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Dry micro-polymeric inoculant of *Azospirillum brasilense* is useful for producing mesquite transplants for reforestation of degraded arid zones

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This study is dedicated for the memory of the Mexican agricultural molecular biologist Alberto Mendoza Herrera (1957–2017) of The Center of Biotechnology on Genomics of the National Polytechnical Institute of Mexico in Reynosa, Mexico.

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ABSTRACT

Massive clear-cutting of wild stands of mesquite trees in the Mexican part of the Sonoran Desert result from high demand for this wood by the charcoal industry. Consequently, there is a need to develop techniques for reforestation of this tree in the desert and maintain its natural diversity at the same time. An outdoor nursery procedure to produce mesquite transplants from diversely originated seeds for reforestation of arid zones was developed. This procedure involved: 1) inoculation of the seedlings in the nursery with the plant growth-promoting bacteria (PGPB) *Azospirillum brasilense* immobilized in dry microbeads of alginate, and 2) developing a reliable way to monitor plant development and aerial volume in the nursery for the entire growth period of seven months before transplantation. Dry microbeads containing the PGPB and maintained at room temperature were tested for survival of bacteria for up to seven months. These dry microbeads maintained sufficient population levels of *A. brasilense* to inoculate the plant for the entire period. Inoculation with the PGPB enhanced all growth parameters of the plants, including biomass, aerial volume, root system, and chlorophyll pigments, but not the auxiliary photosynthetic pigments. The PGPB was specifically identified colonizing the roots of the transplants by fluorescent in situ hybridization for the entire growth period. Measuring a few simple parameters allowed development of a workable model for plant growth. This model was confirmed by data obtained from sacrificed plants whose parameters were measured directly. This study shows that outdoor nursery cultivation of inoculated mesquite transplants is feasible.

1. Introduction

Mesquite legume trees are globally one of the most useful trees of arid zones. About 45 species of the genus *Prosopis* have significant ecological, economic, and social roles for all human communities living in deserts (Felker, 2009). Mesquite shrubs or trees serve as anchor tree in the desert phenomenon of “resource islands”, improve soil fertility, allow other species to grow under its canopy, serve as a refuge for wildlife, and is a source of nectar for foraging insects (Bashan and de-Bashan, 2010; Carrillo-Garcia et al., 1999; Garcia et al., 2018). Economically, some species are used as hard wood for furniture (Felker and Guevara, 2003), intensive charcoal production (Galindo Almanza and Garcia Moya, 1986; Taylor, 2006), livestock feed (Felker and Bandursky, 1979) honey production, a source of food and energy for indigenous and poor communities (Wojtusik et al., 1993), a gum and colorant (Goycoolea et al., 1995), ornamental value, recreation sites and shade in deserts where shade is in short supply. The latter are difficult to quantify economically. In areas such as southwestern USA and parts of northern Mexico where the wild trees are not commercially harvested for wood, they are ubiquitous (Johnson and Mayeux, 1990). The mesquite species used in this study, *Prosopis articulata* S. Watson (Johnston, 1962; León de la Luz et al., 2000), is an endemic, common species of the southern Sonoran Desert and in other parts of Mexico. It is a thorny shrub or small tree (2–15 m tall) and serves as a climax vegetation of this desert. When mature, its main role is as the dominant tree in resource island formation and, therefore, directly contributes to the natural vegetation of these deserts and well-being of the desert. Its role as a common source of charcoal makes it a candidate for desert restoration (Moreno et al., 2017), a trend that gained momentum during the last decade, using many species of desert plants (Armada

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et al., 2014; Bacilio et al., 2006; Bashan et al., 1999, 2009a,b, 2012; de-Bashan et al., 2010; Felker et al., 2005; Founoune et al., 2002; Gao et al., 2002; Grandlic et al., 2008; Medina et al., 2004; Mengual et al., 2014a,b; Puente et al., 1999; Requena et al., 2001; Toledo et al., 1995; Valdenegro et al., 2001; Vovides et al., 2011).

Although mesquite is considered an invasive plant of grasslands in Arizona (Gibbens et al., 1992), in Mexico it is a highly exploitable resource for charcoal production, mainly for export to the US market. Consequently, large stands of wild mesquite have been removed and have become scarce, with no re-planting efforts (Taylor, 2006). Revegetation to conserve the remaining natural stands is necessary. Propagation of mesquite seedlings from seeds is a relatively slow process and faster procedures for mesquite production were proposed, such as rooting of mesquite cuttings (Felker and Clark, 1981; Klass et al., 1984; Leakey et al., 1990), mini-grafting (Ewens and Felker, 2003), and tissue cultures and clonal propagation (Batchelor et al., 1989; Buendía-González et al., 2007; Ramawat and Nandwani, 1991). While these methods allow rapid development of seedlings for re-forestry or industrial plantation, for ecological restoration, seed propagation that maintains the natural diversity of the species is preferable, since natural dissemination and colonization of pastoral land by mesquite is always done by seeds. Any means of shortening the growth period in nurseries and better plant development is economically preferable.

