Chapter 26
Inoculant Preparation and Formulations for *Azospirillum* spp.

Yoav Bashan and Luz E. de-Bashan

Abstract In general, shortly after suspensions of *Azospirillum* spp. are inoculated into soil, seed surface, or root surfaces without a proper carrier, the bacteria population declines rapidly. This phenomenon, combined with poor production of bacterial biomass, makes difficult to sustain activity in the rhizosphere, and the physiological state of *Azospirillum* spp. at application time can prevent the buildup of a sufficiently large bacterial population in the rhizosphere. Consequently, a major role of formulation of inoculants is to provide a more suitable microenvironment, combined with physical protection for a prolonged period to prevent a rapid decline of introduced *Azospirillum* spp. Inoculants for field use have to be designed to provide a dependable source of bacteria that survives in the soil and become available to crops, when needed. This chapter provides technical details on production of several formulations proven useful for *Azospirillum* spp. from propagation of the bacterium in culture medium to final formulation for the field, and the industrial considerations involved in the entire process of inoculant production. These include: media for massive propagation, techniques for useful formulation, mode of application out of the laboratory setting, and industrial consideration regarding production of commercial inoculants.

Dedication: This review 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.

Y. Bashan (✉) • L.E. de-Bashan
Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Av. IPN 195, La Paz, Baja California Sur 23096, Mexico

The Bashan Foundation, 3740 NW Harrison Blvd, Corvallis, OR 97330, USA
e-mail: ybb0001@auburn.edu; ybashan@cibnor.mx

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26.1 Introduction

The end goal and economic justification of any long-term investigation of the effect of the plant growth-promoting bacteria (PGPB) *Azospirillum* spp. on plant growth and productivity is practical application of inoculants by growers. To that end, inoculants are made of viable cultures with high numbers of *Azospirillum* spp. embedded, suspended, or mixed in a carrier of choice. The later can be either solid or liquid. A fundamental requirement of such technology, in an industrial setting, is the production of cultures of *Azospirillum* spp. of high cell number, usually in fermenters using cheap, yet efficient, culture medium for growth and simple procedures for growing conditions (Bashan 1998).

Azospirillum spp. has been commercially used on a relative large scale in Argentina, Mexico, Europe, South Africa, and India, mainly on cereals, but also on other crops (Díaz-Zorita and Fernández-Canigia 2009; Fuentes-Ramirez and Caballero-Mellado 2005; Hartmann and Bashan 2009). Manufacturers are mostly small- to medium-sized companies and government agencies that are involved in inoculant production (Fuentes-Ramirez and Caballero-Mellado 2005).

This chapter provides technical details on production of several formulations proven useful for *Azospirillum* spp. from propagation of the bacterium in culture medium to final formulation to be used in the field and industrial considerations in the entire process of inoculant production.

26.2 Media for Propagation of *Azospirillum* In Vitro

There are several proven media for successfully growing *Azospirillum* spp. in fermenters. Some are less useful for inoculant production, mainly because of low cell counts and costs.

26.2.1 OAB Medium (a.k.a. Modified NFb)

Comments: This is an improved derivative of the original semisolid, buffer-free, nitrogen-free medium (NFb) that was based on organic acids, mainly malate and succinate, the preferred carbon sources of this bacterium in situ (Döbereiner and Day 1976). The OAB medium is improved by increasing its buffering capacity over the original medium and adding microelements, a limited amount of NH_4Cl to initiate aerobic growth, and a small amount of yeast extract to shorten the lag phase and aid vigorous growth (Okon et al. 1977). While OAB medium is well suited and well recommended for laboratory studies, it is not suitable for mass cultivation in inoculant production at larger scales, larger than laboratory experiments.

OAB medium (with latest minor modifications) is composed of: Solution A [(g/L): DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3$, 0.01; (mg/L): $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; 900 mL distilled water] and Solution B [(g/L): K_2HPO_4, 6; KH_2PO_4, 4; 100 mL distilled water]. After autoclaving and cooling, the two solutions are mixed. The pH of the medium pH is 6.8 (Bashan et al. 1993).

