improved seeds." Coated seeds with fertilizers, fungicides, or hormones are commonplace and accepted by most farmers. In developed countries with large-scale agricultural practices, pre-coated seeds eliminate an additional field treatment and provide the most convenience for growers and incentive to use. This significant benefit notwithstanding pre-coating seeds with PGPB/PGPR is not an easy industrial task, considering all the other amendments mentioned above. Some are toxic or not compatible with PGPB/PGPR. Consequently, so far it has been done only on a small experimental scale. Yet, a similar formulating idea, but with a peat inoculant, has been applied commercially for a long time as a pre-inoculation of forage legumes, such as alfalfa. The peat containing the PGPB/PGPR is applied to the seeds as slurry. Later an adhesive is added, and finally inoculated seeds are covered with finely ground calcium carbonate (Brockwell 1977).

The production of alginate microbeads is relatively simple and involves low-pressure spraying through a small nozzle, resulting in small-diameter droplets of an alginate solution mixed with liquid bacterial culture suspended in a very rich medium. These droplets, while sprayed into a slowly stirred solution of $CaCl_2$, immediately solidify into microbeads at diameters ranging between 100 and 200 μm, which entrap ~10^8–10^{10} CFU g^{-1} bacteria, similar to the population levels entrapped in alginate macrobeads (Bashan et al. 2002; Bashan 1986; Campos et al. 2014). Specialized equipment is available (Bashan et al. 2002), and commercial microbead equipment is already available. An alternative and more complicated technique to produce microbeads is that the size of calcium alginate gel beads can be controlled by applying high voltage that affects the size of the alginate solution droplets from a few millimeters to a few 100 micrometers, as voltage is increased. The droplets are then conventionally hardened with $CaCl_2$. So far, this last idea has not been applied to any useful microorganisms, apart from baker's yeast (Murakata et al. 2001). A simple alternative might be milling of dry solid sheets of alginate containing PGPB/PGPR into a powder and using an agricultural adhesive for coating seeds (Fig. 2.1).

Application of microbead alginate formulations to inoculate plants in soils includes: (1) using several transplanted desert tree species and cacti in desert reforestation programs. These successful long-term shade house (8 months) and field experiments (11 years, to 2015) used the PGPB/PGPR *Azospirillum brasilense* and phosphate-solubilizing *B. pumilus* entrapped in microbeads, where the inoculant was added to the planting holes beneath the root balls (Bashan et al. 2009a, b, 2012). (2) A field assay in a semiarid environment to assess the influence of inoculation with a mixture of two immobilized strains of PGPB/PGPR (*A. brasilense* and *Pantoea dispersa*) and olive residue on the growth of Aleppo pine *Pinus halepensis* showed that 28 months after planting, the microbial inoculation was the most effective treatment for stimulating seedling growth and absorbing nutrients. The inoculated plants had low accumulation of proline, less oxidative damage of lipids, and higher potential of water in the shoots (Mengual et al. 2014). (3) *P. fluorescens* was immobilized in 300–700 μm alginate microbeads; the survival of the

