PRODUCTION OF BIOFERTILIZERS

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INTRODUCTION

Environmental issues, for example, freshwater contamination, energy saving, and soil erosion are compelling the farmers to present developmental strategies that have a lower polluting impact. The utilization of environmentally friendly practices is advanced by voluntary certification schemes (e.g., GlobalGAP or organic farming schemes) as well as by legally binding regulations (e.g., the EU Directive 2009/128 aiming at the implementation of sustainable pest management practices). In this context, the diminished utilization of chemical fertilizers with expanded use of organic fertilizers is viewed as compulsory route to improve the pressure on the environment derived from rural practices. In recent year's history, the chemical pesticides and fertilizers have assumed an essential part in boosting the rural development; however, they have a short history in modern agriculture. Their immediate action and low cost succeeded to bring them rapidly in to the centre of attention. On the other hand, their toxic effects on environment, plant, animal and human life diverted the focus on eco-friendly plant protection. Moreover, the development of resistance in insects against common pesticides has not been solved yet. Thus, practices such as Integrated Pest Management (IPM) have gained more importance.

Biofertilizers are vital segment of the IPM. They can be of extraordinary financial significance: they can in part replace different agrochemicals which are turning out to be

increasingly costly and their improvement is in light of expanding requests for all the more ecologically agreeable farming practices. The term "biofertilizer" commonly refers to a product containing soil microorganisms applied to plants to promote their growth. However, it has often also been wrongly used as a synonym for a wide range of products such as green or animal manure, intercropping, or organic-supplemented chemical fertilizer. Vessey (2003) defined a biofertilizer as "a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant". The microorganisms they contain are also called plant growth promoting rhizobacteria (PGPR) and result in benefits to the plant hosts after inoculation.

The enthusiasm for the utilization of these products is ascending due to the improvement in nutrient uptake efficiency and society demands for more green technologies and increased costs of agrochemicals. Moreover, biofertilizers and phytostimulators have optional helpful impacts that would increase their usefulness as bioinoculants. Indeed, microorganisms such as Rhizobium and Glomus spp. have been shown to also play a role in reducing plant diseases. The practice of inoculating plants with PGPM can be followed back to 20th century, when a product containing Rhizobium sp. was patented. Mycorrhizal fungi, even though utilized as biofertilizers since couple of decades, were reported to promote plant growth through P uptake since the late 1950s. Since then, research endeavours in these fields have consistently expanded, resulting in the selection of various strains demonstrating several beneficial characteristics.

The policies supporting sustainable rural development and broad research that has enhanced the adequacy and consistency of microbial inocula have resulted in the enrolment of several strains for both biocontrol and biofertilization, with mycorrhizal and PGPR preparations being marketed in several countries. Yet, a wider use of microbial inoculants, especially those acting as phytostimulators and biofertilizers, has been frequently hindered due to the variability and inconsistency of results between laboratory, greenhouse, and field studies. The explanation behind these discrepancies lies in the fragmented comprehension of the complex relationships established between the components of the system: the plant, the microorganisms, and the environmental conditions, particularly that of soil. In addition, the lack of correct formulations and the costly and tedious procedures of registration are also among the factors holding back the use of PGPM on a more extensive scale.

The real commercialization of PGPR began in 1995 in the USA and UK with the inoculation of legumes with rhizobia. However, the enthusiasm for other PGPR has been increased over time and a range of new products have been developed more recently. Most of the nonrhizobial PGPR inoculants currently available contain bacteria from the genus *Azospirillum* (free living N_2-fixing bacteria) or *Bacillus* (phosphate-solubilizing bacteria (PSB) and biocontrol agents. Products containing arbuscular mycorrhizal fungi (AMF) are also becoming increasingly applicable worldwide. However, the diversity of PGPR and AMF populations potentially available

in soil and the range of their modes of action are very broad and, for the vast part, incompletely understood and thus underexploited. It is also recognized that the various mechanisms involved in plant promotion may be host plant-specific and strain-specific and that the advantageous impacts may vary extraordinarily under various natural conditions. In addition, once introduced to the soil, microorganisms face competitive and often harsh conditions that may severely reduce their beneficial effects.

The four main types of formulation that have been used up to now are liquid, peat, granules, and freeze-dried powders (Fig.1). Their success relies on target crop, cost, market availability, environmental constraints, and usability. One of the real difficulties for the inoculant industry is to develop an improved formulation that combines all the above characteristics and that are suitable for use under field conditions. Moreover, while a microorganism may seem promising in laboratory, producing it commercially in order to obtain similar results under a wide range of field conditions is a difficult step. Some manufacturers included at least two types of microorganisms (e.g., rhizobia and AMF, rhizobia and PSB, various strains of AMF or PSB) in a single product, thus augmenting the subsequent benefits for the host plants. However, only a few reviews reported the positive effects of these co-inoculants. Their efficacy was not proven and their production and commercialization pose a number of technical difficulties. The most important aspect during inoculant development is assurance of the quality in a way that guarantees the reliability of the products with maximal chances for success. The absence of consistency in results obtained under field conditions because of conflicting quality has enormously influenced the commercialization of biofertilizers.

