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ORIGINAL PAPER

Development of a powder formulation based on *Bacillus cereus* sensu lato strain B25 spores for biological control of *Fusarium verticillioides* in maize plants

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Abstract Maize is an economically important crop in northern Mexico. Different fungi cause ear and root rot in maize, including *Fusarium verticillioides* (Sacc.) Nirenberg. Crop management of this pathogen with chemical fungicides has been difficult. By contrast, the recent use of novel biocontrol strategies, such as seed bacterization with *Bacillus cereus* sensu lato strain B25, has been effective in field trials. These approaches are not without their problems, since insufficient formulation technology, between other factors, can limit success of biocontrol agents. In response to these drawbacks, we have developed a powder formulation based on *Bacillus* B25 spores and evaluated some of its characteristics, including shelf life and efficacy against *F. verticillioides*, in vitro and in maize plants. A talc-based powder formulation containing 1×10^9 c.f.u. g^{-1} was obtained and evaluated for seed adherence ability, seed germination effect, shelf life and antagonism against *F. verticillioides* in in vitro and *in planta* assays. Seed adherence of viable bacterial spores ranged from 1.0 to 1.41 $\times$ 10^7 c.f.u. g^{-1}. Bacteria did not display negative effects on seed germination. Spore viability for the powder formulation slowly decreased over time, and was 53 % after 360 days of storage at room temperature. This formulation was capable of controlling *F. verticillioides* in greenhouse assays, as well as eight other maize phytopathogenic fungi in vitro. The results suggest that a talc-based powder formulation of *Bacillus* B25 spores may be sufficient to produce inoculum for biocontrol of maize ear and root rots caused by *F. verticillioides*.

Keywords *Bacillus cereus* · *Fusarium verticillioides* · Spores · Powder formulation · Maize ear · Root rots

Introduction

Fusarium species are widely distributed plant pathogens (Hefny et al. 2012). *Fusarium verticillioides* is responsible for several major diseases in maize cultures, including stalk, root and ear rot (Reyes-Velázquez et al. 2011). In addition to provoking a range of deleterious effects such as reduced crop yields, plant deterioration and poor grain quality, *Fusarium* species produce mycotoxins with potential carcinogenic risks for humans and clear effects on rats, swine, and equines (García-Aguirre and Martínez-Flores 2010; Pereira et al. 2011).

Fusarium verticillioides is an effective pathogen that can use different strategies to infect systemically maize plants, and once the disease occurs; there is no fungicide that can eradicate it (Pereira et al. 2011). The Fungus is persistent in the soil and produces conidia and spores that can be resistant to fungicides and could last for many years making it recalcitrant to fungicide treatments. Fungicide treatments either corrective or preventive are not an option to control maize rots caused by *F. verticillioides* (Bacon et al. 2001). To date, resistant hybrids or genetically

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modified maize lines with resistance to *F. verticillioides* have not been generated (Balconi et al. 2014). Genetically modified maize in some endemic regions such as Mexico could generate problems with use because of the potential risks of horizontal gene flow to the native maize landraces (Serratos-Hernández et al. 2004; Snow 2009).

In field experiments, biocontrol treatments using microbial agents have shown effectiveness against *Fusarium* infections on seeds, plants and soil of maize (Bacon et al. 2001; Cavaglieri et al. 2005; Gerber 2010). The use of biological control is important because it has several advantages over other methods for controlling pests and diseases. Advantages include long-term control of the target pest, limited or no negative effects on the environment; control is directed to only one organism or group of related organisms making it very specific (Bhattacharjee and Dey 2014). The use of bacterial strains as biological control agents has received great attention because of their ability to suppress different plant diseases involving diverse modes of action and opens the possibility to be combined with other control methods (Romero et al. 2007). Products based on spore-producing organisms, as bacteria, have been proposed in the agricultural sector as alternatives to the agrochemicals used in disease control and plant growth promotion (Errington 2003). Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogens growth (Ongena and Jacques 2008). Strains of *B. subtilis* have been reported as powerful biological control agents of plant diseases due to their ability to produce antimicrobial compounds, including among others, lipopeptide antibiotics (Yánez-Mendizábal et al. 2012b). The ability of *Bacillus* species to form endospores ensures that they can endure adverse environmental conditions, making them ideal biocontrol agents in comparison to vegetative cells (Saharan and Nehra 2011). The advantages offered by spore formation include mechanical strength, and a greater resistance to external factors such as desiccation, solar radiation and high temperatures, which makes endospores attractive for commercial application, as they can withstand the aggressive processing steps during large scale production (Sanders et al. 2003). Additionally, spore-based products can be stored in a stable form for long time periods at room condition (Lalloo 2010). Several studies have identified numerous strains of bacteria, fungi and viruses that can act as potential biocontrol agents. However, their commercial success has been limited by the absence of an adequate method for large-scale biomass production in addition to an appropriate formulation technology (Boyetchko et al. 1999; Gašić and Tanović 2013).