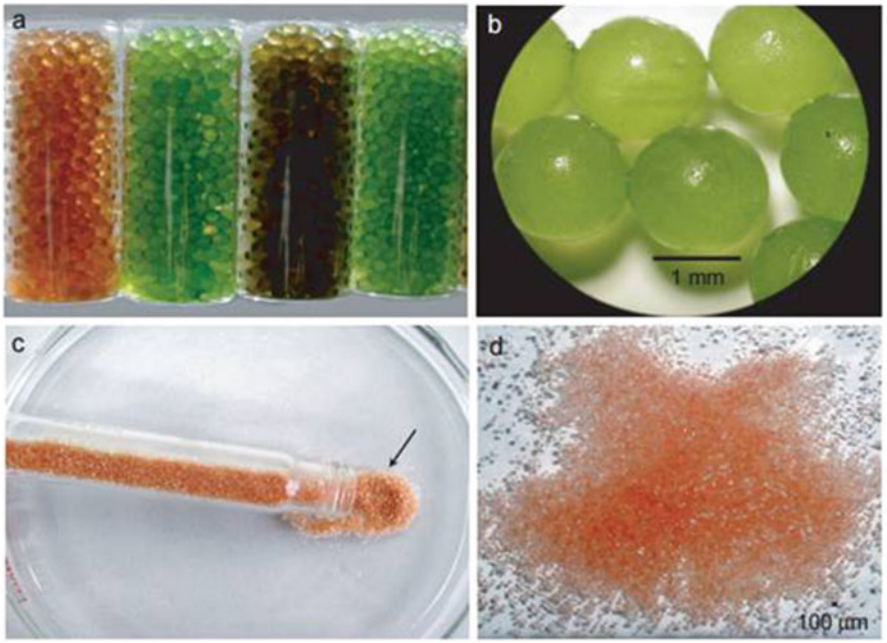

Fig. 2.1 Immobilization of microorganisms in alginate inoculants. (**a**, **b**) Wet macrobead inoculant and (**c**, **d**) dry microbead inoculant. (**a**) Inoculants of (from *left*) *Azospirillum brasilense*, *Chlorella vulgaris*, *C. vulgaris* (in alginate obtained from *Sargassum*), *C. sorokiniana*. (**b**) Macrobeads containing an association between *A. brasilense and C. sorokiniana*. (**c**, **d**) Dry alginate microbeads of *A. brasilense* (*arrow*)

microorganisms and ability to colonize sugar beet were measured after 1 year. Although dried alginate beads reduced the quantity of viable bacteria, the microbeads provided a satisfactory level of root colonization and protection against two fungal pathogens *Pythium ultimum* and *Rhizoctonia solani*. The capability of the immobilized bacteria to produce the antifungal metabolite 2,4-diacetylphloroglucinol was not affected after storage for 12 months (Russo et al. 2001). (4) Alginate beads coated with peanut oil, containing the entomopathogenic fungus *Beauveria bassiana*, were used against the red fire ant *Solenopsis invicta*. Broadcast applications and individual mound treatments with this inoculant reduced activity of the ant populations (Bextine and Thorvilson 2002).

2.4.3 Future Improvements of Micro-alginate Beads

Currently, food, pharmacology, nanotechnology, and cosmetics are far larger research fields employing immobilization than agriculture. Consequently, several technical improvements derived from these fields, aiming to make the polymer more suitable for immobilization of biological materials, were proposed. Although

these techniques were unrelated to agricultural/environmental inoculants, they offer insights for future developments (John et al. 2011; Schoebitz et al. 2013b). A few examples having potential for inoculant production are: (1) Biotin was covalently coupled with alginate in an aqueous-phase reaction that combines the advantages of alginate gelling to entrap cells to provide a gentle hydrated and highly porous environment and the high-affinity interaction of the avidin-biotin complex. The conjugate was successfully used to immobilize bioluminescent reporter cells into microbeads (Polyak et al. 2004). (2) Alginate hydrogels were reinforced at the surface with several secondary polymers to enhance mechanical strength and stability to delay degradation in soil (Bashan 1986; Nussinovitch 2010). Common alginate hydrogels were reinforced with polyethyleneimine, leading to greater elasticity than gels without polyethyleneimine. The stable interactions of the alginate and polyethyleneimine prevented alterations of the pore structure in the gels and slowed deterioration of gel properties, even under continuous agitation in a bioreactor (Kong and Mooney 2003). The high stability of barium alginate beads was improved by a multilayer coating with polyethyleneimine and polyacrylic acid (Gaumann et al. 2000). Short-chain alginate was synthesized and used for coating the membranes of microcapsules to provide high mechanical strength (Chang et al. 2002). To extend degradation time and attain maximum mechanical strength, a chitosan-alginate-$CaCl_2$ system was accomplished to produce water-insoluble membranes of biodegradable polymers (Wang et al. 2001). These ideas may need improvements to adapt them to agriculture/environmental practices. Particularly in agricultural applications, water-soluble membranes on the microcapsules are necessary to release microbes because the solvent in the soil is water. (3) A method to form macroporous beads with an interconnected pore structure in alginate was developed to improve growth and survival of microorganisms by incorporating gas pockets within the beads. This stabilized gas bubbles with surfactants and subsequently removed the gas (Eiselt et al. 2000).