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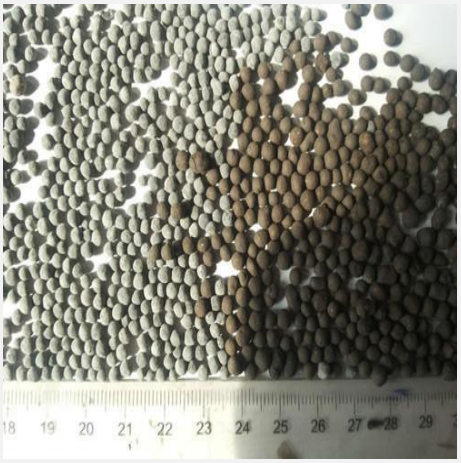

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Fig. 1. Types of biofertilizers formulations: A – liquid; B - peat, C - granules, and D – encapsulated freeze-dried powders.

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PRODUCTION OF INOCULANTS

Development of an effective inoculant represents a multistep procedure comprising the attachment of one or more strains of microorganisms in a particular carrier together with sticking agents or other additives which assure the protection of the cells during storage and transportation. Since the inoculants are often stored under non-optimum conditions (e.g., high temperature, light exposure), they must have an extended shelf life, i.e., the microorganism should be either robust or to have greater capacity to survive in high numbers under harsh conditions. A good formulation will also provide effective introduction of microorganisms in the soil and will enhance their activity in order to obtain the maximal benefits after inoculation to the host plants. To be easily accepted by the farmers, an inoculant must be cost effective and simple to deal with and use, to guarantee that the microorganisms are delivered to the target plant in the most suitable way and form. Formulation is a crucial issue and limited investigations were performed in this subject. Available data showed that since the 1980s, most *rhizobial* research are concentrated on the bacterial genetics and physiology and less than 1 % - on formulation aspects of rhizobia inoculants. In any case, there is a real need for improved formulations of inoculants, to develop and commercialize new biofertilizers that will be more successful, more stable over time, of better quality, and addressing agricultural needs.

The ideal formulation does not exist and obviously every type has its own particular advantages and constrains. However, there are some critical steps which must be precisely considered during the biofertilizers production. The choices made at these steps can lead to the success or the failure of the inoculation. The decision of the microorganisms to be inoculated is of crucial importance. Some of the most important desirable characteristics of the inoculant strain (bacterial or fungal) include its genetic stability, its ability to be beneficial for the target crops, to be competitive to the indigenous populations, to migrate from inoculation site to the hosts, and to survive in hostile soil without the presence of the host. Other important features sought during production is the ability of the strain to grow in laboratory conditions (exception is made for AMF which cannot grow without a host plant), grow or survive in carriers (during curing or storage), on seeds and in soil and to be compatible with agrochemical products that might be applied on seeds. The live inoculant must also be able to overcome the various technological processes during production and maintain its functional properties. Bacterial inoculants are generally cultivated in liquid medium to reach high biomass yields. The composition of the media and growth conditions (temperature, pH, agitation, aeration, etc.) are directly related to the physiology-biochemical properties of the particular strain and the kind of inoculant that is to be produced. Obtained bacterial cultures are then used to inoculate the different carriers (encapsulation or impregnation of peat and granules), or after addition of various additives liquid formulations could be produced. The large-scale production of bacteria in pure cultures using bioreactors is wildly spread common practice (Fig. 2).

Fig.2. Mass-production of *Azolla*

In this way, once the specific strain/s for the inoculum has been chosen, an industrial standardized procedure of production can be defined. However, for biofertilizers, dissimilar to biopesticides, the cost of production is an important limitation. This is due to the fact that the price of the biofertilizer shall not exceed that of the conventional ones. Hence, several cheap raw materials (e.g., whey, water sludges, composts, etc.) have been utilized as growth media for PGPM. Another approach to diminish the production costs is by using agro-industrial residues enriched with rock phosphate. During composting or fermentation, free or immobilized microorganisms that produce organic acids are added to the matrix, enhancing the solubilisation of phosphate and thus making it more available to plants.

Recently, the use of biofilms has also been applied as possible means to produce effective plant inocula. A biofilm comprises of microbial cells embedded into a self-produced polymeric matrix (known as an extracellular polymeric substance—EPS) and adherent to an inert or living surface, which provides structure and protection to the microbial community. Three major types of biofilms are observed in the soil: bacterial (including Actinomycetes), fungal, and fungal-bacterial biofilms). Both bacterial and fungal biofilms are formed on abiotic surfaces, while fungi act as the biotic surface in formation of fungal-bacterial biofilms. The majority of plant-associated

bacteria found on roots and in soil are forming biofilms. Therefore, applying PGPM strains that form biofilms could be a successful strategy in formulation and production of biofertilizers. While ectomycorrhizal fungi can be produced under fermentation conditions, the production of AMF inocula is much more difficult due to the need of a plant host for the multiplication of the mycorrhizal fungi. The first attempts in AMF production are based on pot cultures with soil mixtures, or aeroponics. However, the development of monoxenic cultures in the late 1980s has allowed the production of AMF under strictly controlled conditions. A method was developed for production of spores by using split-plate cultures and Ri T-DNA transformed roots of carrots. However, although the method allows production on average of 15.000 spores per Petri dish in 4-5 months after beginning the production cycle, it has been used mainly for physiological and laboratory studies. The improvement of this method was achieved through replacing the media in the distal compartment every 2 months with parallel replenishing the carbon source in the proximal compartment with glucose. Obtained results lead to the production of about 65.000 spores in 7 months. Yet, such methods are mainly used for experimental batch production of spores or for maintenance of gene banks. The reason is that the estimated annual cost for producing of one spore is up to 30–50 USD, depending on the method utilized. Recently, a large-scale in vitro production of mycorrhizal fungi, feasible for implementation on a commercial scale, has been proposed. It is based on several key points: selection of appropriate Ri T-DNA transformed host roots for different AMF species, selection and maintenance of optimal growth medium, and application of quality assurance procedures.