26.2.2 Modified TYG

Comments: TYG medium, based on tryptone, yeast extract and glucose, allows massive cultivation of many PGPB/PGPR. This medium was further developed to enhance the growth of *Azospirillum* by supplementing it with the buffer capacity and micronutrients of the OAB medium that were specially designed for this species (Bashan et al. 2002). Yet, it has a major deficiency. Glucose is not used by some species of *Azospirillum*, such as the most common species used as an inoculant, *A. brasilense*, and glucose is not a preferred carbon source for this genus.

TYG medium contains (g/L): tryptone, 5 (Difco); yeast extract, 5, D-glucose, 5; NaCl, 1.2; $MgSO_4 \cdot 7H_2O$, 0.25; K_2HPO_4 0.13; $CaCl_2$, 0.22; K_2SO_4, 0.17; Na_2SO_4, 2.4; $NaHCO_3$, 0.5; Na_2CO_3, 0.09; Fe(III) EDTA, 0.07; the pH was adjusted to 7.0 after sterilization (Bashan et al. 2002).

26.2.3 BTB-1 and BTB-2

Comments: These media increase mass production of cells more than what is obtained with modified TYG medium and in the shortest period of time (18 h) to a level of ~10^{11} cells/mL. The variant medium using glycerol is much cheaper because industrial grade glycerol from biodiesel production is readily available. Currently, these are the most suitable media for mass production of *Azospirillum* spp.

These media contained the modified TYG medium, where the glucose is replaced by 5 g/L Na-gluconate or 8 mL/L glycerol (Bashan et al. 2011).

26.2.4 General Commercial Microbiological Media (Nutrient Broth Medium, Luria-Bertani Broth, Tryptone Soy Broth, and a Few More of that Type)

Comments: These media produce less than optimal yields of cells for propagation of the bacteria for inoculants compared to the two BTB media. They are still useful for experimental laboratory propagation of *Azospirillum* spp. All these commercial media are prohibitively expensive for industrial-scale inoculant production.

26.3 Justification for Formulation of Inoculant

In general, shortly after suspensions of *Azospirillum* spp. are inoculated into the soil, seed surface, or root surfaces without a proper carrier, the bacteria population declines rapidly. In plantless soil, they disappeared within 15 days (Bashan 1999). This phenomenon, combined with poor production of bacterial biomass, difficulty sustaining activity in the rhizosphere, and the physiological state of *Azospirillum* spp. at application time, prevents the buildup of a sufficiently large *Azospirillum* population in the rhizosphere. A threshold number of cells, ~10^6–10^7 cells·plant^{-1} for *Azospirillum brasilense* is required (Bashan 1986b). The inherent heterogeneity of the soil is the key obstacle, where introduced bacteria sometimes cannot find an empty niche in the soil. These unprotected, inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by soil microfauna. Consequently, a major role of formulation of inoculants is to provide a more suitable microenvironment, combined with physical protection for a prolonged period, to prevent a rapid decline of introduced *Azospirillum* spp. Inoculants for field-scale use have to be designed to provide a dependable source of bacteria that survives in the soil and become available to crops, when needed (Bashan 1998). Many inoculants do not do this; yet, this is the main purpose of inoculant formulation. The three fundamental and essential characteristics for all inoculants are to: (1) support the growth of the intended microorganisms, (2) support the necessary number of *viable* microbial cells in good physiological condition for an acceptable period of time, and (3) deliver enough microorganisms at the time of inoculation to reach a threshold number of bacteria that is usually required to obtain a plant response, i.e., the inoculant must contain enough *viable* bacteria after the formulation process. Formulation is the crucial issue for commercial inoculants. In practice, the formulated inoculant is the sole delivery vehicle of live microorganisms from the factory to the field.

26.3.1 "Primitive" Inoculants: Culture Media with No Additional Formulation

The oldest method of inoculation of seeds and plants with bacterial culture suspension, as has been practice since the pioneering times of plant inoculation decades ago, still prevails today. It is practiced mostly at research facilities. It is a very common practice, especially among highly trained researchers because it is the least laborious.

Obviously, despite the large number of publications demonstrating repeated success of researchers, "no formulation" inoculants are definitely not a practical inoculation technology. This is mainly because the level of expertise needed for proper delivery of the bacteria, under optimal conditions, is many times beyond the skills and the available time of growers. It is also impractical for large-scale application; the large volume of liquids involved, incubation, and refrigeration facilities

needed to maintain culture medium that have no formulation make it unlikely to meet economic and commercial needs. It will serve mainly in the domain of laboratory and greenhouse initial experimentation in research facilities.