Culture medium composition and fermentation growth conditions both have a determining role on the effectiveness, stability and desiccation tolerance of many spore-producing biocontrol agents during the formulation process. Optimizing these conditions for biomass production will enhance the viability of biocontrol agents during the stabilization, drying and rehydration steps used in the formulation process (Schisler et al. 2004). The formulation process plays a substantial role in the control efficacy of products, by maintaining and improving the survival of plants in field applications (Boyetchko et al. 1999). Nevertheless, the short shelf life of products made with living organisms has held back their proper marketing. Therefore, the support material used in the formulation must be improved in order to maintain product viability for a long shelf life (Nakkeeran et al. 2006).

Our group previously identified *Bacillus cereus* sensu lato strain B25 and demonstrated its control effect against *F. verticillioides* following seed bacterization with live bacteria in the field (Figueroa-López et al. 2016; Lizárraga-Sánchez et al. 2015). The aim of the present work was to develop a talc-based powder formulation of *Bacillus* B25 spores to evaluate its shelf life and efficacy against *F. verticillioide*s, both in vitro and in maize plants.

Materials and methods

Micoorganisms

Bacillus cereus sensu lato strain B25 was obtained from the scientific collection CIIDIR-003 at the CIIDIR-IPN Unidad Sinaloa where it is maintained as a frozen 15 % glycerol stock (−70 °C). B25 strain was originally isolated from maize rhizosphere samples from Guasave, Sinaloa, Mexico. This strain was selected for its antagonistic activity against *F. verticillioides* in in vitro assays, and its efficacy to control infection in maize plants (Cordero-Ramírez 2013; Figueroa-López et al. 2016; Lizárraga-Sánchez et al. 2015).

F. verticillioides strain P03 was isolated from the roots of maize plants exhibiting root rot symptoms from a maize field located in El Fuerte Valley, Sinaloa, Mexico and was kept frozen as a 15 % glycerol stock (−70 °C). P03 strain was previously morphologically characterized, molecularly identified and identified as pathogenic to maize plants (Figueroa-López et al. 2016).

Bacillus B25 antagonistic activity in vitro against eight other maize fungal pathogens was assayed. *Fusarium nygamai* CI61, *F. andiyazi* 106 and *F. thapsinum* F33 were isolated from maize and showed pathogenicity to maize seedlings and plants (Leyva-Madrigal et al. 2015). *Fusarium roseum*, *Macrophomina phaseolina*, *Phomopsis* sp., *Choanephora* sp. and *Alternaria alternata* were isolated from different plant species, although they are all considered to be potential maize pathogens (Ahmad and Mirza 1988; García-Aguirre and Martínez-Flores 2010; Saleh et al. 2010).

Bacterium inoculum

A pre-inoculum of B25 strain was started from a frozen glycerol stock (−70 °C) by growing a single colony in an assay tube with 5 ml of Luria Broth (LB) medium and incubating it in an orbital shaker at 200 rev.min^{-1} and 30 °C for 18 h. After bacterial growth, a 500-ml Erlenmeyer flask containing 100 ml of LB medium was inoculated with 1 ml of this culture (1 % v/v) and incubated at 30 °C and 200 rev.min^{-1} for 24 h, until a final optical density close to 1.0 was obtained. Inoculum was added to attain 1x10^6 colony forming units (c.f.u.) ml^{-1} for the experiments.

Spore production

For spore production, cultures were set up in 500-ml Erlenmeyer flasks containing 100 ml of Difco Sporulation Medium (DSM; 5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 1 g l^{-1} KCl and 0.12 g l^{-1} $MgSO_4 \cdot 7H_2O$). After DSM sterilization (at 121 °C and 1.5 psi for 15 min), 1 ml of each of the following filtered and sterilized solutions were added to the DSM prior to its use: 1 M Ca $(NO_3)_2$, 10 mM $MnCl_2 \cdot 4H_2O$ and 1 mM $FeSO_4$ (Monteiro et al. 2005). Each experimental treatment was inoculated with 1 × 10^6 c.f.u. ml^{-1} of *Bacillus* B25. Culture conditions were at 30 °C and 200 rev.min^{-1} for 72 h (Chen et al. 2010; Rao et al. 2007).

B. cereus B25 spore identification and quantification

Endospores from 72-old culture were identified by differential staining with a solution of malachite green (5 % w/v) and safranin (0.5 % w/v) using the Schaeffer and Fulton method (1933). Mature spores appeared green (whether or not they were free or still in the vegetative sporangium), while vegetative cells and sporangia were stained red (Schaeffer and Fulton 1933).