However, commercial inoculants containing AMF species are still produced mainly by growing host plants in controlled conditions, with the addition to the inoculant of various fungal structures (spores, mycelium hyphae) and containing mycorrhizal roots residues from the plants used as the propagating material (i.e., sorghum, maize, onion, or *Plantago lanceolata*) (Fig. 3). This could be considered a classical method where substrates of sand/soil and/or other materials (e.g., zeolite, perlite) are used to mass-produce AM fungal inoculum in pots, bags, or beds, for large-scale applications. Critical issues in this production strategy are:

(i) usage of known AMF species,

(ii) selection of host species with a short life cycle, adequate development of the root system, a good colonization level by a large range of AM fungi, and tolerance to relatively low levels of phosphorus,

(iii) control of mineral nutrients level in soil,

(iv) suitable combination of AMF species and host plant.

With this technique, it is possible to achieve inoculum densities of 80–100 thousand propagules per liter. This implies the need of diluting the inoculum with a carrier for the preparation of a commercial product.

Fig.3. *Plantago lanceolata* root nodules

Considering that microbial associations between bacteria and mycorrhizal fungi occurring naturally in the soil can promote the mycorrhizal symbiosis, it could be suggested that formulations including two or more species of different PGPM would have enhanced beneficial effect on plants. Microbial consortia can stimulate plant growth through a range of mechanisms that improve nutrient uptake and suppress fungal plant pathogens. The different approaches proposed to explain such growth stimulation are based on the increased rate of nutrients cycling. The last is due to the greater microbial content and biodiversity found in the soil where mycorrhizal plants are grown. Simultaneous inoculation with different PGPR and/or AMF often resulted in increased growth and yield, compared to single inoculation through improved nutrient uptake. Indeed, the interactions between bacteria and AM fungi have positive effect on nutrient uptake, particularly when PGPR and N2-fixing bacteria are combined. Inoculation of maize and ryegrass with *A. brasilense* and AMF resulted in N and P contents comparable to plants grown with fertilizer. Co-inoculation with different AMF species is generally more effective due to the lack of AMF fungi colonization specificity for define plant species/cultivars. Synergistic interaction between AM fungi and several PGPR, including *Azospirillum, Azotobacter, Bacillus*, and *Pseudomonas* species, has also been reported as favourable for plant growth. Improved root colonization by AMF was observed when mycorrhizal fungi were co-inoculated with such PGPR. Four times higher nodule number was reported when plants were inoculated with a mixture containing *Glomus deserticola* and *Rhizobium trifoli*, in comparison to single *R. trifoli*, inoculation, and enhanced mycorrhization and nodulation was observed with co-encapsulated *R. trifoli* and *Yarrowia lipolytica*. Inoculation with

nodule-inducing rhizobia and AM fungi resulted in increasing both P and N uptake efficiency. Application of PGPM as commercial biofertilizers containing consortia of different microorganisms often leads to diminishing the infection rate, better mineral nutrition, and increased plant growth. All these examples are are indicating the convenience and higher adequacy of biofertilizers composed by more species having different mechanisms of growth promotion. The possibility for testing of several strains of PGPR and AMF in different crops species and under different field conditions should allow the definition of consortia suitable for commercial uses.

CARRIERS

The carrier is the delivery material of live microorganisms from the processing plant to the field. It represents the major element (by volume or weight) of the inoculant and has a crucial significance in the delivery of the correct number of viable cells in good physiological condition. It provides a momentarily protective niche to microbial inoculants in soil: physically by provision of a protective surface of pore space (creating protective microhabitats) and nutritionally by provision of a particular substrate. Ideally, a good carrier possesses the following features:

- ✓ Provision of appropriate microenvironment to the target microorganism(s).
- ✓ Possession of appropriate physical and chemical properties: moisture absorption capacity (high water holding capacity), pH buffering capacity, and easy adjustable pH.
- ✓ Stability during the process: the carrier should be chemically and physically stable. It should be sterile or easy to sterilize (autoclaving or other methods), be free of protuberance materials, easily grinding and mixing with other substances (nutrients, adjuvants) using standard machinery equipment. It should also be applicable for as many bacterial or fungal species and strains as possible and simple to deal with and handle.
- ✓ Easy storage and inoculation: a good carrier should guarantee an adequate time span of usability (at least 2–3 months at room temperature), adhere well to and survive on seeds, and permit quick and controlled release of the microorganisms into the soil near the roots of the host.
- ✓ Economically and environmentally sustainable: that suggests a low cost and and reliable accessibility and quality. The carrier should be free of toxic materials, biodegradable, and non-polluting and minimize environmental risks (dispersal of cells to the atmosphere or ground water).

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Selection of a carrier defines the physical form of the inoculant and clearly there can't be a perfect and widespread carrier for all microorganisms (Table 1). The carriers can be of various origins (organic, inorganic, or synthetic) and can be classified into four main categories:

- ✓ Soils: peat, coal, clays, lignite, inorganic soil
- ✓ Plant waste materials: charcoal, composts, farmyard manure, cellulose, soybean meal, soybean and peanut oil, wheat bran, press mud, corn cobs
- ✓ Inert materials: vermiculite, perlite, ground rock phosphate, bentonite, calcium sulfate, polyacrylamide gels, alginate beads
- ✓ Plain lyophilized microbial cultures and old dried bacteria: can be later incorporated into a solid carrier or used as they are

It is also possible to obtain carriers made of a combination of the above: mixture of soil and compost, of soil, peat, bark, and husks among others. Four dispersal forms are generally used: dry inoculant (powders), slurries (powder-type inoculants suspended in liquid), granules, and liquids. Peat is the most commonly used carrier, especially for bacterial inoculants. However, it is not easily accessible worldwide and its use has a undesirable impact on the environment and ecosystem from which it is extracted. This highlights the need of development of new formulations using alternative materials to compete with the existing inoculants.

