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Bacillus cereus sensu lato strain B25 controls maize stalk and ear rot in Sinaloa, Mexico

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ABSTRACT

Fungal pathogens causing maize stalk and ear rot are a potential threat to grain production in regions where monoculture extensions can reach over 500,000 ha per year. This particular problem is observed in northern Sinaloa, Mexico with the fungal pathogen *Fusarium verticillioides*. Three native *Bacillus* spp. strains isolated from the maize rhizosphere were tested for their potential as biocontrol agents (BCAs) against fusariosis in maize, during the 2011–2012 fall-winter growing season. Based on its performance, the *Bacillus cereus sensu lato* strain B25 was selected for further analysis. The effectiveness of maize seed inoculation with this strain was examined in two more consecutive growing seasons. The potential for B25 to control *Fusarium* stalk rot (FSR) and *Fusarium* ear rot (FER) of maize, as well as the accumulation of fumonisins in kernels, was thus assessed with white maize hybrids grown under different field conditions in northern Sinaloa, Mexico. FSR and FER incidence and severity were substantially reduced as compared to controls in all trials conducted. Fumonisin contamination in maize grains was also reduced (up to 93.9%) by B25 application, as compared to the control. Furthermore, B25 significantly increased grain yield in several trial sites or crop seasons, above the average of the untreated controls and consistently above the average of *F. verticillioides*-inoculated controls. Based on these findings, we propose that seed bacterization with strain B25, combined with adequate crop management practices, may become a useful tool for avoiding *Fusarium* stalk and ear rot of maize. This practice will also provide safe fumonisin grain levels for maize production in northern Sinaloa.

1. Introduction

Maize (*Zea mays* L.) is clearly the most important crop in Mexico on two levels: approximately half of the nation's area is dedicated to its cultivation; and it is an integral part of the culture and diet of the population. In 2013, Sinaloa state was the leading producer of maize in Mexico, harvesting 3,627,777.51 t (16% of the national Mexican production) and occupying a field surface of 426,856 ha (SIAP-SAGARPA, 2014). Due to its presence in all regions where maize is cultivated, *Fusarium* stalk, ear and root rot (SERR) of maize is a serious disease that inflicts important economic losses. *Fusarium verticillioides* (Saccardo) Nirenberg (teleomorph *Fusarium moniliforme* (Sawada) Wr), hereafter referred to as *Fv*, is an important fungus involved in the development of SERR on maize (Martínez et al., 2010). Plants infected with *Fv* can show SERR symptoms, as well as wilting, stalk thinning, and reduced aerial and root growth (Oren et al., 2003; Wu et al., 2011). This species produces carcinogenic mycotoxins known as fumonisins, in particular fumonisin B1 (FB1), B2 (FB2) and B3 (FB3), which are all accumulated in maize kernels (IARC, 2002). Fumonisins have been associated with human esophageal cancer, neural tube defects and leukoencephalomalacia in equines, and hepatotoxicity in different animals (Desjardins, 2006). These mycotoxins have been detected in a range of products for human and animal consumption derived from maize (Weidenbörner, 2007).

The incidence of maize SERR in northern Sinaloan fields is low (<10%). The presence of *Fusarium* in SERR symptomatic

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plants is associated with insect attack (Avantaggiato et al., 2003), and once it is present in the kernels, subsequent invasion of the ears by *Fusarium*, *Aspergillus* and yeast is commonly found (Quintero-Benítez and Apodaca-Sánchez, 2008). Rot caused by *Fv* on maize is difficult to control with chemicals, due to multiple factors such as the endophytic nature of the infection (Bacon et al., 2001). Another factor is that chemical control of this pathogen is applied to seeds before their planting, despite reports of the ineffectiveness of fungicides used in this manner and significant increases in fumonisin concentrations in plants resulting from fungicide-treated seeds (Pereira et al., 2007; Falcão et al., 2011). In spite of the significance of this disease to maize production, a more thorough study of the problem and effective control strategies are still necessary. Biological control is proven to be a promising alternative for the control of fungal plant pathogens (Heydari and Pessarakli, 2010). Several bacterial species have been reported to be highly antagonistic against *Fv*, the main causal agent of fusariosis (Bacon et al., 2001; Pereira et al., 2010, 2011). However, most studies have been developed mainly under controlled conditions that differ significantly from what happens in the field.

Bacteria with a potential for *Fv* biocontrol were previously selected both *in vitro* and *in planta* by screening a collection of 11,520 native maize rhizobacterial isolates from northern Sinaloa, Mexico (Figueroa-López et al., 2014; Cordero-Ramírez, 2013). In the present work we have evaluated the potential antagonistic activity of three such previously selected *Bacillus* spp. strains against *Fv* in the field. The goal of this work was to improve our understanding of their plant growth-promoting activities, by assessing their ability to reduce *Fusarium* stalk and ear rot (FSR and FER, respectively) and fumonisin content in maize kernels. *Bacillus cereus sensu lato* B25, the most potentially antagonistic strain during the first year field trials, was further tested for a total of three agronomic cycles. These findings demonstrate that B25 exhibits the best protective effect against SERR in the field.

2. Materials and methods

2.1. Microorganisms

Bacillus strains B5, B25 and B35 were used in the present study. Bacterial isolates were obtained from the maize rhizosphere of commercial maize fields in northern Sinaloa, Mexico (where the fungus displayed more negative effects on maize production). Strains were identified on the basis of their 16S rDNA gene sequence (GenBank accession numbers: *B. megaterium* (B5), JQ830832; *B. cereus sensu lato* (B25) JQ835946) (Cordero-Ramírez, 2013). B35 was only identified at the genus level as *Bacillus* sp. The strains were stored at −70 °C in Luria Bertani medium (LB, Sigma, No. Cat. L3022, USA) supplemented with glycerol (15%, v/v), and deposited in the CIIDIR-003 microorganism collection (CIIDIR – Sinaloa, Mexico).