In summary, based on experiments in the last three decades, it appears that alginate is the most promising polymer. However, with the relatively limited published research on alginate beads tied to agriculture/environment projects and even if the material is currently inexpensive compared to other polymers, it is premature to predict whether alginate will displace peat in the agro-inoculation industry or will remain in the industrial and environmental microbiology setting, where it runs supreme.

2.5 Polymeric Inoculants with Other Materials

Ironically, although commercial alginate preparations are not yet available for PGPB/PGPR, several other polymers that are used in industrial and environmental microbiology may be considered as substitutes when the microorganisms fail to adapt to alginate preparations. Even though all materials are still experimental, it is worth mentioning them to encourage further research on these potential carriers. Earlier cases are listed in Bashan (1998) and Bashan et al. (2014).

Formulation using chitosan as a carrier for several PGPR was mixed with soilless growth medium for successful biocontrol against cucumber mosaic virus in tomato (Murphy et al. 2003). Five PGPB were prepared into several formulations of polymers composed of carboxymethyl cellulose–starch. These formulations maintained the bacterial strains in high numbers during 60–120 days of storage. The formulations were effective in promoting the growth of sugarcane cuttings (da Silva et al. 2012). Rhizobia that were formulated with the same inoculants kept their populations inside the inoculants during 165 days of storage and were still capable of promoting growth of cowpeas (Júnior et al. 2009). *P. fluorescens* was formulated with three polymers: commercial film-forming "methacrylic acid copolymer" (Evonik Industries, Darmstadt, Germany), ethyl cellulose, and a modified starch. The best performer was the commercial polymer, where bacteria survived for 1 year. Survival of bacteria was related to the microspheres' residual moisture; the highest survival of bacteria occurred when the residual moisture was ~25 %. This inoculant was not tested on plants (Amiet Charpentier et al. 1998). With limited information on these carriers, it is impossible to predict their future as bacterial inoculants.

2.6 Dry Polymeric Carriers

A main mission of immobilizing PGPB/PGPR for agricultural and environmental uses, similar to older inoculant types, is to increase shelf life, rather than maintain a high bacterial count, since the number of microorganisms decreases during storage. From commercial and agricultural perspectives, longer survival of bacteria in these polymeric preparations makes dry formulations extremely attractive.

Several studies tested dry alginate inoculants. A microbead formulation containing *A. brasilense* was air-dried at 38 °C to form a powder; each particle contained $>10^9$ CFU g^{-1} bacteria. Alternatively, dry microbeads were produced using a standard freeze-drying procedure. The dry preparation was easily attached to dry seed surfaces by adding a lecithin or a synthetic adhesive. The bacteria in both inoculants were slowly released from the microbeads in concentrations ranging from 10^4 to 10^6 CFU g microbeads^{-1} d^{-1}, depending on the formulation and the time of incubation. Longer incubation periods led to lower numbers of bacteria being released. The dry inoculant enhanced development (dry weight, height of plants) of wheat and tomato seedlings growing in infertile soil and was biodegraded within 15 days in moist soil (Bashan et al. 2002). A similar microbead formulation used for desert reforestation with leguminous trees was air-dried in flat trays at 30 °C for 24 h without losing efficacy. The resulting effect lasted for several years in the field (Bashan et al. 2009a, b, 2012). The efficacy of freeze-dried alginate beads was tested with an agricultural strain of *Pantoea agglomerans*. The dry beads were produced with bacteria supplemented with glycerol and chitin. Glycerol increases pore size within the beads, which affects the slow-release properties, where addition of glycerol and chitin enhanced survival during the freeze-drying process. These beads were able to protect the applied PGPB/PGPR to the soil compared to bacterial suspension