26.3.2 Liquid Inoculants

Liquid inoculants are an upgraded derivative of "no-formulation" inoculants to address some of the limitations listed above. Essentially, they are microbial cultures or suspensions amended with substances that may improve stickiness, stabilization, and surfactant and dispersal abilities (for techniques used originally for rhizobia but valid for *Azospirillum* spp., see: Singleton et al. 2002). The main advantage of these inoculants over solid inoculants is that they are easy to handle. Unlike solid carrier-based inoculants, liquid formulations allow the manufacturer to include sufficient amounts of nutrients, cell protectants, and inducers responsible for cell/spore/cyst formation to improve performance. Liquid inoculants containing concentrations of 2×10^9 cells per mL are now common, allowing for lower application rates and increased efficiency in using inoculants (Schulz and Thelen 2008). Further, it is claimed that these inoculants have no contamination and have longer shelf life for some formulations, greater protection against environmental stresses, and increased field efficacy, compared to peat-based inoculants (Singleton et al. 2002). They are compatible with machinery on large farms, such as air seeders and seed augers.

There are no scientific reports concerning the use of liquid formulation for *Azospirillum* spp. Yet, these formulations are sold commercially without specifying their ingredients. The following compounds, used for other PGPB, can be potentially used as additives to create liquid inoculants of *Azospirillum* spp.: sucrose (Cong et al. 2009; Taurian et al. 2010); carboxymethyl cellulose (Jha and Saraf 2012), glycerol, polyvinylpyrrolidone, trehalose, FeEDTA (Albareda et al. 2008; Manikandan et al. 2010; Singleton et al. 2002), gum arabic (acacia gum; Diouf et al. 2003; Gamal-Eldin and Elbanna 2011; Wani et al. 2007).

26.3.3 Peat Inoculants

Peat is the carrier of choice for rhizobia and many PGPB, including *Azospirillum* spp. in North and South America, Europe, and Australia, and the main ingredient of inoculants that are sold in large volumes. Currently, technical details of production of the basic peat-based inoculant, such as grain size, pH, optimal moisture, several amendments, quality of inoculants, quality control standards, and occupational health and safety are common knowledge (Deaker et al. 2011) The physical state of the formulation of peat (solid [powder], pellet, or liquid [slurry]) can make a difference in the success of inoculation in rhizobia, but such studies are not available for *Azospirillum* spp.

There are many small variations of preparing peat inoculant (depending on the desired formulation required) and most are small modification that are based on the following procedure: for preparation of 100 g of inoculant for *Azospirillum* spp., 45 g of ground peat (40 mesh) is thoroughly mixed with 5 g of $CaCO_3$ and 20 mL of tap water (final pH 6.8) stored in polyethylene bags sealed with a plug. The bags are sterilized (gamma-irradiated or tyndelization in an autoclave), and 30 mL of a 24-h-old bacterial culture (approximately 5×10^9 CFU/mL) are aseptically added to each bag, mixed, and incubated for an additional 7 days at 33 ± 2 °C. Every 2 days, the peat is mixed by shaking the bags. The final number of bacteria in the inoculant range from 5×10^7 to 5×10^8 CFU/g of inoculant. The bags are stored for up to several months at 4 ± °C. One day before plant inoculation, the bags are transferred to 30 ± 2 °C for acclimation before inoculation.

Peat is user-friendly to several amendments used for other PGPB but not tested so far for *Azospirillum* spp. Such amendments include: vermiculite (Kokalis-Burelle et al. 2003), chitin, heat-killed *Aspergillus niger* mycelium or spent compost from *Agaricus bisporus* (Manjula and Podile 2001), pyrophyllite (hydrous aluminum silicate) (Meyer et al. 2000, 2001), and charcoal plus an adhesive (Riggs et al. 2001).

Another option to consider for small-scale inoculant production is that some organic wastes and composts can perform equally well or better than peat as a carrier. The main limitation is availability of the raw material for industry. Compost made of cork, bagasse, sawdust, brewery waste, or banana leaves (tested for other PGPB; Bashan et al. 2014) can sustain a small, local inoculant industry where the materials are available. They cannot form a base for a large nationwide industry, especially when the batch raw material is variable.