Dry inoculants (powders)

Dry inoculants are delivered using soil, organic, or inert carrier. In many parts of the world, inoculants are formulated using peat (soil carrier). Peat is made of partially decomposed flora accumulated over the years. It provides a nutritive and defensive growth environment of an extensive variety of microorganisms which can develop and form microcolonies both on the surface of the particles and in fissures. To be appropriate for inoculant use, peat must be nontoxic (for microorganisms, plant, animals, and human), highly adsorptive and easily sterilized, have a high organic matter content and water-holding capacity, and be available locally at a reasonable cost. Peat has been principally utilized because it is widely available. However, its processing is expensive as it requires several steps before it can be used as carrier for inoculant. Harvested peat must be drained and sieved to remove coarse material before it is slowly dried to around 5 % moisture. This drying step is of crucial significance since it can prompt to the formation of toxic compounds. The drying should be carried out at the lowest possible temperatures and certainly never surpass 100°C. Air drying is the preferable method instead of oven drying. The type of peat and the particle size desired defines the extent of drying. However, the moisture content must be decreased adequately to guarantee that the subsequent addition of liquid culture brings the final moisture content of the inoculant to the sought level. Once dried, peat is ground, commonly to pass through at least a 250-μm sieve. Generally, the peat deposits have a low pH, which must be

corrected to pH 6.5–7.0. The peat is then sterilized and an adequate amount of liquid inoculum is added to it.

In the case of bacterial inoculant, a final moisture content of 40–55 % is generally acceptable. Inoculated peat is incubated for a certain period to allow bacteria multiplication in the carrier. This step, also called maturing or curing is of major importance since it improves the bacteria survival rate during storage and on seeds. Peat can also be used for AMF and ectomycorrhizal inoculants though the latter are not broadly utilized, except for forest regeneration. Ectomycorrhiza generally are grown in glucose containing medium and produced spores are used for inoculation. Pure mycelia cultures are preferred as they suppress growth of pathogens and contaminants. Ectomycorrhizal inoculants may be formulated using a carrier made of vermiculite and 5–10 % peat moisturized with salts and glucose nutrient medium. This formulation provides a strong buffering capacity (keeping pH below 6) and enhances the production of fulvic acid that stimulates growth.

Table 1. Advantages and limitations of the most common carriers

Carrier	Benefits	Restrictions
Peat	➢ Suitable for a wide range of microorganisms: bacteria, AMF, ectomycorrhizal ➢ Protective nutritive environment ➢ Moisture content can be adjusted to improve growth and survival of bacteria during curing, storage, and inoculation ➢ Strong buffering capacity	➢ Not readily available ➢ Strong negative impact on the environment and the ecosystems ➢ Costly investment for extraction ➢ Toxic compounds released during drying and sterilization ➢ Highly variable in composition and quality depending on the origin ➢ Seed application: contact with other chemical compounds (toxicity)
Liquid	➢ Easy to handle and apply ➢ Easy addition of additives to improve growth or survival of the cells ➢ Composition easily defined and controlled ➢ High cells concentration → low application rates	➢ Lack carrier protection: low viability during storage and on seeds ➢ Cool temperatures for storage (4 °C) ➢ Limited shelf life ➢ More sensitive to stressful conditions
Granules	➢ Easy to store, handle, and apply ➢ Less dusty than peat ➢ Application rate easily assessed ➢ Soil application: no direct contact with the other chemical compounds (no toxicity) ➢ Especially efficient under stressful environmental conditions	➢ Bulky: high transport and storage costs ➢ Higher application rates ➢ Often nonsterile carriers
Lyophilized encapsulated cells	➢ Suitable for all types of cells (all sizes) ➢ Cells protected in a nutritive shell against mechanical and environmental stresses and against predators ➢ Slow and controlled release of the microorganisms when the shell is degraded ➢ Wide variety of polymers: nontoxic, biodegradable ➢ High concentration of cells/shell → limited space for storage ➢ Storage at room temperature (dried capsules)	➢ High production cost ➢ More handling work at the industry level ➢ Specific equipment required ➢ Physiological, morphological, and metabolic changes occurring in the shell ➢ Several applications needed if strains cannot establish in soil ➢ No commercial product available

Inoculated peat is typically applied on-site on the seeds just before sowing. The required amount of product is relatively small. However, the quantity of microorganisms used per seed is not well controlled as they are in direct contact with the other chemicals which may have been covered on the seeds. The seed coating can be done by machines (large dough, cement mixers, and mechanical tumbling machines). This procedure allows the inoculation of a large number of seeds. The significant disadvantage of peat originates from the variability in its quality and composition, which are source-dependent. Peat is an undefined and complex material and different sources will vary in their ability to support cell growth and survival. Toxic compounds might also be released during sterilization, negatively influencing the growth and survival rate of desired microorganisms. This may bring about challenges to guarantee reliable quality and results in the field, as well as to identify the optimal storage conditions, or usage instructions. Regardless of these restrictions, peat remains the standard by which every other material is judged.