Spore concentration was evaluated by determining the number of c.f.u. ml^{-1} after heat-shock treatment. The *Bacillus* B25 spore suspension was placed at 80 °C for 10 min, followed by a quick cooling to room temperature (under running water at 25 °C). Serial dilutions were made, spread on LB agar plates and incubated at 25 °C for 24 h. Colonies were counted and reported as c.f.u. ml^{-1} (Chen et al. 2010; Chung et al. 2010).

Powder formulation

The powder formulation was prepared based on a mix composed of talc as a carrier, with carboxy-methyl-cellulose (CMC; 1 % w/w), $CaCO_3$ (15 % w/w) and glucose (0.25 % w/w). All materials were mixed in powder form and autoclaved at 121 °C and 15 psi for 15 min. Subsequently, materials were mixed with the *Bacillus* B25 spore suspension and dried by at 55 °C for 36 h. The formulation was pulverized with a sterile porcelain mortar and pestle, packed in plastic bags and stored at room temperature until use. A formulation mix without spores was used as a control in subsequent in vitro experiments. The number of viable spores was determined using the plate count method with serial dilutions (Chung et al. 2010; Omer 2010).

Shelf life

The shelf life of the powder formulation was determined every 60 for 360 days. Viable *Bacillus* B25 spores were determined by counting c.f.u. g^{-1} of formulation. 0.1 g of powder formulation was weighed and mixed in a 1.5-ml tube containing 0.9 ml of distilled water, and then placed at 80 °C for 10 min to eliminate vegetative cells. Serial dilutions were prepared and 0.1 ml of the suspension was added to an LB plate. The dilution plates were incubated for 24 h at 25 °C until colonies were visible. Two independent subsamples were analysed twice, with three replicates per treatment (Chen et al. 2010; Chung et al. 2010; Omer 2010).

In vitro antagonism tests with the powder formulation

In vitro assays were performed to measure how the formulation process affected the antagonistic activity of *Bacillus* B25 against *F. verticillioide*s P03. One agar plug (5 mm in diameter) containing the mycelia of each fungal pathogen was placed in the centre of a Petri dish (90 mm in diameter) containing potato dextrose agar (PDA) medium. *Bacillus* B25 powder formulation was then rehydrated with sterile distilled water according to (Yánez-Mendizábal et al. 2012a), then five microliters of a 1:10 (w/v) powder formulation suspension (1 × 10^8 c.f.u. ml^{-1}) were placed at four equidistant points, 2 cm away from the central mycelia disc, and plates were incubated at 25 °C for 3–5 days. Five microliters from a cellular suspension of a 24-h culture of *Bacillus* B25 (2.5 × 10^5 cells) were used as a control. Experiments were terminated once mycelia growth in control plates (i.e. containing no *Bacillus* B25) reached 4 cm in diameter. A formulation mix without spores was used as a control. Percentage of inhibition was calculated by Eq. (1), previously used by Paneerselvam et al. (2012).

$$L = [(C - T) / C] \times 100 \quad (1)$$

where L is the inhibition of radial mycelium growth, C is the measurement of radial mycelium growth in the control plate, and T represents radial mycelium growth in the

presence of the powder formulation (Paneerselvam et al. 2012).

Seed adhesion assays

Ten grams of maize seeds were placed in a sterile plastic tube and moistened with 0.2 ml of 1 % (w/v) CMC solution used as an adhesive. Seeds were then coated with 1 g of powder formulation (1×10^9 spores g^{-1}). The mixture was shaken until the seeds were covered. To determine the amount of bacteria adhering to seeds, five seeds were randomly selected and the formulation excess was removed. The seeds were then separately placed in test tubes with 1 ml of sterile distilled water to wash the formulation coat from the seeds. The wash water was then used to set up serial dilutions and aliquots were plated to count the number of *Bacillus* B25 spores per seed (El-Hassan and Gowen 2006).