26.3.4 Synthetic Inoculants

Synthetic formulations based on an assortment of polymers have been continuously evaluated for decades for PGPB because they offer substantial advantages over peat, such as longer shelf life, appropriate survival at the destination field, sufficient cell density, ease of manufacturing, and improved performance in general (Bashan 1998; Bashan et al. 2014; John et al. 2011; Schoebitz et al. 2013). However, the major drawback of polymeric inoculants is that the raw materials for all polymers are relatively expensive compared to peat, soil, and organic waste inoculants and require additional expensive handling by the industry at costs similar to those in the fermentation industry. For agricultural and environmental uses, these polymers include, so far, alginate, agar, λ- and κ carrageenan, pectin, chitosan, bean gum, and proprietary polymers. Several basic requirements for these polymers, which are components of polymeric inoculants, are: (1) nontoxic and free of harmful preservatives that affect bacteria within the inoculant and inoculated plants, (2) slowly degradable in the soil by soil microorganisms, thereby gradually releasing the

bacteria in the needed quantities, usually at the time of seed germination and emergence of seedlings, (3) physical protection for the inoculated bacteria from soil competitors and many environmental stresses (Covarrubias et al. 2012, Schoebitz et al. 2012; Zohar-Perez et al. 2003), (4) sufficient water for survival of the bacteria, and (5) dispersive in water to allow movement of the bacteria from the polymer to the plants. For *Azospirillum* spp. the polymer of choice is alginate (Bashan 1986a; Bashan and Gonzalez 1999; Bashan et al. 2002).

26.3.5 Macro- and Micro-formulations of Alginate

The advantages of alginate formulations are their nontoxic nature, biodegradability, availability at reasonable costs, and slow release into the soil of the entrapped microorganisms, which is controlled by the polymeric structure (Bashan et al. 2002, 2014). Two basic formulations for *Azospirillum* spp. exist: macrobeads (1–4 mm dia.) and microbeads (50–200 μm in dia.) used for several PGPB.

Alginate macrobeads may have solved many of the problems associated with common peat inoculants; yet, their application as agricultural and environmental inoculants have two major disadvantages. First, an additional treatment during sowing is needed even if the inoculant is planted by the seeding machine. In developed countries, the grower who is busy during sowing might be pressed for time and reluctant to incur additional expense and time. In developing countries, the farmer might not inoculate the seeds at all. This happens because of insufficient agricultural education and conservative cultural traditions that make some small-scale farmers suspicious of new technologies, especially those involving live bacteria. Second, the bacteria released from macrobeads need to migrate through the soil toward the plants. Under agricultural practices, when beads are loosely mixed with seeds and sown together by planters, the inoculant beads might fall far from the seeds (up to a few centimeters). Thereafter, the bacteria released from the beads must migrate through the soil, facing competition from the native microflora, sometimes more aggressive and more adapted to the soil than the inoculated strain. Sometimes, the absence of a continuous film of water needed for their movement is an additional limiting factor. These distances, large on a microbial scale, might prove prohibitive for many PGPB, even those with a proven motility in soil, such as *Azospirillum* (Bashan and Levanony 1987).

The microbead concept was conceived to overcome these two fundamental difficulties (Bashan et al. 2002). The underlying hypothesis is that, if the beads are small enough, yet still 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 this "bead powder" at the seed-handling facility and sold to the farmer as "improved seeds." Alternatively, the farmer can coat the seeds, as done with peat inoculants.

26.3.5.1 Production of Macrobeads

Basic preparation: A bacterial culture (logarithmic or stationary phase) is aseptically mixed with 2 % (w/v) sodium alginate powder and stirred gently for 1/2–2 h to ensure complete dissolution of all ingredients. The mixture is added dropwise with the aid of a 10-mL sterile syringe into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature. Beads are immediately formed. The resulting alginate beads (mean diameter, 2 mm) entrap the bacterial cells. The beads are cured in the solution at room temperature for additional 1–3 h. The $CaCl_2$ solution is pumped out, and the beads are washed twice with sterilized tap water. After washings, the beads are incubated in fresh BTB medium (described above) for an additional 24 h for *A. brasilense* in a rotary shaker at 30 ± 2 °C to allow bacteria to multiply further inside the beads (secondary multiplication). Then, the beads are washed twice and collected. The wet beads are kept at 4 ± 1 °C in hermetically sealed flasks under moist conditions for several days. Dry preparation can be achieved by lyophilization or drying at 40 °C in thin layers in trays (Bashan 1986a). After complete dryness, the macrobeads can be stored in hermetically sealed flasks containing silica gel for years (Bashan and Gonzalez 1999).