Coal, clays, and inorganic soils (i.e., lapillus, volcanic pumice or diatomite earths) are available in different areas and could be utilized as carriers. Their microbial load depends on the deriving place (about 102-103 CFU g−1), but it is generally lower than in organic carriers. Vermiculite, perlite, and bentonite are also available in different countries, but their application in general is restricted due to the difficulties in preparing an effective formulation. In reality, the impact of these carriers on bacteria viability and growth is dependent on the pH, ion strength, and the electrolyte in solution. Expanded clay has been tested as a carrier for AMF and mycorrhized roots mixed with soil are also used for AMF inocula. Among other inorganic compounds, glass beads have also been proposed for AMF inocula. A mixture of organic and inorganic materials has been demonstrated successful in increasing activity and shelf life of *Burkholderia sp*. The majority of the previously mentioned carriers depend on the absorption of the microorganisms by the substance/matrix of the carrier. This strategy for incorporation has some disadvantages, especially in relation to the survival of the microorganisms and their protection during transport, storage, and handling. Nevertheless, some procedures with different carriers using such approach have been patented:

(i) the Belgian patent no. 521.850 for use of diatomaceous earth and colloidal silica for Rhizobium,

(ii) the British patent no. 1.777.077 for the use of bentonite for *Rhizobium*,

(iii) French Patent no. 1.180.000 using a must juice, to which substances with an adsorbing action are added, such as cellulose, bone meal, kaolin, or silica gel, in the manufacture of preparations rich in bacteria of the *Azotobacter* group,

(iv) 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 of low moisture absorbing capacity together with a minor proportion of a silica gel absorbent. The inorganic salts may be sodium or calcium carbonates, bicarbonates, sulfates, or phosphates.

Granules

To overcome the disadvantages in application of peat, the interest in other types of formulations and especially in granular inoculants is increasing. Granules are made of peat pill or small marble, calcite, or silica grains that are wetted with an adhesive material and then mixed with a powder-type inoculum. Thus, the granules are coated or impregnated with the target microorganism(s). The size of the granules varies, however the relation between initial microbial population density and finished product quality is direct: the better the initial microbial population, the better the product. Granules have many advantages over peat. They are less dusty and easier to handle, store, and apply. The placement and the application can be easily controlled and the limitations of seed applications are overcome: the inoculant is placed in a furrow near to the seed to facilitate lateral–root interactions but is not in direct contact with the chemicals or pesticides potentially toxic for the microorganisms. Limits in granules applications are related to the fact that they are bulkier and the transport and storage costs are therefore higher.

The prevalence of *rhizobial* granular inoculants over peat and liquid inoculants has been evaluated in several studies and obtained results are variable. A few reviews demonstrated that granular application of rhizobia did not display predominant nodulation or biological N_2 fixation compared with the other formulations (peat and seed coating), while other studies on inoculation of legumes showed that granular formulations are superior to peat-based products and liquid inoculants in terms of number of nodule formation and weight, N accumulation, N_2 fixation (% Ndfa), and total biomass generation. The benefits of using granular inoculants are particularly advantageous under soil stress conditions like high acidity, moisture stress, or cool, wet soils.

Liquid inoculants

Liquid inoculants are based on aqueous (broth cultures), mineral or organic oils, oil-in-water or polymer-based suspensions. Liquid products have been elevated as being simpler to handle and apply either on seeds or in soil. So, their ubiquity has expanded in the most recent decade. They are currently popular and have been applied for legume inoculation (in the USA and Canada for instance) due to their high cell concentrations. This characteristic allows the application of a lower quantity of inoculant for a similar efficiency. However, a number of limits blocked their utilization: inoculants based on liquid cultures lack carrier protection and quickly lose viability on the seed. They require more particular storage conditions (cool temperatures) and generally have

a limited shelf life. It was additionally revealed that liquid inoculants were more sensitive to environmental stresses and poorly survived in the carrier. Application of some other components (sucrose, glycerol, gum arabic, PVP) may improve survival of microorganisms in liquid inoculants.

Polymer-based carriers (cell immobilization)

The advance made in formulation improvement has led to new types of microorganism entrapment and immobilization processes that seem particularly promising. Immobilization encompasses the different forms of cell attachment or entrapment into a matrix. These include flocculation, adsorption on surfaces, covalent binding to carriers, cross-linking of cells, and encapsulation in a polymer gel. Encapsulation has proven to be the most promising technique for development of microbial carriers. Once encapsulated, the living cells are protected in a nutritive shell (or capsule) against mechanical and environmental stresses (such as pH, temperature, organic solvent, or poison) and predators. When placed into the soil, soil microorganisms slowly degrade the capsules and the target cells are gradually released in large quantities. Usually this happens during the time of seed germination or seedling emergence. Different kinds of cells could be encapsulated, including bacteria, fungal spores, or small hyphal segments. In this way, the encapsulation procedure represents a promising technology for development of single and multiple strain products, such as PSB–AMF or rhizobia–AMF-based ones.