Fungal isolate *Fv* P03 was used in experiments. It was isolated from infected maize roots and identified on the basis of partial sequences of the calmodulin (GenBank accession number KF641082) and elongation factor 1α (GenBank accession number KF640976) genes (Leyva-Madrigal et al., 2014). The pathogenicity of this isolate has been tested in multiple assays and in different maize hybrids (Figueroa-López, 2011; Leyva-Madrigal et al., 2014). A frozen stock (−70 °C) maintained in Potato Dextrose Agar (PDA; BD Bioxon, Edo. de México, México, Cat. No. 211900) and supplemented with 15% glycerol since 2009 was used as a starter inoculum for experiments conducted during this study.

2.2. Bacterial and fungal inocula

Bacterial isolates were reactivated on LB medium and grown at 25 °C for 24 h. A single colony was inoculated in 5 mL of LB broth and incubated for 18 h at 25 °C and 250 rpm. 1 mL of the latter culture was transferred to 50 mL of LB broth and incubated for 20 h at 25 °C and 250 rpm. Finally, 5 mL of the previous culture were transferred to an Erlenmeyer flask with 250 mL of LB broth and incubated for 24 h at 25 °C and 250 rpm. The concentration of the bacterial cultures (CFU/mL) was assessed by the serial dilution method (Supplementary Table 1). Maize seeds were treated with the corresponding inoculant and soaked in the bacterial suspension for 5 min prior to sowing. Bacteria remaining on maize seeds were assessed by the serial dilution method and reported as CFU g^{-1} of seed (Luna and Sánchez-Yáñez, 1991) (Supplementary Table 1). The inoculated seeds were sowed at a depth of 5–8 cm.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2015.02.015.

Fv isolate P03 was reactivated on PDA plates by incubation at 25 °C for 14 days. Twenty mycelial plugs (1 cm-diameter) were transferred to sterile plastic bags containing 500 g of sterile cracked maize, hydrated with 200 mL of sterile distilled water, and incubated at 25 °C for 14 days. Three kg of the maize–fungus mixture were manually inoculated to the furrows of each plot (four furrows of 10 m separated by a distance of 0.8 m between them). The control treatment received 3 kg of sterile non-inoculated cracked maize. Inoculum concentrations of *Fv* in the soil (CFU g^{-1}) were determined 6 days before sowing (Supplementary Table 1). To determine the natural population levels of *Fusarium* before inoculation with the fungus, soil samples were collected at three points from the usable area a depth of 0–30 cm. Subsamples were mixed, and one subsample was obtained for each treatment plot. Natural *Fusarium* populations were then quantified using the serial dilution method and cultivated on Nash–Snyder agar plates (Nash and Snyder, 1962), with incubation at 25 °C for 6 days. To determine the population levels of *Fusarium* after soil inoculation, three plants per treatment were randomly selected 20 days after emergence of maize plants. Rhizosphere samples of these plants were collected and *Fusarium* populations were quantified by serial dilutions, as described by Nash and Snyder (1962) (Supplementary Table 1).

2.3. Field trials

Three field trials were conducted during the three consecutive fall-winter growing seasons of 2011–2012, 2012–2013 and 2013–2014. Trials were conducted at four locations in northern Sinaloa, Mexico: El Realito (site A), La Noria (site B), Santa Rosa (site C) and El Burrión (site D). Geographic information for each experimental field is reported in Table 1.

Experimental plots in each trial consisted of four furrows of 10 m separated by 0.8 m. The usable area consisted of the two central rows, so as to avoid "border effects" for each experimental plot. Seeds were mechanically sown (1053P 1010 MaxPlanter Seeder, John Deere) during the recommended dates for this region (November 1–December 31), with the following exceptions: sowing was manually performed for field trial I; and experiments commenced after the recommended dates in site A, for two growing seasons. Maize sowing and harvest dates for each growing season and field are provided in Table 2. A density of 90,000 plants ha^{-1} was used in all field trails (INIFAP, 2002). During the tested crop cycles four maize hybrids: Garañón, Cebú, and Gorila (Asgrow), and DK2038 (DeKalb) were evaluated. These maize hybrids are the most commonly used in the region due to their agronomic characteristics:. According to the FAO classification, they all belong to maturity class 900 (ultra-late with a vegetative cycle ≥150 days).

Nitrogen was supplied in two applications using ammonia as the source: 220–250 kg N ha^{-1} before sowing and 60–80 kg N ha^{-1}, 60 days after sowing (according to the soil analysis results; data not shown).

Table 1
Geographic and agronomic information for the experimental fields from Sinaloa, Mexico.

Site	Location	Geographic coordinates	Soil classification	Altitude (m)
A	El Realito, El Fuerte	26°22′47″ N, 108°41′3″ W	Cambisol	80
B	La Noria, Guasave	25°41′22″ N, 108°30′28″ W	Vertisol	50
C[a]	Santa Rosa, Ahome	25°50′45″ N, 105°54′40″ W	Vertisol	10
D	El Burrión, Guasave	25°29′9″ N, 108°47′7″ W	Vertisol	50

[a] This field was selected on the basis that during the 2012–2013 fall-winter season it experienced total losses by SERR associated with *F. verticillioides*, as confirmed by microbiological and molecular identification in our laboratory (data not shown).

In all fields, irrigation was applied during the periods of high water demand for maize: at the germination, vegetative (V3 and V7) and reproductive stages (R1 and R3) (Abendroth et al., 2011). No agrochemical applications were used in El Realito (site A) during the three experimental years; in the other fields (B, C and D), agrochemical applications included chemicals used for weed control, and for common diseases and plagues that are normally present in this region (Table 2). All ears from each usable area were collected by hand at a grain moisture content between 14 and 18% (SW 20300 John Deere model). Ears were shelled using an electric corn sheller machine (Maize sheller, mod. M2010, Agripak International). Kernels were mixed thoroughly to obtain a random sample distribution. Seven-kg grain samples were used for fumonisin content analysis.

2.3.1. Field trial I

The first field trial was performed on site A during the 2011–2012 growing season, using the Garañón (Asgrow®) white maize hybrid. This trial was designed to evaluate the ability of the three bacterial isolates (B5, B25 and B35) to promote maize growth and reduce FSR and FER. Each bacterial strain was tested individually and in all possible combinations with each other. The bacterial inoculum was applied on maize seeds before sowing, as described in Section 2.2. Additionally, a commercial formulation of *Bacillus subtilis* Q11 was used as the control. Eleven treatments were evaluated in randomized blocks with three replicates, including an untreated control (without bacteria or fungus), a pathogenicity control (soil inoculated with *Fv* P03; control + *Fv*), and a LB medium control.