Based on this principle, equipment were developed to produce macrobeaded inoculants (de-Bashan and Bashan 2010, http://www.bashanfoundation.org/device.html, http://www.bashanfoundation.org/beads/macrobead.html).

26.3.5.2 Production of Microbeads

Production of alginate microbeads is 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 (a unit for this purpose is described in Bashan et al. 2002). These droplets, sprayed into a slowly stirred solution of $CaCl_2$, immediately solidify into microbeads at diameters ranging between 100 and 200 μm, which entraps a large number of bacteria (~10^9–10^{11} CFU g^{-1}) (Bashan et al. 2002). Like macrobeads, a description of automated production of microbeads is available (Bashan et al. 2002; de-Bashan and Bashan 2010 http://www.bashanfoundation.org/bead.html). Microbead inoculant of *A. brasilense* Cd is presented in Fig. 26.1.

26.4 Quality Control and Detection of the Strain in the Inoculant

Quality control of inoculants ensures that the declared content of *Azospirillum* spp. is present and that it is alive. Several countries now have guidelines that state how many cells should be in the inoculant. In other countries, this is left to the

Fig. 26.1 (**a**) Dry microbead inoculant of *Azospirillum brasilense* Cd. (**b**) Magnification of single microbeads. *Arrow* indicates a single microbead

manufacturer's discretion. The method for detection of the genus/species/strain varies and depends on the microbiological/molecular analytical capacity of the manufacturer. Generally, traditional, immunological, and molecular methods are employed for *Azospirillum* spp.

26.4.1 Traditional Microbiological Techniques

26.4.1.1 Cultivation of Samples of the Inoculant on Semi-selective Media

The limit of detection depends on the level of contamination by other microorganisms because these methods are based on serial dilutions. Lower limits of detection by these methods are ~10^3–10^5 cfu/g inoculant. Because regulations require that the inoculant will contain at least 10^8 cfu/g, these cultivation methods, despite being almost obsolete in other diagnostic fields, are still useful. These techniques require minimal microbiological infrastructure.

26.4.1.1.1 Semi-selective Media for *Azospirillum*

26.4.1.1.1.1 Congo Red-NFb

This medium is basically the original NFb medium supplemented with 15 mL/L medium of 1:400 aqueous solution of Congo red, autoclaved separately, and added just before using (Rodriguez-Caceres 1982).

Note: This medium permits the recognition of *Azospirillum* colonies on plates and facilitates the isolation of pure cultures since the colonies appear dark red or scarlet with typical colony characteristics, whereas many soil bacteria do not absorb Congo red.

26.4.1.1.1.2 BL and BLCR Media

These two semi-selective media are based on the OAB medium. The BL medium is the OAB medium supplemented with (mg/L) streptomycin sulfate, 200; cycloheximide, 250; sodium deoxycholate, 200; and 2,3,5-triphenyltetrazolium chloride, 15. The BLCR is the BL medium supplemented with an aqueous solution of Congo red (approximately 1 mL of a 1 mg/mL solution per liter) (Bashan and Levanony 1985).

Note: These media are very suitable for the isolation of *Azospirillum* from the rhizosphere since the colonies are easily recognizable, especially on BLCR medium. However, some strains of *A. brasilense* failed to grow on this medium, and the growth of *Azospirillum* on BLCR medium is significantly slower compared to the original OAB medium (about 10 days incubation time). Lower limits of detection are ~10^2–10^3 cfu/g inoculant when the inoculant has a low contamination load. Contaminants are easily recognizable.

26.4.2 Immunological Methods

For immunological detection of *A. brasilense,* only the enzyme-linked immunosorbent assay (ELISA) was developed (Levanony et al. 1987). Although highly specific for the species, lower limit of detection is 10^4 cfu/g inoculant, still useful for inoculant evaluation for the reason explained above for bacteria load in inoculants. When a production of inoculant fails, and the number of *Azospirillum* spp. is below the detection level, but still the manufacturer needs to know the number of *Azospirillum* spp. in the inoculant, this method can be combined with limited enrichment (Bashan et al. 1991). Minor limitations of the immunological method are an access for antibody facility (many commercial facilities are available) and access to plate count reader equipment that reads the ELISA plates.