Plant growth-promoting bacteria (PGPB) of the genus *Azospirillum* are motile (Bashan and Levanony, 1987), non-pathogenic bacteria (Bashan, 1998), known to promote growth and performance of over 130 species of plants (Pereg et al., 2016), affecting the entire plant growth, but especially enhances root growth (Bashan and Dubrovsky, 1996; Levanony and Bashan, 1989). This includes cultivating mesquite in hydroponic growth solution (Leyva and Bashan, 2008), in small growth cell masses (Dominguez-Nuñez et al., 2012), or root cuttings dipped in bacteria culture (Spiekermannm et al., 1999). All these studies used inoculation without any formulation of the culture medium or formulated into macro-beads of alginate (2–3 mm in dia.) together with *Bacillus pumilus,* AM fungi, and compost in screen houses (Bashan et al., 2009a), or in the field (Bashan et al., 2012; Lopez-Lozano et al., 2016; Moreno et al., 2017). However, long-term greenhouse and field experiments (1–E12 years) did not measure the shorter net effect of formulated *A. brasilense* for mesquite seedling production for a duration of about six months, common for production of transplants in nurseries. Formulation is an essential process because this bacterium is motile, can be easily adsorbed by soil particles (Bashan and Levanony, 1988a,b), and has weak initial adsorption to roots (Bashan et al., 1986) that later develops into a very strong attachment process (Levanony et al., 1989).

A synthetic inoculant made of macrobeads of alginate (2–3 mm in dia.) for PGPB was developed over 30 years ago (Bashan, 1986a), and is used for wastewater treatment (de-Bashan et al., 2004), agricultural experimentation worldwide (Bashan et al., 2014, 2016; Schoebitz et al., 2013), studies of bacteria microalgae interaction (de-Bashan and Bashan, 2008, de-Bashan et al., 2015) and bacterial diversity studies in mesquite roots (Galaviz et al., 2018). A derivative of this inoculant, microbeads of 100–200 μm, was developed for use in agriculture and environmental re-vegetation (Bashan et al., 2002) but, so far, has limited experimental experience. The usefulness of this dry inoculant for mesquite transplants production has not been tested, as well as some of its inherent characteristics, such as long-term survival of bacteria after long storage at ambient temperatures.

The two hypotheses of this study were: (1) it is feasible to construct a dry, pulverized, synthetic inoculant of *A. brasilense* and use it successfully to promote growth of mesquite transplants destined for environmental restoration, and (2) it is feasible to develop a reliable technique to measure development of mesquite in nurseries without the need to sacrifice plants for analytical measurements. To that end, we checked survival and quantity of bacteria in dry inoculant for up to 210 days, measured the effect of inoculation on plant parameters, presence on roots, and content of photosynthetic pigments for 210 days, a period common for transplant production in nurseries. We also developed a method of estimating biomass and aerial volume of the plant without the need to uproot the plant, which is appropriate for monitoring mesquite planted in reforestation areas.

2. Materials and methods

2.1. Bacterial strain, growth medium, and cultivation

The plant growth-promoting bacterium *Azospirillum brasilense* Cd (DSM 1843, Leibniz-Institut DMSZ, Braunschweig, Germany) was used. The bacterium was mass cultivated in BTB-1 medium (Bashan et al., 2011) and incubated at 36 ± 2 °C at 120 rpm for 24 h. The culture was centrifuged at 2817*g* for 5 min, the supernatant was discarded, and the bacteria re-suspended in 200 mL sterile saline solution (0.85 NaCl, w/v) at concentration of 10^9 cfu·mL^{-1}.

2.2. Formulation of dry microbead inoculant

All procedures were done under sterile conditions. The bacteria suspension (200 mL) was mixed with 800 mL of high viscosity sodium alginate (MP Biomedicals, Santa Ana, Ca.) at a concentration of 1.4% and viscosity of 280 mPa. This specific combination was chosen because spraying of alginate is easy, it forms spherical beads, is consistent, and keeps its shape once dried (described later, Fig. 1a). The two solutions were mixed with a stirrer for 2 min and, once homogenized, connected to a microbeads-producing device (Bashan et al., 2002). Droplets were formed at room temperature at a pressure of 20 psi and deposited in a tray containing 2 L of 2% $CaCl_2$ under agitation of 40 rpm. Polymerization with calcium ions created beads of 200 μm average size. After curing for 1 h in this solution, the beads were filtered with a paper filter, washed three times with saline solution, and transferred to BTB-1 medium for 18 h at 30 °C and 120 rpm for one period or two periods. This step was necessary to maintain the same concentration of bacteria in the microbead that is reduced by the polymerization process. Then, the beads were washed again, as described above.

Once washed, the beads were transferred to a stainless steel tray covered with paper towels and dried in a convection oven at 37 °C for 24 h. The dried bead clumps were pulverized with a hand mortar, pass through 250 μm meshes to dust consistency, and maintained in sealed serological flasks containing silica gel (Fig. 1b).

2.3. Counting A. brasilense in beads and quality control of the inoculant

This was done during formation of the inoculant and up to 210 days later. For each sample, 100 mg of beads was dissolved by immersion for ~30 min at ~28 °C in 20 mL citrate buffer, containing (in mM): sodium citrate (55), EDTA anhydride (30), and $NaCl_2$ (150), and adjusted to pH 8 with NaOH. The solution was shaken at 120 rpm for 20 min and centrifuged at 6000*g*; the supernatant was decanted, the pellet was suspended in 1 mL 0.85% saline solution, serially diluted, and plated on Nutrient Agar medium (Sigma-Aldrich, St. Louis, MO). After incubation for 48 h at 35 °C, colony-forming units were counted. Further validation of the viability of the inoculant were done at 120 and 210 days, using the fluorescein diacetate method (Chrzanowski et al., 1984) and analyzed under a fluorescent microscope (Olympus BX41, Tokyo, Japan) equipped with FITC filter (range 460–490 nm) and connected to an image analyzer (Image Pro Plus 6.3.1.542). Verification of species identity as *A. brasilense* was done by fluorescent in situ hybridization (FISH), based on the method of Daims et al. (2005), and modified for *A. brasilense* (Bashan et al., 2011, Bashan and de-Bashan, 2015), using the specific probe Abras 1420 (Stoffels et al., 2001).