Fv isolate P03 was inoculated in soil 20 days before sowing, as described in Section 2.2.

2.3.2. Field trial II

The second field trial was performed simultaneously on sites A and B during the 2012–2013 growing season. This trial evaluated the effect of single (seed bacterization) and double (seed bacterization plus foliar spread 10 days after plant emergence) applications of the selected *Bacillus* strain B25 in different white maize hybrids, as described in Section 2.2. Soil at site A was inoculated with *Fv* P03 as described in Section 2.2. Two maize hybrids were tested (Garañón and DK2038) at site A. Four treatments per maize hybrid were evaluated in a completely randomized block design with three replicates.

The trial at site B was conducted under natural *Fusarium* population conditions, in which three maize hybrids were tested (Garañón, Cebú and Gorila). Three treatments per maize hybrid were evaluated in a completely randomized block design with three replicates.

2.3.3. Field trial III

The final field trial was conducted to confirm the growth-promoting and protective effects of strain B25 against FSR and FER. This was performed simultaneously on sites A, C and D during the 2013–2014 growing season. This trial was conducted under natural *Fusarium* population conditions, using the Garañón white maize hybrid. The bacterial inoculum was applied only once on seeds before sowing, as described in Section 2.2. Two treatments per maize hybrid were evaluated in a completely randomized block design with three replicates.

2.4. Morphometric measurements

Morphometric measurements were taken (six plants per plot) at 50, 100 and 150 days after emergence of plants. Height was measured from the base of the stalk to the apex of the plant using a measuring tape (reading error: 0.05 cm). Stalk diameter was measured using a Vernier Calipers (Series 530 – Standard Model). Grain yield was measured as total grain production adjusted to 14.0% moisture for each treatment and repetition of the usable area.

2.5. Disease rating

Incidence and severity of FSR were evaluated in 30 plants per treatment, randomly selected from the center of each usable area at reproductive stage R1 (Abendroth et al., 2011). Plants were removed with a shovel and the stalk was dissected longitudinally with a knife to observe the damaged tissues. FSR incidence was assessed by visually estimating presence or absence of disease signs for each of the sampled plants. FSR severity was assessed according to the severity scale reported by Hines et al. (2001).

FER incidence and severity were evaluated in 30 plants per treatment, randomly handpicked from the center of each plot at full maturity of maize ears (stage R6). FER incidence was calculated by dividing the number of ears showing symptoms (discolored kernels, mycelial growth, *etc.*) by the total number of ears sampled and multiplying by 100. FER severity was calculated using a six-class scale based on the percentage of kernels visibly damaged, as proposed by Briones-Reyes et al. (2007). The FSR and FER severity data were converted to disease percentage using the formula reported by Towsend and Heuberguer (1943).

2.6. Incidence of fungi associated with maize kernels

Samples of 120 seeds per treatment were used to determine the presence of associated fungi in the harvested maize kernels. Maize kernels were surface disinfected with a 0.75% sodium hypochlorite solution and washed three times with sterile distilled water. Disinfected seeds were tested for *Fusarium* infection using the freezing blotter test (Warham et al., 1999). Briefly, the technique consists in the disinfection of maize seeds with a 10% sodium hypochlorite solution, followed by incubation at 25 °C for 2 days. Maize seeds were then frozen at −20 °C for 1 day and finally kept at 25 °C for 11 days. Fungal colonies developed on the surface of the seeds were examined microscopically as described in Section 2.7.

2.7. Morphological identification

Fungal colonies obtained from freshly harvested grain were isolated on plates with PDA (BD Bioxon, Estado de Mexico, Mexico, Cat. No. 211900). Plates were maintained at 25 °C for 20 days to observe

Table 2
Maize sowing, harvest dates and agricultural management in the field trials conducted at four sites in three consecutive growing seasons.

Site	Growing season	Hybrid (FAO rating[a])	Sowing and harvest date	Agrochemicals					
				Weeds	AI[c] and dosage	Application time	Plagues	Insect incidence (%)	AI and dosage
A (El Realito)	2011–2012	Garañón (900)	01/21/2012–06/28/2012 (159 DAS[b])	Broadleaf weeds	–[f]	–	*Spodoptera frugiperda*[d]	25	–
	2012–2013	Garañón (900) Dk2038 (900)	01/18/2013–07/22/2013 (185 DAS)		–	–		20	–
								35	–
	2013–2014	Garañón (900)	12/16/2013–06/06/2014 (172 DAS)		–	–		40	–
B (La Noria)	2012–2013	Garañón (900)	11/29/2012–06/04/2013 (158 DAS)	Broadleaf weeds	Atrazine + terbutryne 3 kg ha^{-1}	Pre-emergence	*S. frugiperda*	40	Cypermethrin 0.25 L ha^{-1}
					Atrazine + 2,4-D 1.0 kg + 0.5 L	Post-emergence	*Heliothis zea*[e]	20	–
		Cebú (900)		Broadleaf weeds	Atrazine + terbutryne 3 kg ha^{-1}	Pre-emergence	*S. frugiperda*	45	Cypermethrin 0.25 L ha^{-1}
					Atrazine + 2,4-D 1.0 kg + 0.5 L	Post-emergence	*Heliothis zea*	50	–
		Gorila (900)		Broadleaf weeds	Atrazine + terbutryne 3 kg ha^{-1}	Pre-emergence	*S. frugiperda*	60	Cypermethrin 0.25 L ha^{-1}
					Atrazine + 2,4-D 1.0 kg + 0.5 L	Post-emergence	*Heliothis zea*	45	–
C (Santa Rosa)	2013–2014	Garañón (900)	11/25/2013–05/23/2014 (179 DAS)	Broad and narrow leaf weeds	Atrazine + terbutryne 3 kg ha^{-1}	Pre-emergence	*S. frugiperda*	40	Cypermethrin 0.25 L ha^{-1}
					Atrazine + 2,4-D 2.0 kg + 0.5 L	Post-emergence			
D (El Burrión)	2013–2014	Garañón (900)	12/06/2013–06/06/2014 (182 DAS)	Broadleaf weeds	Atrazine + terbutryne 3 kg ha^{-1}	Pre-emergence	*S. frugiperda*	20	Cypermethrin 0.25 L ha^{-1}
					Atrazine + 2,4-D 3 kg ha^{-1}	Post-emergence			