26.4.3 Molecular Methods

Many molecular methods were developed and are capable of detecting specific *Azospirillum* spp. Yet, only one technique, fluorescent in situ hybridization (FISH) has been shown, so far, to detect this species in inoculants (Bashan et al. 2011) and

later on the roots of inoculated plants (Trejo et al. 2012). Because many FISH procedures are available, the technique described by Stoffels et al. (2001), but with numerous small modifications was used for detection of *A. brasilense* and *A. lipoferum*: Hybridization was performed at 35 % formamide stringency at 46 °C for 2 h. The final concentration of the probe was 3 ng μL^{-1}. Samples were then washed at 48 °C for 5 min with 50 mL of pre-warmed washing buffer. The slides were rinsed for a few seconds with ice cold, deionized water and then air dried. Slides were stored at −20 °C in the dark until observation. Three types of probes were used: an equimolar mixture of probes EUB-338 I (Amann et al. 1990), and II and III (Daims et al. 1999) that cover the domain of bacteria. An *Azospirillum brasilense-specific* probe (Abras 1420, Stoffels et al. 2001) was used for the two *A. brasilense* strains and Alila 1113 (Stoffels et al. 2001) for *A. lipoferum*. The probes Abras 1420 and Alila 1113 were labeled with the fluorochrome FITC (green) and the mix of EUB I, II, and III was labeled with the fluorochrome Cy3 (red). All fluorescent-labeled probes were purchased from Integrated DNA Technologies (Coralville, IA). Before visualization, the slides were mounted in AF1 anti-fading reagent (Citifluor, London, UK). Visualization was done with an epifluorescent microscope (Olympus) with two filters, the Cy3 filter (maximum excitation at 552 nm with maximum emission of light at 565 nm, red fluorescence (Olympus America, Center Valley, PA)) and the FITC filter (maximum excitation at 490 nm with maximum emission of light at 520 nm, green fluorescence (Olympus America)). Positive fluorescent signals that identify the bacteria are a combination of red and green that yields a green-yellow-orange tone, depending on the intensities of the individual color channels. Evaluation of *Azospirillum* spp. in the inoculant can be done either by fluorescent microscopy where images in red and green are superimposed to create a green-yellow-orange tone that provide absolute detection of the species (Fig. 26.2; Bashan et al. 2011) or directly by confocal laser microscopy and quantification by an image analyzer (Trejo et al. 2012).

The main advantage is specific identification of the species. The main limitation of this technique is the skill of the technician and the availability of equipment, which is expensive. Technologies for identifying specific strains of *Azospirillum* spp. in inoculants have not been developed.

26.5 Inoculation Techniques

Azospirillum spp. can be inoculated directly on the seed surface or in the soil. Seed applications greatly outnumber soil applications. This happens because it is easy to use and requires a relatively small amount of inoculant and because *Azospirillum* do not survive well in soils.

