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ORIGINAL PAPER

Endophytic bacteria from wheat grain as biocontrol agents of *Fusarium graminearum* and deoxynivalenol production in wheat

D. Pan[1] · **A. Mionetto**[1] · **S. Tiscornia**[1] · **L. Bettucci**[1]

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Abstract In Uruguay, *Fusarium graminearum* is the most common species that infects wheat and is responsible for *Fusarium* head blight (FHB) and contamination of grain with deoxynivalenol (DON). The aim of this work was to select bacterial endophytes isolated from wheat grain to evaluate their antagonistic ability against *F. graminearum* and DON production in vitro and under field conditions. Four strains identified as *Bacillus megaterium* (BM1) and *Bacillus subtilis* (BS43, BSM0 y BSM2) significantly reduced fungal growth and spore germination of *F. graminearum*. This antagonist activity remained unchanged after the bacterial cultures were heat treated. Under field conditions, treatments with antagonist BM1 was the most effective, reducing the FHB incidence and severity by 93 and 54 %, respectively, and the production of DON by 89.3 %.

Keywords Biological control · *Fusarium graminearum* · Deoxynivalenol · Endophyte

Introduction

In Uruguay, wheat is one of the most important crops for human food, and the principal fungal contaminant is *Fusarium graminearum* Schwabe (teleomorph=*Gibberella zeae* (Schwein) Petch being the most common casual agent of *Fusarium* head blight (FHB) (Pereyra and Dill-Macky 2010). This is an important disease responsible for extensive yield and quality losses in wheat in several regions of the world (Bai and Shaner 1994; Mc Mullen et al.. 1997; Goswami and Kistler 2004; Starkey et al. 2007). In addition to decrease yields, *F. graminearum* can also produce deoxynivalenol (DON), a potent mycotoxin that affects human and animal health (Marasas et al. 1984). DON is associated with vomiting, food refusal, and neurotoxic and immunotoxic effects (Pancaldi et al. 2004). In Uruguay, DON has been regulated at 2 mg/Kg for wheat and 1 mg/Kg for finish flour products (Decreto Ministerio de Salud Pública 2001).

In the last years, several FHB outbreaks with varying degrees of severity have been reported in wheat growing areas of Uruguay. In 2001 and 2002, FHB was particularly devastating, with estimated yield losses of 60 and 25 %, respectively. During these severe epidemics, 100 % of the samples of wheat contained detectable levels of DON were the mean DON contents were 6.6 and 5.9 mg/Kg, respectively (Pan et al. 2009).

Several strategies are used to reduce the impact of FHB including crop rotation, tillage practices, fungicides application, and planting less susceptible cultivars. None of these strategies by itself is able to reduce the impact of this disease (Dill-Macky and Jones 2000; Matthies et al. 2000; Khan et al. 2001; Yi et al. 2001; Schisler et al. 2002; Hollins et al. 2003; Vogelgsang et al. 2011). Thus, the biological control using antagonistic microorganisms, or as supplements to minimize the use of chemical fungicides in a system of integrated plant disease management, have become more important in recent years (Dal Bello et al. 2002; Kim et al. 2003).

There has been increasing interest in using endophytic bacteria in plant protection and plant growth promotion towards evolving environment-friendly technologies, opening newer areas in microbial exploitation. Endophytes are microorganisms that grow within plants without causing any obvious

✉ D. Pan
dpan@fing.edu.uy

[1] Laboratorio de Micología, Facultad de Ciencias – Facultad de Ingeniería, UdelaR, Julio Herrera y Reissig 565, 11200 Montevideo, Uruguay

symptoms of infection or diseases. The advantages to use endophytes as biocontrol agents are that they are well adapted to live inside the plants and therefore they can provide reliable suppression of disease. Some of the endophytes are thought to protect their host from attack by fungi, insect, and mammals by producing secondary metabolites or suppressing phytopathogens by competence of invasion sites (Wang et al. 2007). In particular, endophytic bacteria are thought to interact closely with their host plants, and could therefore potentially be used as biological control agents in sustainable crop production (Hallman et al. 1997; Sturz and Nowak 2000; Ait Barka et al. 2002; da Luz et al. 2003; Bacon and Hinton 2007; Wang et al. 2009; Zhao et al. 2010; Ohike et al. 2013; Szilagyi-Zecchin et al. 2014; Tao et al. 2014).

Since 1990, *Bacillus* strains have been intensively investigated as biological control agents, but no studies have been done using endophytic strains of Bacillus to control FHB. Bacillus is well-known as antibiotic producers, which have advantage over other biocontrol microorganisms due to their inherent property to form endopores and resistance to extreme conditions. The antagonistic effects of *Bacillus* strains have been shown by in vitro antibiosis and in situ reduction of disease severity (Chan et al. 2003).

As the anthesis is considered the period of greatest susceptibility for *F. graminearum* infection, and it is presumed that anthers are the common route of entry into the plant and it is also the moment for antagonist application to prevent infection when conditions for the disease exist (Fernando et al. 1997; Stockwell et al. 1997; da Luz et al. 2003).

In an attempt to develop biological controls of *F. graminearum* and DON contamination, the aims of this study were (1) to isolate endophytic bacteria from wheat grains and to evaluate the inhibitory effect on *F. graminearum* mycelial growth and DON production, (2) to evaluate the ability of the selected bacteria to reduce the impact of FHB and DON production under field conditions, and (3) to determine if the bacterial thermostable metabolites had antagonistic effect on *F. graminearum*.