(Zohar-Perez et al. 2002). Dry seed coats of various formulations of alginate, either alone or with bran and chitin additives, but not containing a PGPB, did not affect the viability or percent of germination of seeds of wheat, basil, cabbage, and radish (Sarrocco et al. 2004), but Na alginate supplied as an additive to liquid formulation without polymerization significantly extended the shelf life of *Sinorhizobium meliloti* (Rouissi et al. 2014). Dry beads (by hot temperature) made of alginate–bentonite were very efficient in slow release of the PGPR *Raoultella planticola*, but this formulation was not tested on plants (He et al. 2015). Other combinations of alginate with pea protein of *B. subtilis* protect the cells in the soil; the PGPR was capable of colonizing two model plants (Gagné-Bourque et al. 2015).

2.7 Shelf Life of Inoculants

Liquid inoculants produced in the field by in situ fermenters and immediately applied are uncommon, and only a few exist for turf grass for golf courses and hydroponic cultivation. For conventional agricultural applications, inoculants made of peat or other organic and inorganic materials are used, and a designated shelf life between manufacturing and application is usually required. The shelf life of inoculants for more than one growing season, while retaining its biological traits intact, is a major challenge for any formulation. So far, the most common solutions to this problem of extending survival time have been to (1) reduce moisture in the formulation and produce dry formulation by drying in either a fluidized bed, air-drying, or freeze-drying. These processes reduce water content in the final product or (2) store under refrigeration. In completely dry formulations, bacteria remain in a dormant form, its metabolism is very slow or even halted, and in this form, they are resistant to environmental stresses, are insensitive to contamination, and are more compatible with fertilizer applications.

The main difficulty with dry polymeric formulations is the survival of the microorganisms from the three stresses they have to endure: immobilization stress, desiccation, and rehydration before application on plant roots. During stress and storage, the mortality rate reaches >90 % of the initial incorporated population from the fermenter. The dehydration phase is perhaps the most critical and most stressful for microbes during the formulation process. This is particularly difficult for nonspore-forming Gram-negative bacteria, which are the majority of species among PGPB/PGPR. Additional stress occurs when reviving the bacteria at the time of inoculation, which produces hydration stress on the cells. Survival in formulated inoculants is affected by several factors: the culture medium used for cultivating the PGPB/PGPR, physiological state of the bacteria when harvested from the medium, process of cell immobilization, use of protective materials, type of drying procedure, and speed of dehydration. Drying during formulation is a crucial step. The highest death rate occurs either soon after manufacturing, while in storage, or immediately after application to the seeds or soil (Date 2001). Yet, if properly dehydrated, shelf life of the dried formulation is much longer than any liquid, wet, or moist inoculant.

A comparison between two PGPB/PGPR showed that using various organic, inorganic, and polymeric formulations, *Bacillus subtilis* survived at room temperature (~22 °C) for 45 days, but *P. putida* required refrigeration (~0 °C) and depends on the type of carrier that is used (Amer and Utkhede 2000). When γ-ray irradiated cork compost or perlite inoculants, with zero contamination, were stored at 25 °C, rhizobia in these inoculants remained unchanged for 90–120 days of incubation; inoculants composed of two clays maintained a high bacterial population for more than 5 months (Albareda et al. 2008). This is also typical for polymeric inoculants that are essentially sterile.