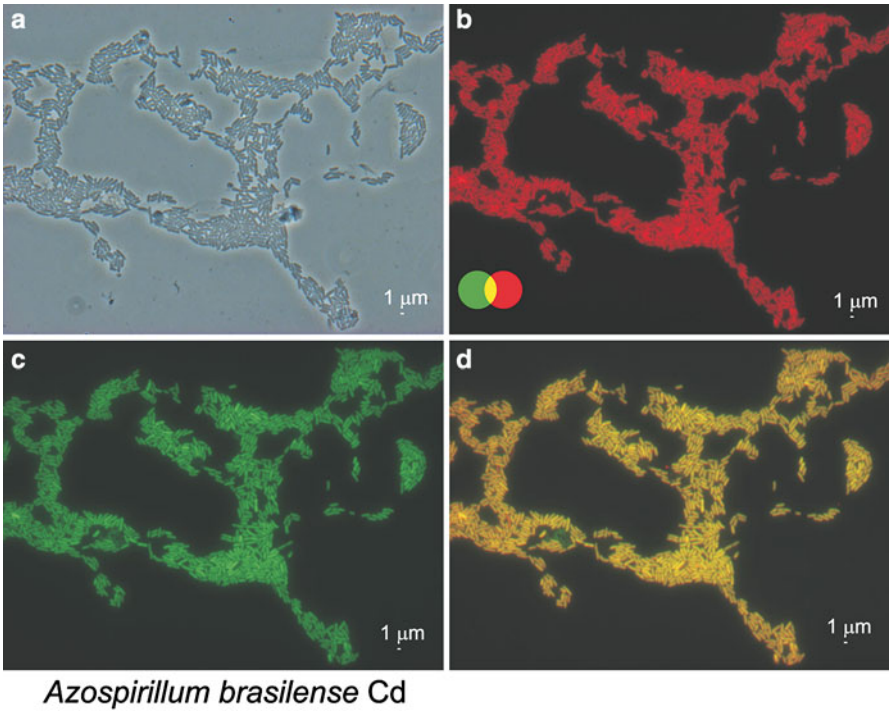

Fig. 26.2 Determination of the quality of the inoculant. Fluorescence in situ hybridization (FISH) of *Azospirillum brasilense* Cd. (**a**–**d**) after formulation and release from microbead alginate inoculant. Bars represent 1 μm. Original figure published in Bashan et al. Biology and Fertility of Soils 47: 963–969 (2011)

26.5.1 Seed Inoculation

There are many small variations of the basic technique. Using a variety of machinery, the basic technology of even seed-coating has not changed for decades. Briefly, prior to sowing, seeds are dusted with peat inoculant, with or without water or adhesive. For small seeds, this is followed by superfine, ground limestone, with or without adhesive, and allowed to dry. Drying can be done in situ or when the coating is applied prior to sowing. The seeds, held in shallow trays, are air-dried or dried by forced air. Coating and drying is also possible by using fluidized beds, where the seeds are floating on a cushion of pressurized air and then sprayed with inoculant. The inoculant is mixed with seeds either by hand, rotating drums that are cheap to operate, large dough or cement mixers, or mechanical tumbling machines. Alternatively, large farm operators use automated seeders fitted with an inoculant

tank, pump, and a mixing chamber commonly used for applying chemical coatings. The latter are not specialized equipment for microbial inoculation, so inoculant may be dislodged from the seeds.

Because every seed needs to be coated with a threshold number of *Azospirillum* spp., adhesives (the same level that were mentioned before for liquid inoculants) are used. A second role of an adhesive is to prevent the inoculant (either dry inoculant as powder or wet inoculant once the moisture evaporates) from dislodging during sowing with the seeding equipment, especially the powdered type, when applied with air seeders. The seeds are then sown with common seeding equipment. While it is commonly agreed that one essential condition to seed coating is adding adhesive materials, there is no agreement on what are the best adhesives. Each manufacturer or experimentalist empirically evaluates which adhesive best fits seeds and inoculants. When seeds are inoculated with liquid inoculant, with or without dissolved adhesive, the inoculant is sprayed directly onto the seeds. After drying, the seeds are sown.

Seed inoculation, despite their popularity in the marketplace, has several significant disadvantages. (1) Each seed, especially small ones, can be coated only with a limited amount of inoculant, which may be a limiting factor because a threshold of *Azospirillum* spp. (10^6–10^7 cfu/g, Bashan 1986b) may be needed for successful inoculation. (2) If an inoculant is not attached well with an adhesive or by pelleting, it may be dislodged by the sowing machinery. (3) In some seedlings, the seed coat is lifted out of the soil during germination, causing desiccation and death of the inoculant bacteria if the inoculant had been applied directly to the seeds. (4) Some species release anti-bacterial compounds from their seeds, which can inhibit the inoculant. (5) Some fungicides and insecticides commonly applied to the seeds may be detrimental to the inoculant (Bashan et al. 2004; Bashan and de-Bashan 2010).

26.5.2 Soil Inoculation

This technique is popular with rhizobia but is far less useful for *Azospirillum* spp. Only a few publications, mostly old, support this application. Therefore, at the state-of-the-art of our knowledge, this technique is not recommended for inoculation of *Azospirillum*.