Different kinds of polymers may be used for encapsulation: natural (polysaccharides, protein material) or synthetic (polyacrylamide, polyurethane) and homo-, hetero-, or co-polymers. There are more than 1,350 possible combinations of polymers which can be applied for encapsulation. Selection generally is made on the basis of their chemical composition, molecular weight (too low or too high molecular weights being considered as a disadvantage), and their ability to interact with other components. Polyacrylamide and alginate are the most commonly used polymers for cell encapsulation. However, alginate is preferred since polyacrylamide requires more specific handling precautions due to its toxicity. Alginate is a natural, biodegradable and nontoxic substrate which forms a 3D porous gel when mixed with multivalent cations (Ca^{2+}). To form beads, microorganism cells are dispersed into the polymer matrix and the mixed solution is simply dropped in the cationic solution. Nutrients and other supplements can be included to prolonged shelf life and inoculation efficacy. The beads are then dried for simplicity of packaging and handling. Different technologies are applied (including spray drying, extrusion, emulsion technique, coacervation, solvent extraction/evaporation, thermal gelation, pre-gel dissolving technique) to control the size, the shape, and the texture of the beads. Smaller beads of 10–100 μm (microencapsulation) are preferred since they offer direct contact with seeds, while macroencapsulation (larger size, extending from a few millimeters to centimeters) requires the released cells to move through the soil toward the plants.

Inclusion of bacteria in alginate beads has been used for various species, either spore forming or not. Different AMF have also been entrapped into alginate matrixes or in beads formed with different polymers. Spores of mycorrhizal fungi were entrapped in alginate film formed in a PVC coated fiberglass screen. Roots of leek seedlings inoculated with this alginate film containing *G. mosseae* spores were heavily colonized after few weeks of growth in greenhouse conditions. Similar results were obtained with spores obtained from monoxenic cultures embedded into beads. Inclusion of filamentous microorganisms such as Aspergillus and Actinomycetes has been also proved possible.

Several positive effects over free cells (conventional formulations) have been reported. Besides the cell protection provided by the shell, different studies under numerous conditions have revealed that encapsulation has numerous advantages during storage and field applications. This process is not stressful to cells, aseptic conditions minimize contamination, and the carriers are biodegradable and nontoxic. As the beads can be highly concentrated, their volume is very low, and thus, limited space for storage is required and transportation and handling are facilitated. They have an extended shelf life, can even be stored dried at room temperatures for relatively long periods, are easy to use, and are of consistent quality. When are microencapsulated the cells are distributed uniformly to the targeted site, even on small seeds, thus enhancing the application efficacy. As a result, the cell movement through soil and the possibility of off-site drift during application are significantly reduced. It was also demonstrated that encapsulation of PSB microorganisms increased their P solubilization capacity and their potential to promote plant growth compared to free cells. Limitations include a high production cost, more handling work at the industry level, and special equipment requirements. It was also mentioned that physiological, morphological, and metabolic changes may occur in encapsulated cells and that repeat applications of beads may be required since cells may not establish outside of beads.

Even though encapsulation seems to have a relative success, the vast majority of the research was performed in laboratory conditions and up to now no commercial bacterial product is available on the market. One of the explanations of the non-adoption of the technology by the inoculant industry might be the high production costs and technical handling. New technologies must remain affordable and cost effective to be easily implemented by manufacturers and farmers.

Reducing the cost of the production process and improving the quality of the beads were achieved by encapsulation and air-drying of bacteria into a mixture made of alginate (3%), standard starch (44.6%), and modified starch (2.4%). This process permits to obtain beads that after drying have a water content of 7%, size of 4 mm, and a mechanical resistance of about 105 Newton (features like that of grain seeds). Encapsulated bacteria can be stored at room temperature or at 4◦C without losing their viability - they are able to survive up to six months maintaining a final population size of about 108 CFU g−1 (corresponding to about 105 CFU bead−1). However, with this composition, some problems can arise when standardizing and automating the beads formation due to the viscosity of the mixture and the need of a continuous agitation of the stock

medium. Recently, a new procedure was proposed, using starch industry wastewater as a carbon source for the production of *Sinorhizobium meliloti* with simultaneous addition of alginate and soy oil as emulsifier. Results obtained showed a cell viability of more than 109 CFU mL−1 after 9 weeks of storage. Addition of synthetic zeolite to the alginate mixture did not improve the survival of the embedded microbial cells, nor the physical structure of the beads.

Different other polymers have also been tested with AMF. Carrageenan was used to encapsulate AMF communities while hydroxyethylcellulose was used as a gel carrier. Two patents have also been registered:

(i) 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,

(ii) 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.

Other carriers

An extensive variety of materials, both natural and artificial, have been tested and assessed as alternative carriers for diverse microorganisms. The principle drivers for the utilization of another carrier appear to be its supply and cost rather than a requirement for better quality and that works against their more widespread adoption.

Several cheap organic matrixes including water sludge, composts, sawdust, sugarcane bagasse, whey, or enriched agro-industrial residues have been proposed. Sludge wastewater might be an appropriate carrier but it contains heavy metals and this pose legal problem in respect to its utilization. Good alternative to peat is the compost from the cork industry. It is better in maintaining the survival of different rhizospheric bacteria during 6 months of storage as well as survival on seeds. However, organic composts may not be applicable for AMF formulations as they can decrease the mycorrhization rate.

Coal, clays, and inorganic soils (lapillus, volcanic pumice, or diatomite earths) can be used where available, though microbial concentration is lower than in organic carriers. In Madagascar, AMF production was done using Pouzzolane, a volcanic rock. Utilization of perlite as an inoculant gave variable outcomes. It is a suitable carrier but less efficacious than cork- and peat-based inoculants. Its effectiveness was increased when sucrose was employed as adhesive.

Gels of various chemical compositions (including magnesium silicate, fluidized bed or cellulose-based gel) is regarded as having a potential but none of them have been adopted on-farm till now.