[a] Ultra-late with a vegetative cycle ≥150 days.
[b] Days after sowing (DAS).
[c] Active ingredient (AI).
[d] Plagues in crop development, growth stages of maize (V5–V7).
[e] Plagues in crop development, reproductive stages of maize (R1 and R2).
[f] Letter denotes the absence of application of agrochemicals.

colony color and morphology, according to Leslie and Summerell (2006). The isolates were placed on Carnation-Leaf-Agar medium (CLA) (Nelson et al., 1983) to obtain the macro- and microconidia structures. Plates were kept at room temperature (25 °C) with exposure to light for 25 days, and then permanently mounted for microscopic evaluation. Isolates were grown in KCl medium (Nelson et al., 1983) to stimulate the formation of microconidia chains. Plates were kept at room temperature (25 °C) for 10 days in darkness, and the aerial mycelium was microscopically examined. Morphological identification of the isolates to the genus level was performed following the Barnett and Hunter (1998) manual; the Booth (1971) and Leslie and Summerell (2006) manuals were used for species identification. Isolates morphologically identified as *Fusarium* sp. were kept cryopreserved in potato dextrose broth (PDB; BD Difco, Le Pont de Claix, France, Cat. No. 254920) with 15% glycerol at −70 °C.

2.8. Molecular identification

2.8.1. DNA extraction

Fungal isolates obtained from trial III were grouped according to mycelial growth on PDA; two isolates per group were used. Isolates were separately cultured in 5 mL of PDB and were incubated at 28 °C in an orbital shaker at 200 rpm during 72 h, followed by centrifugation (13,000 rpm for 10 min). The pellet obtained was used for genomic DNA extraction using the yeast protocol from the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Genomic DNA quantity was measured in a Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE).

2.8.2. PCR amplification

Isolates were identified using the partial sequence of the EF-1α gene amplified with the primer pair EF1/EF2 (O'Donnell et al., 1998). PCR reactions were performed in a 25 μL volume containing: 1 μL of DNA (10 ng), 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of each primer, and 1.25 U of Taq polymerase (Invitrogen, Brazil, Cat. No. 11615-050). The amplification program sequence included: an initial denaturation at 94 °C (5 min); 35 cycles of denaturation at 94 °C (30 s), annealing at 60 °C (30 s) and extension at 72 °C (1 min); followed by a final extension step at 72 °C (5 min). PCR products were separated by electrophoresis in a 1% agarose gel and were visualized by ethidium bromide staining. PCR products were purified using the QIAquick PCR purification kit (Qiagen Cat. No. 28106) and quantified in a Nanodrop 2000c spectrophotometer.

2.8.3. Sequencing and phylogenetic analysis

The amplified PCR products were sequenced in both directions in an ABI 3730xl sequencer (Applied Biosystems, USA). Sequences were edited in a CHROMAS Pro 1.6 (Technelysium Pty. Ltd., South Brisbane, Queensland, Australia) and compared to the NCBI (National Center for Biotechnology Information) database using the BLAST-N software and the Megablast algorithm. All sequences were deposited in the GenBank database (Table 7). The MEGA 6.0 software (Tamura et al., 2013) was used for phylogenetic analyses, and the sequences were aligned using the MUSCLE alignment program (Edgar, 2004). Multiple alignments were subjected to a DNA substitution model analysis in MEGA 6.0, to select the model that best fits the data.

2.9. Fumonisin analyses

Seven kg of grain were harvested per treatment and subsequently pulverized into flour (sieve #20) using a mechanic mill (1/3 HP electric). The flour samples were then divided into eight subsamples, and small portions of each subsample (approx. 250 g) were homogenized to obtain 2 kg of flour per sample. Five grams from each of the 2-kg flour samples were used to quantify fumonisins with the Reveal Q+ fumonisins kit and the AccuScan® III Reader, 4.22 version (Neogen) following the manufacturer's protocol.

2.10. Statistical analyses

All data obtained were subjected to variance analysis (ANOVA) using the program SAS 9.0 (SAS Institute, Inc., Cary, NC). Mean comparisons were made using Tukey's test; all statistical tests were conducted at a probability level of $P \leq 0.05$. All percentage values were previously converted to arcsine ($\sqrt{(x\%/100)} + 0.5$) for data normalization and to proceed with the analysis of variance (Dughetti and García, 2004).

3. Results

3.1. Trial 1 (fall-winter 2011–2012)

Trial 1 examined the growth-promoting and protective effects of three *Bacillus* spp. isolates (both individually inoculated and combined) against FSR and FER. Maize plants inoculated with bacterial strains (either alone or in combination) did not affect stalk thickness, as compared to the untreated control (Table 3). However, maize plants inoculated with the strain B35 and the B5 + B25 + B35 combination were significantly taller than all control treatments (Table 3). Strains B5, B25 and the B5 + B25 combination also increased plant height in comparison to the untreated control, as well as maize plants inoculated with the commercial product (Table 3). FSR incidence ranged from 100% in the untreated control and control + *Fv* plants, to 50% on maize plants inoculated with B25, the only bacterial strain that significantly reduced FSR incidence (Table 3). The untreated control and control + *Fv* displayed 77.8% and 80% FSR severity, respectively. In contrast, bacterial treatments with the strains B5, B25 and the combinations B5 + B35, B25 + B35 and B5 + B25 + B35 significantly reduced FSR severity by up to 27.8% (Table 3). The untreated control and control + *Fv* displayed 76.6% and 81.1% FER incidence, respectively; this incidence was significantly reduced by all strains and combinations, except B25 + B35. FER severity was reduced in all treatments, except B35 and B25 + B35 combination treatments (Table 3). Bacterial inoculation did not increase grain production as compared to the untreated control. However, treatments with B5, B25 and the combinations B5 + B25 and B5 + B25 + B35 significantly increased yield (up to 11.3 t ha^{-1}), as compared to the control + *Fv* (7.1 t ha^{-1}). Isolation of *Fv*-like isolates from maize kernels was significantly reduced in all bacterial treatments except in the B5 + B35 and B25 + B35 combinations. Inoculation of *Fv* increased the concentration of fumonisins (2.8 μg g^{-1}) in comparison to the contamination observed in untreated control kernels (1.3 μg g^{-1}). Strain B25 and the combination B5 + B25 significantly reduced total fumonisins on maize kernels to 0.2 and 0.8 μg g^{-1}, respectively (Table 3).