Effect of powder formulation on seed germination

Maize seeds were surface-sterilized using a chlorine solution (0.75 %), incubated for 15 min at 52 °C, washed three times with sterile distilled water, and left to dry in a laminar flow hood for 2 h before use. Maize seeds were moistened with 0.4 ml of CMC 1 % (w/v) and coated with 2 g of powder formulation (1×10^9 spores ml^{-1}). The mixture was stirred until the formulation completely covered the seeds, after which the excess was removed and seeds were placed on water-agar plates at 25 °C for germination. Treatments included in the assay were: (1) untreated seeds (control 1), (2) seeds covered with the formulation without *Bacillus* B25 spores (control 2) and (3) seeds treated with the formulation containing *Bacillus* B25 spores. For the plate assay, six seeds per replicate and ten replicates per treatment were plated on water-agar in Petri plates by duplicate. All plates were incubated in a growth room at 25 °C for 5 days to calculate the final germination percentage (FGP) (Almaghrabi et al. 2014), based on Eq. (2). The assay was performed twice independently.

$$\text{FGP} = (\text{number of germinated seeds/total seeds}) \times 100 \quad (2)$$

Pathogenicity assays

Pathogenicity assays for the powder formulation against *F. verticillioide*s P03 were performed twice using the rolled paper towel technique (Warham et al. 1996) with some modifications, as described in detail in Leyva-Madrigal et al. (2015). Maize seeds (Garañón hybrid from Asgrow) were inoculated at planting time with 3.5×10^6 c.f.u. of formulated B25 spores per seed. A completely randomized design with five replicates per treatment was used. Treatments containing the fungal pathogen *F. verticillioide*s P03 were inoculated with 2.5×10^4 conidia per seed. Experiments were maintained at 25 °C for 10 days after emergence of seedlings.

The following treatments were included: (1) untreated maize seeds (control 1); (2) maize seeds treated with the powder formulation mix without *F. verticillioide*s P03 (control 2); (3) maize seeds treated with the powder formulation and *F. verticillioide*s P03; and (4) untreated maize seeds containing *F. verticillioide*s P03 (pathogenicity control).

We used a disease rating system modified from the scale previously described by du Toit et al. (1997), in which 0 = 0 % root rot; 1 = 1–25 % root rot; 2 = 26–50 % root rot; 3 = 51–75 % root rot; 4 = 76–100 % root rot; 5 = root system dead and fragmented; and 6 = whole seedling dead. The readings were converted to a disease index using Eq. (3).

$$\text{Disease index}\,\% = [\Sigma(\text{R} \times \text{N})] \times 100/\text{H} \times \text{T} \quad (3)$$

where R = disease rating, N = number of plants with this rating, H = highest rating category (i.e. 5), and T = total number of plants counted (Asran and Buchenauer 2003; du Toit et al. 1997).

Length and dry weight measurements of plant roots and shoots were also taken. Maize plants were excised at the seed junction, and their shoot length was measured by extending the leaves and roots and measuring the maximum distance between the excision site and the root or leaf tip. Root and shoot dry weight was determined after drying at 80 °C in a forced-air oven for 72 h or until a constant weight (Cohen et al. 1980; Harman et al. 2004; Sharp et al. 1988).

Statistical analysis

Rolled paper towel assays were analysed by one-way ANOVA using the Statistical Analysis System (SAS 9.0) program. The disease severity index data was arcsine-transformed and then analysed using the PROC GLM from SAS (SAS Institute Inc.). Differences among treatments were determined using Duncan's multiple range tests (DMRT) at a significant level of $P = 0.05$. Data are presented as mean ± standard errors (SE).

Results

Shelf life of the powder formulation

The amount of *Bacillus* B25 spores in the powder formulation remained relatively high throughout the first

6 months after the formulation process. Spore counts decreased from 1.4×10^9 c.f.u. g^{-1} on the day of preparation to 1.1×10^9 c.f.u. g^{-1} after 180 days of storage at room temperature (Fig. 1), which corresponds to 78.57 % viability. The spores' viability in formulation was 52.85 % at 360 days of storage. A second experiment ran in parallel gave similar results, but only results from one experiment is shown in Fig. 1.

In vitro antagonism tests with the powder formulation

The powder formulation inhibited growth of *F. verticillioide*s P03 (Fig. 2) and all of the fungal pathogens tested in vitro. This demonstrates that the antagonistic activity of the bacteria against *F. verticillioide*s P03 remained stable, even after the formulation process. It was also effective in controlling the eight other maize phytopathogenic fungi in vitro, as measured by the dual culture assays, whose inhibition percentages ranged from 22.85 to 42.85 % depending on the fungus (Table 1). This experiment was repeated twice. The formulation mix without spores used as a control showed no effect on *F. verticillioides* growth (Fig. 2; Table 1).

Seed adhesion effectiveness of the powder formulation and its effect on seed germination

The talc-based powder formulation was examined for spore viability by comparing c.f.u. counts before (2×10^9 c.f.u. g^{-1}) and after the formulation process (1.4×10^9 c.f.u. g^{-1}), with a final recovery of 50 %. Regarding adherence of the powder formulation applied to seeds, the viable spores ranged from 1.08 to 1.41×10^7 c.f.u. g^{-1} seed ($3.6–4.7 \times 10^6$ c.f.u per seed) in three independent experiments. The powder formulation had no negative effect on seed germination, since seed germination remained at 100 % in comparison to control treatments.