The commercial literature we found on the internet agrees that shelf life of 1–2 years under warehouse conditions for peat inoculants is desirable, even though, in practice, this is not true for many contemporary PGPB/PGPR inoculants. Storage time is even longer for polymerized and synthetic inoculants. Longer period of storage was tested on PGPB/PGPR. Dry alginate beads containing *A. brasilense* stored for 1 year at room temperature retained significant growth promotion effects on sorghum plants even though the populations of *A. brasilense* within the dry beads declined with time (Trejo et al. 2012). Identical data was found in a dry alginate–starch inoculant, where 76 % of viable cells of *A. brasilense* survived more than 1 year of storage (Schoebitz et al. 2012). Survival time of 3 years, without losing viability of the entrapped bacteria, occurred in wet alginate beads at 4 °C for *B. subtilis* and *P. corrugata* (Trivedi and Pandey 2008). The longest survival time, without losing efficacy, was 14 years for *A. brasilense* and *P. fluorescens* in dry alginate beads (Bashan and Gonzalez 1999).

In summary, a practical formulation must maintain enough viable bacteria over acceptable periods of time to ensure successful seed inoculation. Longer shelf life can be obtained by either increasing the number of microbes in the inoculant, so despite a decline in population over time, enough cells remain alive at seeding time. Alternatively, use an additive in the formulation to increase growth during storage or maintain cold storage that reduces the rate of decline in bacteria. In this case, even formulations with lower starting populations can be acceptable (Xavier et al. 2004). Yet, polymerized dry formulations are far superior for extending shelf life.

2.8 Emerging Innovative Products: Cell-free Microbial Products

The inherent limitations of live cell-based inoculants are still a strong bottleneck in widespread application of bioproducts and continue to delay commercialization and use of PGPB/PGPR on a larger scale. Evidently, there is growing interest to produce and market bioproducts that are not affected by soil and weather conditions and are capable of consistent performance. “Inoculants” made of active fragments and metabolites of PGPB/PGPR in the absence of living cells are emerging. Many elicitors of plant defenses and secondary metabolites of PGPB/PGPR are documented. These microbial products, if produced commercially, have potential as “inoculants”

for reducing agrochemical use (Compant et al. 2013). There are growing numbers of commercial, cell-free microbial products based on metabolites, which can be integrated with growers' standard practices and are generally not affected by soil or weather conditions. These products are based on the assumption that all beneficial microbes act on plants through bioactive molecules (Mabood et al. 2008; Zhuang et al. 2013). Microorganisms have been the source of some highly active biomolecules of significant commercial importance, and, due to their chemical complexity, biological fermentation processes remain the best means of production (Warrior 2000).

Several natural products from microbes, such as harpin proteins, have found commercial applications in improving crop health (Copping and Duke 2007). Lipochitooligosaccharides are nodulation factors secreted by rhizobia that induce nodules on the roots of legumes (Truchet et al. 1991). Although lipochitooligosaccharides are important signal molecules for plant–symbiont interactions, they can directly impact general nonlegume plant growth and development (Prithiviraj et al. 2003; Tanaka et al. 2015). Potential commercial lipochitooligosaccharide products for seed and foliar applications in legumes and nonlegumes, such as corn, are currently available in North America. Concentrated metabolites containing lipochitooligosaccharides, exopolysaccharides, and hormones produced from rhizobia have a shelf life of 24 months. This product improved yields of soybean and corn and is currently registered for use in Brazil (Marks et al. 2013). Lipochitooligosaccharides are known to improve plant symbiosis with vesicular–arbuscular mycorrhizal fungi (Xie et al. 1995). More practical uses of this chemical are expected in coming years.

2.9 Future Prospects

Even though the prevailing opinion in many new companies using PGPB/PGPR is that formulation is a side issue and quick to develop once the right PGPB/PGPR is selected, realistically, developing new and effective formulations for inoculation of PGPB/PGPR and rhizobia is a very slow process and consumes resources. Creation of new formulations is a challenge in practical microbiology, and shortcuts usually lead to failure of the inoculant in the field. Improvements in formulations are key to the development of enhanced high-end inoculants. Literature surveys show that the identification of new isolates having PGPB/PGPR capacities is often not difficult, and many are identified annually. Yet, development of most PGPB/PGPR strains stops there, without ever reaching the formulation stage.