These findings indicate that the *B. cereus sensu lato* strain B25 has a major protective effect against FSR and FER, and that it efficiently reduces fumonisin contamination. Based on these results, B25 was selected for further analysis.

3.2. Trial 2 (fall-winter 2012–2013)

In this trial, the effect of a second application of strain B25 was evaluated on maize plant growth and protection against FSR and FER. Two maize fields were used for this trial (sites A and B).

Two maize hybrids were evaluated in site A: Garañón (Asgrow) and DK2038 (DeKalb). In the Garañón hybrid plants, double inoculation of B25 did not increase stalk thickness or plant height in comparison to plants inoculated only once (seed) (Table 4). The control + *Fv* and the untreated control displayed FSR incidence values

Table 3
Effect of *Bacillus* isolates B5, B25 and B35 on Garañón maize hybrid growth and protection against *Fusarium* stalk and ear rot in El Realito field (A; El Fuerte, Sinaloa) during the 2011–2012 crop season.

Treatment	Stalk thickness (mm)	Plant height (cm)	FSR[a] incidence (%)	FSR[b] severity (%)	FER[c] incidence (%)	FER[d] severity (%)	Yield (t ha^{-1})	*Fv*-like isolates from kernels[e] (%)	Total fumonisins (μg g^{-1}) (FB1 + FB2 + FB3)
Strain B5	22 a	210 b	72.2 ab	33.3 bc	6.0 d	4.1 d	11.0 a	15.4 d	2.6 a
Strain B25	26 a	226 ab	50.0 b	27.8 c	4.7 d	3.6 d	11.2 a	11.4 d	0.2 c
Strain B35	26 a	234 a	100.0 a	70.0 ab	40.5 c	15.2 b	9.7 ab	24.1 c	1.2 b
B5 + B25	24 a	206 b	66.7 ab	52.2 abc	39.9 c	8.0 c	10.5 a	22.7 c	0.8 c
B5 + B35	24 a	200 bc	72.2 ab	37.8 bc	44.7 bc	8.9 c	9.6 ab	32.6 b	1.5 b
B25 + B35	18 b	172 cd	72.2 ab	37.8 bc	69.0 abc	14.8 b	9.6 ab	32.4 b	1.2 b
B5 + B25 + B35	25 a	236 a	55.6 ab	33.3 bc	8.6 d	3.6 d	11.3 a	23.2 c	1.3 b
Untreated control	21 ab	169 c	100.0 a	77.8 a	76.6 a	16.1 b	9.4 ab	58.8 ab	1.3 b
Control + *Fv*	23 a	197 bc	100.0 a	80.0 a	81.1 a	34.2 a	7.1 b	66.7 a	2.8 a
LB control	23 a	206 bc	77.8 ab	46.7 abc	58.5 abc	18.9 b	9.0 ab	56.7 ab	2.6 a
Commercial formulation[f]	19 b	195 c	100.0 a	81.1 a	73.5 ab	18.6 b	9.5 ab	43.8 ab	1.2 b

[a] *Fusarium* stalk rot incidence.
[b] *Fusarium* stalk rot severity.
[c] *Fusarium* ear rot incidence.
[d] *Fusarium* ear rot severity.
[e] Percentage of isolates identified as *Fv sensu lato* based on their morphological traits.
[f] Commercial formulation of *Bacillus subtilis* (Q11).
Means with different letters in the same column are significantly different at a probability level of 0.05, according to Tukey's test. The reported means of FSR and FER incidence and severity were arcsine transformed ($\sqrt{(x\%/100)} + 0.5$) to normalize the data and to proceed with the ANOVA.

Table 4
Effect of the number of applications of strain B25 on maize growth and protection against *Fusarium* stalk and ear rot for different maize hybrids in El Realito field (A; El Fuerte, Sinaloa) during the 2012–2013 crop season.

Maize hybrid	Treatment	Stalk thickness (mm)	Plant height (cm)	FSR[a] Incidence (%)	FSR[b] Severity (%)	FER[c] Incidence (%)	FER[d] Severity (%)	Yield (t ha^{-1})[i]	*Fv*-like isolates from kernels[e] (%)	Fumonisins (μg g^{-1}) (FB1 + FB2 + FB3)[j]
Garañón	Strain B25	26 a	234 a	0.0 b	0.0 b	32.0 c	15.0 cb	15.3 a	10.7 b	0.0 c
	Strain B25 (2)[f]	24 a	236 a	11.1 b	2.2 b	28.1 c	14.1 c	14.6 ab	16.2 b	0.0 c
	Untreated control	22 b	210 b	88.9 a	35.6 a	52.8 b	22.9 b	12.2 b	42.0 a	14.2 b
	Control + *Fv*	16 c	196 c	61.1 a	24.4 a	74.3 a	42.9 a	9.4 c	58.9 a	17.3 a
Dk2038	Strain B25	25 a	236 a	11.1 a	12.2 a	61.9 a	13.6 b	13.1 a	21.5 b	1.3 c
	Strain B25 (2)[f]	21 b	219 b	16.7 a	14.4 a	26.2 a	14.7 b	13.4 a	22.5 b	0.9 c
	Untreated control	20 b	200 bc	61.1 a	4.4 a	51.5 a	43.9 a	12.5 ab	48.6 a	11.2 b
	Control + *Fv*	18 c	172 c	72.2 a	5.6 a	55.8 a	42.7 a	11.2 b	42.5 a	15.2 a