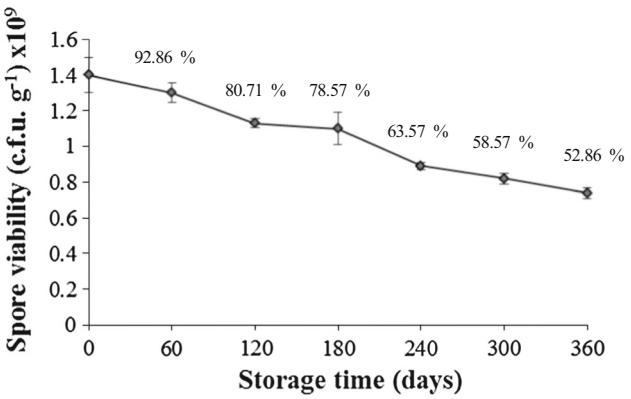

Fig. 1 Spore viability of a *Bacillus* B25 powder formulation, stored at room temperature and evaluated at 60-day intervals post-formulation. The c.f.u. values for each sampling date represent the mean of three replicates ± SE. The percentage values on each point represent the viability percentage

Pathogenicity assays

We next examined the efficacy of the *Bacillus* B25 powder formulation on the suppression of *F. verticillioide*s P03 root rot in maize plantlets (Fig. 3). No significant effect was observed ($P \leq 0.05$) in the dry weight of roots or shoots when the seeds were treated with the powder formulation (Table 2). In contrast, maize seeds treated with the powder formulation showed a significant reduction in the root rot disease index (55.45 %) as compared to the pathogenicity control (68.35 %) (Fig. 4). This is probably due to the increase in the *Bacillus* B25 population in the rhizosphere coming from the powder formulation. Overall, the results indicate that seed treatment with the *Bacillus* B25 powder formulation was effective in controlling *F. verticillioide*s P03 in maize plants.

Discussion

The ability of any formulation to successfully suppress disease or enhance plant growth depends on the availability and shelf life of microbes, since the shelf life of the formulation has a decisive effect on the commercialization of bioinoculants. This emphasizes the formulation process as an essential step in determining the quality and commercial success of the final product (Shaikh and Sayyed 2015).

There are ample studies in the literature on the screening of microorganisms that possess pesticidal activity. However, very few of these reports have considered formulating the microorganisms for commercial applications (Hynes and Boyetchko 2006). In particular, information regarding the formulation of microorganisms has been highly limited due to secrecy issues imposed by commercial companies (Herrmann and Lesueur 2013). Although several bacteria and fungi have been isolated to the point where they are now being evaluated for commercial development as biopesticides, one of the main constraints of these biopesticides is their limited tolerance to fluctuating environmental conditions, as well as the difficulties in developing a stable formulated product (Palazzini et al. 2013).

Formulation components should help support the viability of the biocontrol agent for an increased storage period, taking into account a desirable shelf life of 6–12 months for industrialization; moreover, carriers and other components should not affect the viability and activity of the biocontrol agent over time (Shaikh and Sayyed 2015). In the present study, a talc-based powder

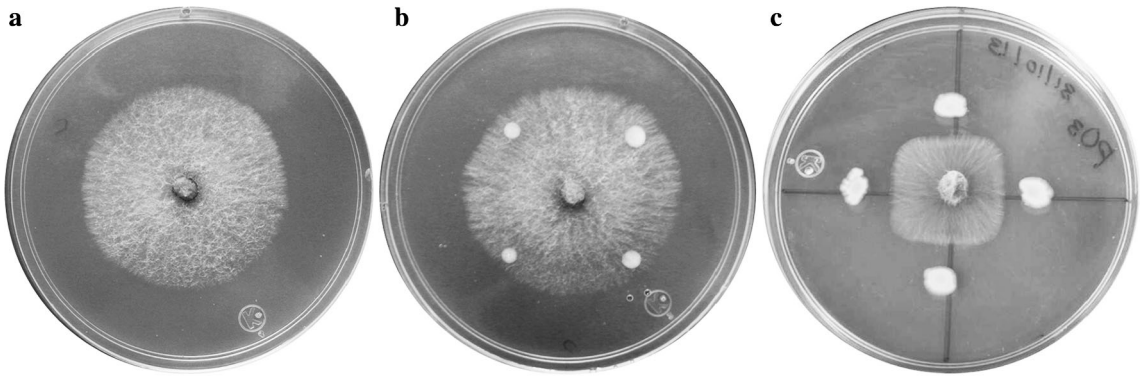

Fig. 2 In vitro inhibition assay of powder formulation against *F. verticillioide*s P03. **a** Mycelial growth of *F. verticillioide*s P03 in the control plate. **b** Mycelial growth of *F. verticillioide*s P03 in response to the powder formulation without *Bacillus* B25 spores. **c** Inhibition of *F. verticillioide*s P03 mycelial growth by a powder formulation of *Bacillus* B25 spores after 4 days of incubation in PDA medium