While several thousand articles in the literature describe inoculation of plants with PGPB/PGPR, only a handful focused on delivery systems. Mostly, formulations and application techniques, if described at all, are hidden within the Materials and Methods section of publications on other topics. Grant applications in the developed countries regarding formulations are rarely successful. This topic, which spans fundamental microbiology and industrial technologies done simultaneously in

research facilities and commercial agricultural fields, is mostly neglected. For the future of plant inoculation, this topic cannot be ignored.

The following research topics should be top priorities of research on new or improved delivery systems for PGPB/PGPR and rhizobia, excluding peat formulations, which have already reached their peak:

- In-depth evaluation of known carriers. Periodically, a new formulation, involving a known carrier, such as alginate or liquid inoculant containing a supplement, is presented as a solution to all the maladies of previous carriers. In-depth analysis of the pros and cons for each formulation is seldom investigated and, if done by the industry, is mostly unpublished. Because numerous carrier materials of inoculation practices were proposed in recent decades, fair and honest evaluation of the most common formulations, liquid, organic wastes, and polymeric, is needed.
- Improvement of formulations that showed positive field results needs fine-tuning of key ingredients (quantities, conditions, ratio of mixtures) or improvements in the process of production. Although evaluation is assumed to be done by the industry, specific information is not available.
- Improve survival of the PGPB/PGPR in the inoculant. Reducing the decline of the population of the PGPB/PGPR during formulation and application should be a major target of research. If cell mortality can be significantly reduced, it may be possible to raise the number of cells applied per seed by two or three orders of magnitude. This includes physiological age of cells (growth phase) and relative humidity and water activity during storage, which is species dependent. Slow drying is usually superior to fast drying. Lower water activity brings better survival because it is a restraint on survival, especially at high temperatures. Optimizing the rate of rehydration should be a research target.
- Shelf life is an essential commercial concern because application time in the field is short, while production time in the factory of large quantities is long and usually cannot be done close to application time. Current shelf life is relatively short or too short. Maintaining efficacy for 2 years is optimal. Yet, various experimental formulations, such those involving alginate, show that PGPB/PGPR survival, without losing efficacy for several years, is achievable. This objective deserves better attention, even though it takes years to obtain the data.
- Multi-strain inoculants and combination of inoculants containing rhizobia and PGPB/PGPR. Numerous studies have shown the advantages of these combinations, usually demonstrated only in the laboratory without any formulation. While formulation of several microorganisms does not add additional significant technical difficulties compared to formulation of one microorganism, the interaction of the partners within these formulations is largely unknown. Development of such consortia inoculants require explorations regarding (1) compatibility between microbial populations, (2) symbiosis or interaction with plants, (3) efficiency of their resulting plant growth-promoting effects, (4) their growth rate when together, (5) potential biofilm formation, and (6) technical difficulties of culturing microorganisms in a fermenter, where each has different nutritional

requirements or other in vitro cultivation conditions (Reddy and Saravanan 2013). Two (or more) different microorganisms grown (and formulated) separately or mixed inoculant that are developed after a mixed fermentation can be developed. The former would be called "compounded inocula" while the latter "complex inocula." As multi-strain applications appear to be the current frontier for PGPB/PGPR (Vassilev et al. 2015), appropriate formulation should be explored.