[a] *Fusarium* stalk rot incidence.
[b] *Fusarium* stalk rot severity.
[c] *Fusarium* ear rot incidence.
[d] *Fusarium* ear rot severity.
[e] Percentage of isolates identified as *Fv sensu lato* based on their morphological traits.
[f] Double application of the bacterial inoculum (1.7×10^7 CFU/mL). The first application was performed on maize seeds before sowing, and the second was performed 10 days after maize plant emergence.
Statistical analyses were performed on each hybrid separately. Means with different letters in the same column are significantly different at a probability level of 0.05, according to Tukey's test. The reported means of FSR and FER incidence and severity were arcsine transformed ($\sqrt{(x\%/100)} + 0.5$) to normalize the data and to proceed with the ANOVA.

of 61.1% and 88.9%, respectively. No incidence of FSR was recorded for Garañón plants inoculated only once, whereas plants inoculated twice exhibited 11.1% FSR incidence and 2.2% FSR severity. FER incidence and severity were equal in the two inoculation groups (*i.e.* in plants inoculated once and twice), but were significantly different from the untreated control plants and control + *Fv*. The yields obtained from plants inoculated either once or twice were similar; these were significantly different from the *Fv* inoculated treatment yield (Table 4). Fumonisin concentrations for the two control treatments were in the range of 14.2 $\mu g\,g^{-1}$ to 17.3 $\mu g\,g^{-1}$, whereas no fumonisins were detected on kernels from the maize plants inoculated (either once or twice) with B25 (Table 4).

Double inoculation in DK2038 plants did not improve plant growth, whereas a single application of strain B25 had a significant increase on plant height and stalk thickness (Table 4). FSR incidence and severity were equal in all treatments, as well as FER incidence. FER severity was equal in both control plants (*i.e.* 42.7% and 43.9%); this severity was significantly reduced in plants that were inoculated either once (13.6%) or twice (14.7%) with B25. Single and double inoculation with B25 reduced fumonisin concentration on maize kernels (Table 4). Yield behavior was similar as that previously observed with Garañón.

Three maize hybrids were evaluated in site B: Garañón, Cebú and Gorila (Asgrow). In contrast to the previously described field experiments, this field was not inoculated with *Fv*. No differences were observed on plant growth in any treatment of the different hybrids (Table 5). Likewise, no FSR incidence was detected in site B. In the untreated control plants, the Gorila and Cebú hybrids displayed 72.3% and 77.1% FER incidence, respectively. Single and double inoculation with B25 did not reduce FER incidence in any maize hybrid (Table 5). However, FER severity was significantly reduced in all maize hybrids by both single and double B25 inoculation, although no differences were observed between these two treatments (Table 5). Both inoculations of B25 similarly increased yield in Cebú and Gorila hybrids, as compared to the untreated control. Nevertheless, a single inoculation of B25 in the Garañón hybrid had a greater effect on maize yield (11.4 $t\,ha^{-1}$) than the double inoculation (10.9 $t\,ha^{-1}$) (Table 5). Fumonisin contamination was reduced by both types of B25 application, and no differences were observed between these two treatments (Table 5).

Overall, the results obtained from both fields indicate that double inoculation with B25 does not improve maize plant growth or the protective effect against FSR and FER. Therefore, a single inoculation of the seed prior to sowing is sufficient for a positive effect on the crop.

3.3. Field trial III (fall-winter 2013–2014)

No effect on plant growth (*i.e.* height and stalk thickness) was observed in any of the cultivation sites (Table 6). FSR incidence and severity of the treated plants did not differ from the untreated control plants for any cultivation site, except site A in which seed bacterization significantly reduced (24.4%) FSR severity (Table 6). FER incidence was highest in site C (88.4%); application of B25 reduced this incidence by up to 35.3%. No differences between treatments were observed for FER incidence in sites A and D. Ear rot severity was highest in sites A (46.1%) and C (42.5%) and was reduced by seed inoculation with B25 (12.8% and 10.2%, respectively). Moreover, maize yield was increased in treated plants from sites A and C by 2.6 $t\,ha^{-1}$ and 1.4 $t\,ha^{-1}$, respectively (Table 6). Counts of *Fv*-like isolates were significantly reduced in maize kernels from treated plants (Table 6). Contamination from fumonisins was significantly reduced by B25 in all sites (Table 6).

Results from this trial confirm the efficiency of strain B25 in reducing maize fusariosis and fumonisin grain contamination in different field sites. Furthermore, B25 does not promote maize

Table 5
Effect of the number of applications of strain B25 on maize growth and protection against *Fusarium* stalk and ear rot for different maize hybrids in La Noria field (B; Guasave, Sinaloa) during the 2012–2013 crop season.

Maize hybrid	Treatment	Stalk thickness (mm)	Plant height (cm)	FSR[a] Incidence (%)	FSR[b] Severity (%)	FER[c] Incidence (%)	FER[d] Severity (%)	Yield ($t\,ha^{-1}$)[j]	*Fv*-like isolates from kernels[e] (%)	Fumonisins ($\mu g\,g^{-1}$) (FB1 + FB2 + FB3)[j]
Garañón	Strain B25	37 a	239 a	0.0 a	0.0 a	71.5 a	38.5 b	11.4 a	11.7 b	0.0 b
	Strain B25 (2)[f]	32 a	226 a	0.0 a	0.0 a	58.3 a	33.9 b	10.9 b	18.2 b	0.0 b
	Untreated control	33 a	222 a	0.0 a	0.0 a	74.9 a	64.7 a	9.4 c	42.0 a	14.2 a
Cebú	Strain B25	36 a	226 ab	0.0 a	0.0 a	68.0 a	35.7 b	10.2 a	17.0 c	0.6 b
	Strain B25 (2)[f]	35 a	236 a	0.0 a	0.0 a	74.3 a	28.0 b	10.6 a	27.5 b	0.9 b
	Untreated control	32 a	210 b	0.0 a	0.0 a	77.1 a	71.9 a	9.2 b	52.5 a	7.2 a
Gorila	Strain B25	37 a	220 a	0.0 a	0.0 a	54.4 b	33.9 b	10.3 a	12.5 b	1.6 b
	Strain B25 (2)[f]	34 a	227 a	0.0 a	0.0 a	58.1 b	31.7 b	10.9 a	18.4 b	1.4 b
	Untreated control	32 a	224 a	0.0 a	0.0 a	72.3 a	65.8 a	9.3 b	39.2 a	11.6 a