Table 1 Antifungal activity of the *Bacillus* B25 powder formulation against *F. verticillioide*s P03 and other maize fungal pathogens assessed after 3–5 days of incubation on PDA medium plates

Phytopathogenic fungi	Mycelia growth radius (mm)		% inhibition
	Control	*Bacillus B25*	
PF	17.5	17.5*	0
VC	17.5	14.0	20
Fusarium verticillioides	17.5	12.1	30.8
Fusarium nygamai	17.5	12.3	29.71
Fusarium andiyazi	17.5	10.7	38.85
Fusarium thapsinum	17.5	11.6	33.71
Fusarium roseum	17.5	11	37.14
Macrophomina phaseolina	17.5	10	42.85
Alternaria alternata	17.5	12	31.4
Phomopsis	17.5	11.1	36.57
Choanephora	17.5	13.5	22.85

The inhibition values represent the average of four replicates repeated twice independently

PF stands for powder formulation without *Bacillus* B25 spores. VC means unformulated *Bacillus* B25 vegetative cells

* This control contains only the powder formulation without *Bacillus* B25

formulation containing *Bacillus* B25 spores was prepared and evaluated for its shelf life, as well as its seed adherence ability, effect on seed germination, and antagonistic ability against *F. verticillioides* P03, in both in vitro and *in planta* assays. The *Bacillus* B25 powder formulation has notable features for use at the industrial level, including its control of *F. verticillioides* P03 and eight other potential maize fungal pathogens in vitro and in maize plants.

Treating seeds with biocontrol agents is one of the most suitable application methods for biocontrol of soilborne pathogens in plants (Ugoji et al. 2006). This approach is a reliable way to apply biocontrol agents, as it places them in close proximity with the germinating seed (Bardin and Huang 2003). Furthermore, this method may be especially effective for controlling pre- and post-emergence plant diseases (Ugoji et al. 2006). Indeed, application of *Bacillus* to seeds has been used for the biological control of soilborne phytopathogens affecting many host plants, including *F. verticillioides* in maize.

The process of seed coating usually involves the rehydration of the formulated bacteria (Yánez-Mendizábal et al. 2012a) as well as the use of adhesives such as methyl cellulose, gelatine, dextran, gum arabic, polyvinyl derivatives, polyethylene derivatives and casein. In this work we re-hydrated the spores in water according to Yánez-Mendizabal et al. (2012) who evaluated the effect of rehydration medium on viability of *B. subtilis* CPA-8 formulations finding no significant differences in the survival of CPA-8 formulations rehydrated with 10 % skimmed milk (SM), 10 % sucrose, potassium phosphate buffer (PB) or water. Carboxy-methyl cellulose has frequently been reported to be a good spore adhesive in biocontrol agent formulations

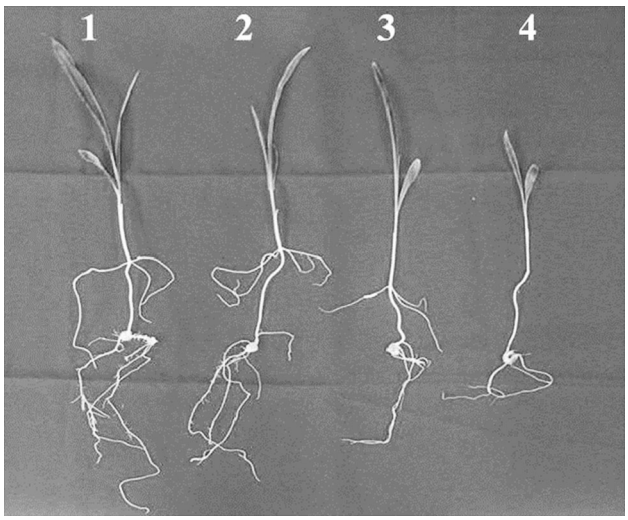

Fig. 3 Effect of the powder formulation on maize disease symptoms caused by *F. verticillioide*s P03. (*1*) Untreated maize seed (absolute control). (*2*) Maize seed treated with the *Bacillus* B25 powder formulation without *F. verticillioide*s P03. (*3*) Maize seed treated with *Bacillus* B25 powder formulation and *F. verticillioide*s P03. (*4*) Maize seed treated with *F. verticillioide*s P03 (pathogenicity control)