Promising New Technologies for Carriers Development

Water-in-oil emulsions seem to be a good, yet underutilized, method for storing and delivering microorganisms through liquid formulations. The oil traps the water around the organism and, therefore, slows down water evaporation once applied. This is especially helpful when microorganisms sensitive to desiccation are used or in case of horticultural crops where irrigation systems are in place. Water-in-oil emulsions permit the addition of substances to the oil and/or aqueous phases. In this way both cell viability and release kinetics are improved. However, cell sedimentation during storage is a major issue to be considered. Several studies are carried out trying to solve this problem through application of nanomaterials. Thickening the oil phase using hydrophobic silica nanoparticles essentially diminished cell sedimentation and enhanced cell viability during storage.

Recently, a new procedure for encapsulation of virus formulations based on the application of supercritical fluid properties has been proposed. Same idea could also be applied to prepare bacterial inocula. The process, named PGSS (Particles from Gas Saturated Solutions), is carried out at low temperatures and uses carbon dioxide as a supercritical fluid. Main advantages of proposed technic would be lack of negative effects on the microorganisms' viability, and the low cost of production. 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.

Another interesting innovation is the exploitation of the natural production of bacterial biofilms as a possible carrier. It could be applied not only for the production of the bacterial inoculum but also for fungi-bacteria consortia. Biofilms are already obtained for different industrial applications (e.g., wastewater treatment, production of chemical compounds). Two types of biofilms are considered: biofilms growing onto inert supports (charcoal, resin, concrete, clay brick, and sand particles) and biofilms that are formed as a result of aggregate formation. In the first case, microorganisms grow all around the particles, and the size of the biofilm grows with time usually to several mm in diameter. Biofilms formed by aggregation is called granular biofilms and their formation may take from several weeks to several months.

There are four phases in the development of a mature biofilm: i) initial attachment, ii) irreversible attachment, iii) early development, and iv) maturation. Particularly critical is the irreversible attachment when cells bind to the surface and extracellular polymeric substances (EPS) are generated. Thus, microorganisms are protected from the surrounding environment. EPS generally are composed form polysaccharides, proteins, nucleic acids, or phospholipids. A typical EPS excreted by bacterial cells in biofilms is the exopolysaccharide alginate (Fig. 4 and 5).

The rate of biofilms formation and maturation is affected by surface, cellular, and environmental factors. Rough surfaces, porous, and less hydrophobic materials tend to improve

the biofilm formation. Biofilms tend to form more readily in the presence of optimum nutrients availability, particularly of phosphorous which increases the adhesion ability of cells. Other factors positively influencing the biofilm formation are high temperature, EPS production, and surface adhesion. Biofilm reactors can be assembled in a number of configurations including batch, continuous stirred tank, packed bed, trickling bed, fluidized bed, airlift reactors, up flow anaerobic sludge blanket, and expanded bed reactors.

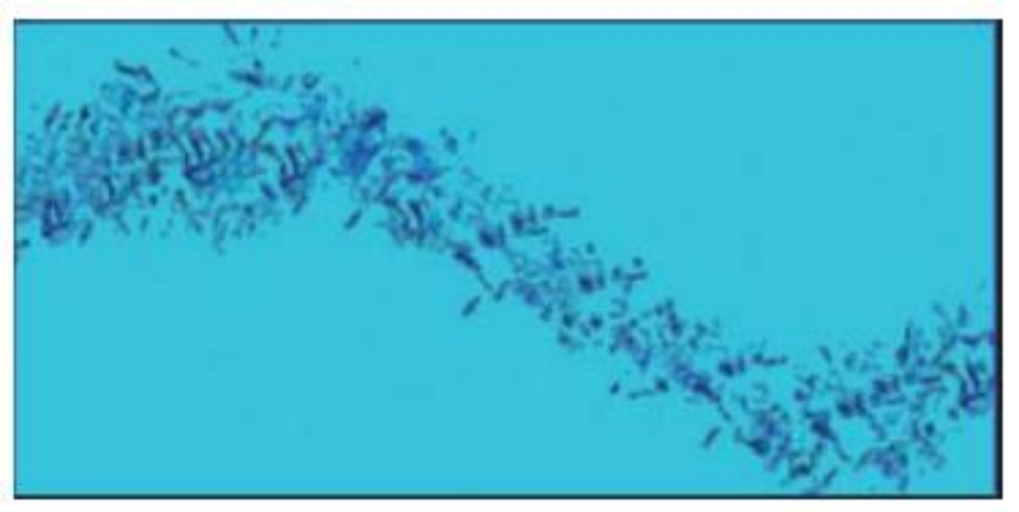

Fig. 4. Fungal –bacterial biofilm (FBB)

Fig. 5. A fungal–rhizobial biofilm (FRB) on a wheat root.

Recently, with good practical efficacy for nonlegume species biofilms were used that were developed in in vitro cultures containing both fungal and bacterial strains. Application of this biofilmed fungalrhizobia consortium led to significantly increased N2 fixation in soybean compared to a traditional rhizobium inoculant. Wheat seedlings inoculated with biofilm-producing bacteria exhibited an increased yield in moderate saline soils. Moreover, experimental data showed that biofilms protect microorganisms and assure their survival even under stress conditions. The last issue is from key importance for the effectiveness of PGPM inoculation under agricultural conditions. It was reported that biofilmed inocula allow rhizobia strains to survive at high salinity (400mM NaCl) by 105-fold compared to rhizobial monocultures. Interestingly, it was observed that beneficial endophytes in biofilms produce higher acidity and plant growth-promoting hormones than their mono- or mixed cultures.