[a] *Fusarium* stalk rot incidence.
[b] *Fusarium* stalk rot severity.
[c] *Fusarium* ear rot incidence.
[d] *Fusarium* ear rot severity.
[e] Percentage of isolates identified as *Fv sensu lato* based on their morphological traits.
[f] Double application of the bacterial inoculum (1.7×10^7 CFU/mL). The first application was performed on maize seeds before sowing, and the second was performed 10 days after maize plant emergence.
Statistical analyses were performed on each hybrid separately. Means with different letters in the same column are significantly different at a probability level of 0.05, according to Tukey's test. The reported means of FSR and FER incidence and severity were arcsine transformed ($\sqrt{(x\%/100)} + 0.5$) to normalize the data and to proceed with the ANOVA.

Table 6
Effect of seed bacterization with *Bacillus cereus sensu lato* B25 on maize growth and protection against *Fusarium* stalk and ear rot, on three different northern Sinaloa fields during the 2013–2014 crop season.

Site	Treatment	Stalk thickness (mm)	Plant height (cm)	FSR[a] Incidence	FSR[b] Severity	FER[c] Incidence	FER[d] Severity	Yied (t ha^{-1})	*Fv*-like isolates from kernels[e] (%)	Fumonisins (μg g^{-1}) (FB1 + FB2 + FB3)
A (El Realito)	Strain B25	25 a	226 a	61.1 a	24.4 b	41.3 a	12.8 b	12.1 a	13.3 b	0.2 b
	Untreated control	25 a	222 a	83.3 a	50.0 a	57.9 a	46.1 a	9.5 b	72.2 a	23.8 a
C (Santa Rosa)	Strain B25	27 a	220 a	61.1 a	24.4 a	35.3 b	10.2 b	12.1 a	18.5 b	1.7 b
	Untreated control	25 a	214 a	83.3 a	33.33 a	88.4 a	42.5 a	10.7 b	58.9 a	37.7 a
D (El Burrión)	Strain B25	31 a	234 a	38.8 a	15.5 a	44.9 a	9.5 a	11.1 a	22.2 b	0.0 b
	Untreated control	29 a	228 a	72.2 a	30.0 a	60.1 a	13.2 a	10.8 a	66.4 a	16.5 a

[a] *Fusarium* stalk rot incidence.
[b] *Fusarium* stalk rot severity.
[c] *Fusarium* ear rot incidence.
[d] *Fusarium* ear rot severity.
[e] Percentage of isolates identified as *Fv sensu lato* based on their morphological traits.
Statistical analyses were performed on each hybrid separately. Means with different letters in the same column are significantly different at a probability level of 0.05, according to Tukey's test. The reported means of FSR and FER incidence and severity were arcsine transformed ($\sqrt{(x\%/100)+0.5}$) to normalize the data and to proceed with the ANOVA.

growth, although in some cases it increases yield, making it an excellent candidate for fusariosis control in maize.

3.4. Molecular identification of Fusarium isolates

Six *Fusarium* isolates obtained in trial III were identified as *Fv* (nucleotide identity ≥98%; Table 7) by partial sequencing of the EF-1α gene. All sequences were deposited in the GenBank database under accession numbers KM598772–KM598777.

4. Discussion

The current study is concerned with FSR and FER, two phytopathological problems in some maize fields of northern Sinaloa. The high rates of FSR and FER incidence under natural conditions can reach up to 100% and 88.4%, respectively. Nevertheless, our observations do not accurately reflect the situation throughout the whole region since they are limited to three field sites. Among them, site C has a history of SERR from a previous crop cycle, and for site A *F. verticillioides* was inoculated in the soil during experimentation. Likewise, fumonisin contamination of freshly harvested grain can reach levels (*e.g.* 37.7 mg g^{-1}) above the permitted range. Small surveys in Mexico have reported similar results since 1994 (Desjardins et al., 1994; Cortéz-Rocha et al., 2003; Sánchez-Rangel et al., 2005). Nevertheless, this problem has not been thoroughly studied and effective control strategies are still lacking. In response to this, the present study investigated the potential use of a biological control agent against *Fv* in maize.

Three bacterial strains previously tested *in vitro* against *Fv* infection (Figueroa-López, 2011; Cordero-Ramírez, 2013) were evaluated in the field as a seed coating during the 2011–2012 growing season. Of the three tested strains (including four different combinations), the single inoculation of strain B25 reduced both incidence and severity of FSR and FER, as well as fumonisin contamination. This effect was maintained in the following growing seasons (2012–2013 and 2013–2014) in different fields, using different *Fv* infection conditions (*i.e.* soil inoculation and natural infection) and hybrids. Seed coating of bacteria has proven to be an effective method for suppressing plant pathogens (Estevez de Jensen et al., 2002), including *Fv* in maize (Pereira et al., 2007, 2010, 2011). Our results demonstrate that a single application of the bacterial inoculum is sufficient to induce and maintain the protective effect against *Fusarium* throughout the entire growing season. This is in agreement with Pereira et al. (2010), who reported an improved control of *Fv* and fumonisin B1 contamination on maize ears through seed bacterization, as compared to direct spraying of the same bacteria on maize ears.