Table 2 Effect of *Bacillus* B25 powder formulation on length and dry weight of leaves and root systems of maize grown under greenhouse conditions

Treatment	Length (cm)		Dry weight (g)	
	Leaf	Root	Leaf	Root
1	28.44[a]	17.79[a]	0.079[a]	0.031[a]
2	29.54[a]	19.06[a]	0.077[a]	0.024[b]
3	28.15[a]	13.48[c]	0.050[b]	0.019[c]
4	24.50[b]	10.00[d]	0.031[c]	0.013[d]

The different treatments consisted of: (1) untreated maize seeds (absolute control); (2) seeds treated with the powder formulation without *F. verticillioide*s P03; (3) seeds treated with the powder formulation and *F. verticillioide*s P03; and (4) seeds treated with *F. verticillioide*s P03 (pathogenicity control)

Different letters indicate significant differences ($P \leq 0.05$) according to DMRT. Values indicate the average of five replicates repeated twice

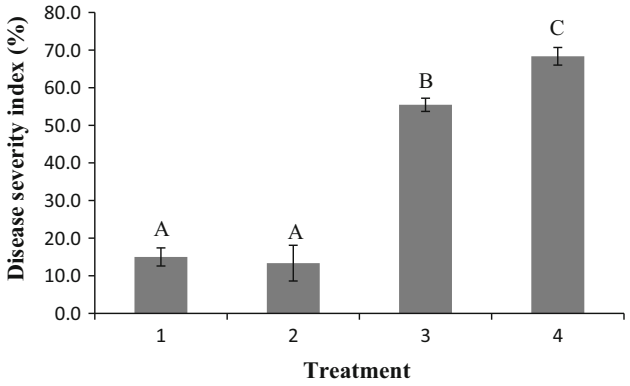

Fig. 4 Effect of the *Bacillus* B25 powder formulation on the disease severity index of maize grown under greenhouse conditions. *Bars* represent the mean values ± SE. *Different letters* indicate significant differences ($P \leq 0.05$) according to DMRT. Numbers along the X-axis indicate the different treatments: (*1*) untreated maize seeds (absolute control); (*2*) maize seeds treated with the powder formulation without *F. verticillioide*s P03; (*3*) maize seeds treated with the powder formulation and *F. verticillioides* P03; and (*4*) maize seeds treated with *F. verticillioide*s P03 (pathogenicity control)

(Chakravarty and Kalita 2013). Its low cost coupled with a low working concentration has also contributed to its wide use (Bardin and Huang 2003).

In the present work, the adherence of a viable bacterial population, using CMC as the adhesive, ranged between 1.0 and 1.41 $\times$ 10^7 c.f.u. g^{-1} seed and displayed no negative effects on maize seed germination. These data are in agreement with Ugoji et al. (2006), who used *Bacillus* spp. in maize seeds and a commercial adhesive. In the latter work, seeds treated with a cell suspension (1 $\times$ 10^9 c.f.u. ml^{-1}) permitted obtaining spore counts from 1 $\times$ 10^6 to 1 $\times$ 10^7 c.f.u. g^{-1} seed, and no negative effects were observed in seed germination (Ugoji et al. 2006).

Talc has been traditionally used as carrier materials for effective bacteria formulations, in fact, stable bacterial populations have been reported for up to 240 days in talc-based formulations (Vidhyasekaran and Muthamilan 1995). The most commonly used formulations involve strains of *Pseudomonas fluorescens*, *P. aeruginosa*, *P. putida*, *B. subtilis* and *B. amyloliquifaciens*. Among these species, *Pseudomonas putida* strains 30 and 180 have survived for up to 6 months in talc-based formulations (Dey et al. 2014).

Talc, the carrier in this work, is a natural product composed of various minerals in combination with chloride and carbonate (Nakkeeran et al. 2005). It has very low moisture equilibrium, relative hydrophobicity and reduced moisture absorption; in addition, its properties prevent the formation of hydrate bridges, enabling longer storage periods. The chemically inert nature and easy availability of talc make it a frequently used carrier for formulation development (Kloepper and Schroth 1981), and its potential as a carrier for formulating rhizobacteria has already been demonstrated (Reddy 2013). Our results using talc as a carrier maintained a 78.57 % *Bacillus* B25 viability (1.1 $\times$ 10^9 c.f.u. g^{-1} seed) after 180 days of storage at room temperature (Fig. 1). These values are consistent with previously reported viabilities in talc-based formulations after 180 days of storage at room temperature for *B. subtilis* (78.13 %) (El-Hassan and Gowen 2006) and *B. megaterium* (68 %) (Omer 2010). In this sense, our particular interest was to evaluate only

spores' viability in the formulation since they are the living component that can survive for long periods of time and are able to endure environmental field conditions. This is the reason why we have measured only endospores at the beginning of the shelf-life experiment to clearly show their survival and efficacy in the formulation. Even though viability in formulations is initially evaluated with all the involved components such as vegetative cells and metabolites (Yánez-Mendizábal et al. 2012b). While our results in the present work are very promising, the product could be further optimized to improve its long-term stability during storage.