- Additives to many formulations are given. However, studies of additives used in formulations have been done ad hoc and are largely empirical. Seldom is their mode of action understood. This part of the formulation process is a virgin field and deserves more attention.
- Polymeric inoculants. Even though many studies pointed out that this is the future of inoculants, no such formulation for PGPB/PGPR or rhizobia has passed the threshold of industrial approval. Immobilization of microorganisms is a large emerging field in pharmaceutical, nanotechnology, medicine, aquaculture, and cosmetics. Many different and efficient immobilization techniques were developed for those purposes [Schoebitz et al. 2013b; Bioencapsulation Innovations (http://bioencapsulation.net)]. Almost none of these technologies were tested in the inoculant field apart from simple polymerization of a few polymers. Many of these emerging technologies from other fields merit testing in the agricultural inoculant industry.
- It is doubtful that commercial farming practices will significantly change, even to accommodate a technology that delivers a high-quality inoculant. Consequently, the goal should be to create formulations that are farmer friendly, as some of the contemporary inoculants are. The best approach will be those formulations which do not require additional effort by the farmer.
- Agriculture and common environmental uses cannot use inoculants with high production costs or expensive carrier materials. Consequently, because large quantities of inoculants are used for staple food crops, cereals, legumes, and not more expensive cash crops, any inoculation technology must be developed with low costs in mind. It is highly unlikely that an outstanding formulation with a high price will find a niche.
- Local strains should be used for improved performance because no PGPB/PGPR strain can perform best under all farming conditions. Since the effectiveness of inoculation depends on multiple factors, including the target plant species and soil and weather conditions, inoculants, in theory, should also be differentiated and matched appropriately for ever-changing cultivation conditions. This situation complicates the task of providing effective inoculants because production economics dictates keeping the variety of inoculants small. This industrial requirement is contrary to the reality of diversity among crop species, inoculant species, climates, and soil biotic communities that otherwise support the production and distribution of multiple inoculants.
- For transplanted crops, inoculation in the nursery is far simpler and usually yields better results. The cost of product and its application is far less because the

volume of the inoculant is small and growth conditions are easier to control. Hence, more research and transfer of technologies should be targeted to nursery-grown plants. For example, seedling roots dipped in microbial inoculants without formulation are effective and easy for microbial inoculation of transplanted rice (Choudhury and Kennedy 2004). This treatment needs to be modified and applied to other transplanted crops.

- A number of PGPR produce lipopeptides with biosurfactant activity (Raaijmakers et al. 2010) that directly inhibit pathogens and provide systemic-induced resistance in crops (Ongena et al. 2007; Ongena and Jacques 2008). There are prospects of developing cell-free lipopeptide product biofungicides where part of the efficiency of the product is due to lipopeptides supplied along with live cells. More exploration of microbial metabolites is required to obtain more benefits from such PGPB/PGPR.
- Microbiome studies produced so far only fundamental knowledge regarding the complexity of the microbial world. Beneficial microbial strains capable of beneficially changing soil microbial community structure have been isolated (de-Bashan et al. 2010a, b; Kang et al. 2013; Lopez et al. 2013). However, this fundamental information is yet to serve as a basis for the next generation of inoculants (Berg et al. 2013, 2014; Massart et al. 2015).
- Information regarding formulation and application techniques should be precisely described in the literature. Specifically, each new manuscript should contain the precise formulation of the inoculants in quantitative details, including all non-active materials and active supplements. In cases where the inoculant is proprietary or is intellectual property, the serial registration number of the patent or the intellectual property and the country of registration should be disclosed. All bacterial species and variants, not just the genus, should be disclosed and be available from microbial collections that are available to the public. When strains are intellectual property of an organization, the name of the organization holding the rights should be disclosed. If specific sequence(s) of a strain is publically known, this should be disclosed as the definitive identification of the strain. All microbial strains in a consortium must be listed. Inoculation techniques should be described in detail sufficient to allow repetition of the experiment (Bashan et al. 2016).

In summary, formulation and field application of inoculants are a pure technological platform that is based on fundamental principles of microbiology and material sciences. The joining of these fields creates useful products that will continue to be an important input in sustainable agriculture and remedial environmental solutions.

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Dedication This chapter is dedicated to the memory of the Israeli soil microbiologist Prof. Yigal Henis (1926–2010) of the faculty of Agriculture, the Hebrew University of Jerusalem in Rehovot, Israel, one of the pioneers of studies of inoculants in Israel.

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