Recent reports with pot assays have demonstrated the potential for strain B25 to control *Fv* in maize (Figueroa-López, 2011). Probable mechanisms utilized by B25 to control fungal growth have been tested *in vitro*, including the production of siderophores, proteases, chitinases, and glucanases (Figueroa-López, 2011). Members of the *Bacillus* genus produce many secondary metabolites with antifungal effects on diverse plant pathogens (Raaijmakers and Mazzola, 2012). This includes siderophores, which chelate iron and prevent fungal proliferation (Yu et al., 2011), and the antifungal lipopeptides zwitermicine and kanosamine (Silo-Suh et al., 1994; Milner et al., 1996). For example, a protein produced by *B. subtilis* may inhibit mycelial growth of *F. oxysporum*, *Rhizoctonia solani*, *F. moniliforme*, and *Sclerotinia sclerotiorum* (Li et al., 2009). *Bacillus* spp. that have colonized the rhizosphere can inhibit a fungal pathogen by secreting different lytic enzymes such as proteases (Sierecka, 1998; Khosravi-Darani et al., 2008) or chitinases (Chan et al., 2003; Chang et al., 2009). Different *Bacillus* species provoke a decrease in different plant fungal diseases such as crown and root rot of tomato

Table 7
Fungal isolates identified using the partial sequence of the EF-1α gene.

Isolate	Site	Treatment	Plant material	GenBank accession number
1	D	Untreated control	Seed	KM598772
13	C	Untreated control	Stalk	KM598773
14	C	B25	Stalk	KM598774
15	C	B25	Stalk	KM598775
T1	C	Untreated control	Stalk	KM598776
T6	C	B25	Seed	KM598777

caused by *F. oxysporum* (Omar et al., 2006). Cavaglieri et al. (2005) demonstrated that *B. subtilis* can help prevent the vertical transmission of *Fv* in maize, in addition to reducing the *Fusarium* wilt disease.

Some *Bacillus* species act as plant growth-promoting rhizobacteria (PGPR), and thus promote plant development due to the synthesis of auxin, cytokinin, vitamins and ethylene (Schippers et al., 1987). In addition, they can produce organic acids and phosphatases that make phosphate available to plants (Richardson et al., 2009). Members of this genus can be found within the rhizosphere in close association with plant roots, where they promote their growth and nutrition (Ahmad et al., 2008). Bacon et al. (2001) demonstrated that *B. subtilis* is an endophyte of maize roots that promotes aerial growth and increases seed germination on soil infested with *Fv*.

The plant growth-promoting effect of B25 was not clear in our field trials. Nevertheless, there was no evidence of growth retardation caused by B25 in any hybrid or at any site. Similar results were previously reported by Pereira et al. (2011). Interestingly, we observed that strain B25 was able to significantly increase maize yield in some of the studied fields or sites. This is a desirable feature that not all control agents possess. For example, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* can control *Fv* on maize ears and are even able to reduce fumonisin contamination in grain, although no effect was found on maize yield (Pereira et al., 2010, 2011). We currently do not know which mechanisms are used by B25 to cause growth promotion and protection against *F. verticillioides* in maize in the fields, although a combination of the above mechanisms may be involved.

Bacillus cereus has been reported as a food-borne pathogen causing diarrheal and emetic syndrome (Kotiranta et al., 2000; Senesi and Ghelardi, 2010). Severe infections with this bacterium have been only reported among immuno-compromised people (Kotiranta et al., 2000; Senesi and Ghelardi, 2010). It is worth noting that strain B25 previously tested negative in hemolysis assays using agar blood tests, suggesting that it is not pathogenic to humans (Figueroa-López, 2011). Although blood hemolysis is an important feature displayed by human pathogens, other types of assays are necessary to complement hemolysis tests, as proposed by Zachow et al. (2009) before ruling out any possible human pathogenicity.

Fumonisins are an imminent risk to all maize crops, and represent a threat to human and animal health (Bacon et al., 2001; Leslie and Summerell, 2006). Currently, there is no regulation in Mexico that defines acceptable levels of these mycotoxins in maize and/or maize products. In 2001, the Food and Drug Administration (FDA) established two thresholds for the total value of FB1 + FB2 + FB3: up to 4 ppm for maize intended for human consumption; and between 5 and 100 ppm in maize for animal feed (FDA, 2001). As stated earlier, we found fumonisin contents up to 37.7 mg g^{-1} in freshly harvested maize from Sinaloa, exceeding the limits established by the FDA. Seed treatment with strain B25 significantly reduced fumonisin contamination in all trials, regardless of the white maize hybrid used. This reduction was also observed in fields with a high inoculum of *Fusarium* (*i.e.* sites A and D). FER and fumonisin contamination have also been significantly reduced by chemical control (De Curtis et al., 2011) and appropriate crop management (Blandino et al., 2008; Blandino et al., 2009). It may be possible to improve the control of this disease by combining the appropriate crop management and seed inoculation with B25 before sowing. This can also reduce the use of agrochemicals, in line with demands from environmental protection authorities and consumers.

Fusarium isolates obtained from maize kernels in all trials were morphologically identified as *Fv sensu lato*. Morphological traits are not sufficient for the accurate identification of species belonging to the *Fusarium fujikuroi* species complex (Kvas et al., 2009). Six fungal isolates from the third trial were identified as *Fv* based on the partial sequence of the EF-1α gene. This is the most frequent pathogen in maize ears, as previously reported in Mexico (Figueroa-Rivera et al., 2010; Reyes-Velázquez et al., 2011; Leyva-Madrigal et al., 2014) and worldwide (Covarelli et al., 2012; Madania et al., 2013). Our current understanding of the fungi associated with SERR in maize in northern Sinaloa suggests the presence of four different species of the *Fusarium fujikuroi* complex: *Fv*, *F. nygamai*, *F. andiyazi* and *F. thapsinum*, all of which have tested positive as pathogenic to maize (Leyva-Madrigal et al., 2014). Importantly, B25 has been tested *in vitro* on 83 isolates of *Fv* as well as with isolates of the other three species detected in this region, and has caused growth inhibition in all of them (data not shown). These findings suggest that B25 potentially offers protection against many of the common pathogens found in northern Sinaloa. Current work in our laboratory includes the genome sequencing of *B. cereus sensu lato* strain B25, and the investigation of its biocontrol mechanisms used to control *Fv* in maize.

In conclusion, the present work suggests that incorporating B25 into integral management practices will become an effective tool in combatting maize FSR and FER. This strain will also help contribute to safe fumonisin grain levels for maize production in northern Sinaloa, and possibly other maize producing areas.

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