One of the most important prerequisites for the commercial use of microbial formulations is that they should retain their biocontrol efficacy (Yánez-Mendizabal et al. 2012). In this work, we determined that the *Bacillus* B25 powder formulation inhibited the growth of *F. verticillioide*s P03 and eight other fungal phytopathogens. Four of the *Fusarium* species tested (*F. verticillioides*, *F. nygamai*, *F. thapsinum* and *F. andiyazii*) have already been identified by our group as associating with *Fusarium* rots in northern Sinaloa, Mexico (Leyva-Madrigal et al. 2015). Previously, two *B. subtilis* strains isolated from the eggplant rhizosphere were assayed against ten important fungal phytopathogens, revealing that both strains inhibit these phytopathogens to a considerable extent (Saha et al. 2012). The authors suggested that this effect was due to general mechanisms of biocontrol in bacteria, including competition for an ecological niche or substrate, as well as the production of inhibitory compounds and hydrolytic enzymes that are often active against a broad spectrum of fungal pathogens.

Previous results obtained by our group have demonstrated chitinase activity, siderophore production, cellulolytic activity and proteolitic activity in the *Bacillus B*25 strain (Figueroa-López et al. 2016). One or more of these mechanisms could thus have possible antifungal activity. Our results are supported by numerous other studies that have established the control by a single bacterial isolate or filtrate against many fungal pathogens. Specifically, it has been shown that an isolate of *B. subtilis* tested in vitro was capable of inhibiting the mycelium growth of such phytopathogenic fungi as *Gaeumannomyces graminis* var. *tritici*, *Coniothyrium diplodiella*, *Phomopsis* sp. and *Sclerotinia sclerotiorum* (Cubeta et al. 1985; Liu et al. 2009). Moreover, one antifungal protein (designated as bacisubin) isolated from culture filtrates of this *B. subtilis* strain was observed to exhibit an inhibitory activity against mycelium growth of *Rhizoctonia solani*, *Magnaporthe grisease*, *S. sclerotiorum*, *Alternaria oleracea*, *A. brassicae* and *Botrytis cinerea* (Liu et al. 2007).

Here, we demonstrated the efficacy of the *Bacillus* B25 powder formulation on the suppression of *F. verticillioide*s P03 root rot in maize plants under greenhouse conditions, which revealed a significant reduction in the root rot disease index in comparison to the pathogenicity control (Fig. 4). *Bacillus* as a seed treatment has been used for biological control of soilborne phytopathogens that affect many host plants (El-Hassan and Gowen 2006; Morsy et al. 2009; Zhang et al. 2009), including *F. verticillioides* in maize. Cavaglieri et al. (2005) demonstrated the ability of *B. subtilis* CE1 to reduce rhizoplane and endorhizosphere *F. verticillioides* colonization at different maize root levels, using a concentration of 1×10^8 cells ml^{-1} as inoculum. Other recent work has shown that applying *B. amyloliquefaciens* as a seed coating at a concentration of 1×10^7 c.f.u. ml^{-1} reduces *F. verticillioides* counts, as well as lowering fumonisin B1 and B2 content from maize grains; similarly, when the authors added *B. amyloliquefaciens* at a concentration of 1×10^9 c.f.u. ml^{-1} to seeds, it significantly reduced the *F. verticillioides* count, but at the inner root tissues of resulting seedlings (Pereira et al. 2009). Furthermore, in soil containing the pathogen *F. moniliforme*, seed inoculation with *Bacillus* spp. isolates has effectively reduced the disease by 79.6 % as compared to the control (Bressan and Figueiredo 2010). More recently, our group has demonstrated that seed bacterization using *Bacillus* B25 protects against *Fusarium* ear and stalk rot in maize fields ((Lizárraga-Sánchez et al. 2015).

The results obtained in this work demonstrate the potential for a powder formulation based on *Bacillus* B25 spores to effectively combat *Fusarium verticillioides* in maize plants. The effect exhibited by the *B. cereus* sensu lato strain B25 powder formulation, whether applied in vitro or *in planta*, supports its use for the control of *F. verticillioides* (and potentially other fungal pathogens) in maize. Further studies on maize fields, cost effectiveness, and performance evaluation in other crops and regions of northern Mexico are currently under way, which will contribute to exploring the potential for commercialization of this formulation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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