Another new frontier in the development of carriers for PGPMs is production of hybrid materials for inoculating microorganisms. Silica has appeared as a promising host for encapsulation: technic is based on dispersing of bacterial population into a silica gel and its immobilization. Cell can be either entrapped into alginate microbeads coated with silica membranes or into macrocavities created inside the silica matrix. Such hybrid material improves the mechanical properties of the alginate bead, reduces cell leakage, and enhances cell viability.

The application of bionanotechnologies could also provide new directions in the development of carrier-based microbial inocula. Nanoparticles made of inorganic or organic materials are employed in dimensions 100 nm and less. The integration of whole cells within

hybrid nanostructures have numerous applications in many fields including agriculture. Already macroscopic filters, made of radially aligned carbon nanotube walls, able to absorb Escherichia coli, were fabricated. This technology was applied to collect bacterial cells from fermentation processes and deliver them to the plant. The physical stability and the high surface area of nanotubes, together with the ease and cost-effective fabrication of these membranes, may also expand in the production of biofertilizer.

The use of nanoformulations may improve the stability of biofertilizers and biostimulators with respect to desiccation, heat, and UV inactivation. The addition of hydrophobic silica nanoparticles of 7–14 nm to the water-in-oil emulsion formulation of the biopesticide fungus *Lagenidium giganteum* reduces the desiccation of the mycelium. The physical features of the formulation are improved and the microorganism are still viable and active after 12 weeks of storage at room temperature.

STICKERS

Often in peat sticking agents are incorporated thus enhancing its uniformity of coverage on seed. The adhesives used in current agricultural practices are different polymers: polysaccharides (such as gum arabic or carboxymethylcellulose), polyalcohol derivatives, or caseinate salts. Important prerequisites are:

- nontoxic to seed or microorganisms,
- easily dispersible in water
- offering a better adhesion and survival to microorganisms on seed.

They have been for the most part for their ability to maintain the viability of rhizobia on the legume seed. However, little is known about the exact mechanisms responsible for the assurance of the enhanced survival by these polymers. The significant disadvantage is that, when applied with stickers, more peat is retained on the seed coat, resulting in a more extended time of contact between the bacteria and the toxic compounds of the coat.

ADDITIVES

Other materials added to the inoculant formulation include macro- and micronutrients, carbon or mineral sources, hormones, and even fungicides. The aim is to supply microorganisms

with protective and/or a nutrient source, to assure better adhesion to seed thus improving the inoculant quality, to make the product more stable, to inactivate the toxins, or to enhance the strain(s) survival during storage and after exposure to environmental stress conditions (high temperature, desiccation). There is a critical interrelation between the strains survival rate and used additives. Some of them (such as glycerol) improve cell viability by protecting cells from desiccation through holding considerable amounts of water. Thus, the drying rate is significantly reduced. Each additive should be selected for individual strain in order to provide maximal performance. Moreover, their chemical nature should be complex to prevent them from rapid degradation. Several components have been tested, such as clay and skim milk, xanthan, or sodium alginate with variable results on strain(s) survival during storage and field application. Furthermore, certain signaling molecules added in the growth media and inoculants have been shown to provoke desired physiological activities of used microorganisms. Recently, it was reported that some *rhizobial* metabolites enhance the performance of *Bradyrhizobium spp*. and *Azospirillum brasilense* inoculants when soybean and maize are treated. These metabolites include mainly lipochitooligosaccharides (LCOs also called Nod factors) but also exopolysaccharides and plant hormones. Nod factors were shown to be produced by most rhizobia and are mandatory for the root legume infection and nodule formation. To our knowledge, the use of signaling molecules for improving the crop performance is still limited. However, several legume inoculants containing stimulators of nodulation (flavonoids or Nod factors) are commercially available in North and South America. Stimulators of the mycorrhizal symbiosis have also been identified. Strigolactones are of fundamental and practical interest as they are supposed to play a key role in the establishment of the mycorrhizal symbiosis. It was reported that they act as hormones in plants, and they may also have a role in the presymbiotic growth of AMF. Application to crops could result in beneficial effects on plant development. However, more investigations are needed to assess the potential of these stimulators for the development of a new generation of mycorrhizal inoculants.

PACKAGING

Packaging material is another important issue to be consider when biofertilizer is produced as it can affect inoculant quality. It must allow some exchange of oxygen but restrict the passage of water. Particular care must be taken when choosing a material for a product that is supposed to be sterilized. Some materials are suitable for autoclaving but might break during irradiation and vice versa.

REFERENCES

1. Herrmann L & Lesueur D. Challenges of formulation and quality of biofertilizers for successful inoculation. Appl Microbiol Biotechnol, 2013, 97:8859–8873

2. Malus E, Sas-Paszt L, Ciesielska J. Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. The Scientific World Journal, 2012, Article ID 491206

3. Yadav AK, Chandra K. Mass Production and Quality Control of Microbial Inoculants. Proc Indian Natn Sci Acad, 2014,80 (2): 483-489

4. Organic Farming:: Organic Inputs and Techniques: http://agritech.tnau.ac.in/org_farm/orgfarm_biofertilizertechnology.html

5. Biofertilizer Manual by FNCA Biofertilizer, 2006, Japan Atomic Industrial Forum (JAIF)

6. Borkar SG. Microbes as Biofertilizers and their Production Technology, 2015, Woodhead Publishing India Pvt. Ltd.