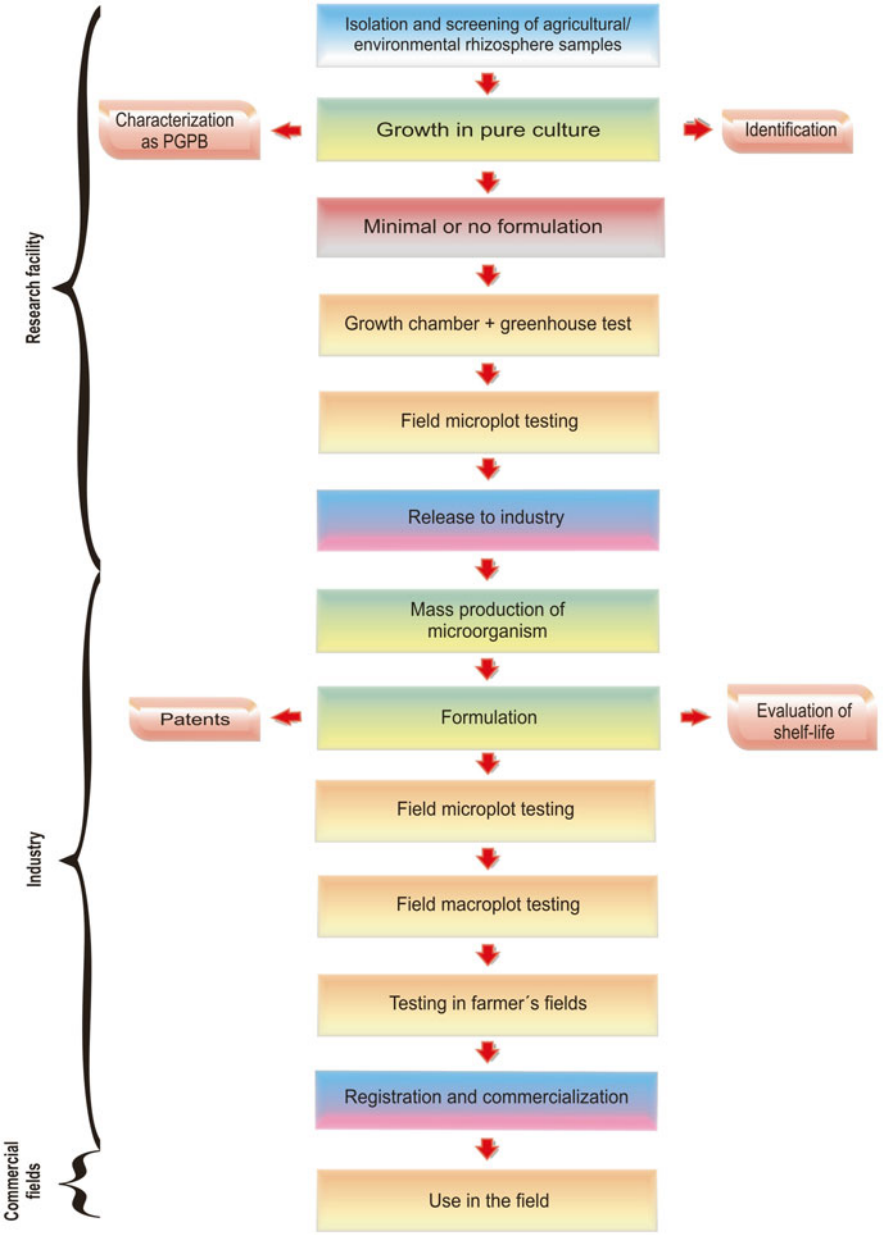

Fig. 26.3 Flow diagram of procedures for developing bacterial inoculants (Original figure published in Bashan et al. Plant and Soil 378: 1–33 (2014))

26.5.3 Industrial Point of View and Industrial Considerations on Inoculants

The industrial point of view of producing *Azospirillum* inoculants is no different from production of any other PGPB inoculant. A flow diagram, showing the procedures for developing microbial inoculants by the industry, is presented in Fig. 26.3. In practical industrial terms, the chosen formulation and method of application determine the potential success of the inoculant more than the specific strain used.

26.6 Conclusions

Inoculation of culture medium as inoculants is a recipe for failure under field condition. Proper formulation of *Azospirillum* is essential and irreplaceable for any successful inoculation beyond the level of test tubes and in vitro studies.

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