Materials and methods

Isolation of endophytic bacteria

Bacteria were isolated from 150 samples of wheat grain collected from commercial fields located in the mayor wheat production area of Uruguay. Samples weighting 500 g, obtained after harvesting operations, were lifted into paper bags to the laboratory. From each of the 150 samples about 200 grains were randomly taken and surface disinfected in 70 % ethanol during 1 min followed by thorough wash with sodium hypochlorite solution at 1 % (amended with 0.1 % Tween 80) for 10 min. Subsequently, grains were rinsed with sterile distilled water (three washes) and dried with sterile filter paper. One hundred grains were randomly selected and placed in Petri dishes (10 grains per plate) containing culture medium Potato Dextrose Agar (PDA), and incubated for 3 days at 25 °C. To test the effectiveness of surface disinfection, imprints were performed. Five grains per sample were placed on Petri dishes containing PDA for 20 min, then the wheat grains were removed from the Petri dishes and these were incubated at 25 °C for 3 days. If there were no microbe colonies on the PDA medium, the grains surface was considered well disinfected.

Bacterial colonies emerging from the wheat grains were subcultured and purified. Individual strains were placed in tubes containing tryptic soy agar (TSA) and stored in 10 % glycerol at−80 °C until required.

Fungal isolates

Eleven *F. graminearum* strains (FgC03.001, FgC03.002, FgC03.003, FgP03.001, FgP03.002, FgSJ03.001, FgSJ03.002, FgS03.001, FgS03.002, FgRN03.001, and FgRN03.006) were obtained from the same wheat samples used for bacterial isolation. The isolates were maintained as spore suspension in 10 % (wt/vol) glycerol at−80 °C. Duplicate subcultures of each isolate were included in the culture collection of the Laboratorio de Micología, Facultad de Ciencias - Facultad de Ingeniería, Universidad de la República, Uruguay.

Determination of antifungal activity in vitro

Fifteen endophytic bacteria were screened for their antagonistic activity against eleven *F. graminearum* strains. From each strain of *F. graminearum*, one mycelium disk was placed at the center of Petri dishes, and bacterial isolates were inoculated at four equidistant sites at 2.5 cm from the center. Other plates were inoculated with *F. graminearum* in the absence of bacterial strains as control. The growth inhibition halo of *F. graminearum* was measured. Three replicates per treatment were carried out.

Mycelial growth inhibition

To evaluate the mycelial growth inhibition by selected bacterial strains, three loops of 2-day-old bacteria cultures on TSA medium were transferred separately to flasks containing 50 ml of potato dextrose broth (PDB) and incubated in shaker at 180 rpm, 28 °C for 5 days. The cells were removed by centrifugation and filtration through a 0.22-μm membrane filter, and then the culture filtrate was mixed with potato dextrose agar (PDA) medium at 10 % (*v*/*v*) and poured into Petri dishes. To detect the absence of bacteria in the cell-free culture filtrate, it was inoculated on solid medium. A disk of 7 mm of each strain of *F. graminearum* was inoculated in the center of the

Petri dish and incubated at 25 °C for 7 days. The diameter of *F. graminearum* colonies was measured daily. The growing radius of the cultures containing both microorganisms was compared with the control cultures. The experiment was repeated three times. The radial growth rate (mmh^{-1}) was subsequently calculated by linear regression. Analysis of variance was carried out on mycelial growth and means were separated with Tukey's test using SigmaStat Version 3.0.

Inhibition of *F. graminearum* spore germination

The inhibitory activity of cell-free culture filtrate against macroconidia germination was assayed. Macroconidial suspension of *F. graminearum* was prepared from the eleven strains. Each strain was grown on Mung Bean Agar (MBA) at 25 °C for 7 days, resulting in heavily sporulating cultures, which were flooded with 10 ml of distilled water and the spore dislodged by gently rubbing the surface with a sterile glass spreader. The suspensions were filtered through sterile gauze to remove occasional mycelial or media fragments, and the conidial suspension was counted using a hemocytometer chamber.

To evaluate the conidial germination inhibition, suspensions with 100 macroconidia were inoculated to Petri dishes containing PDA medium added with 10 % of bacterial filtrate and incubated at 25 °C for 3 days. Microscopic examination was performed 3 days after inoculation at ×100 magnification by using a Nixon Labophot-2 compound microscope. The inhibitory activity was calculated counting the number of spore germinated in comparison with the control. The experiment was repeated three times. Analysis of variance was carried out on spore germination, and means were separated with Tukey's test using SigmaStat Version 3.0.

Field trial of antagonists against FHB and DON production

The isolates that evidenced the greatest biocontrol activity against *F. graminearum* in vitro were selected to evaluate their ability to reduce FHB in wheat cultivar Baguette 9 (susceptible to FHB) at San José Department, Uruguay (S34° 20′, O56° 43′). Plants were grown in 10 row plots, 2-m long with 20 cm row spacing. A randomized complete block design with five replicate rows per treatments was used. A border row of Baguette 9 surrounded the experiment site was not treated. Plots were fertilized based on soil recommendations.

At anthesis time (Zadoks growth stage 65), 1 ml of conidial mix of 11 strains of *F. graminearum* (10^5 conidia /ml) and 1 ml of cells of selected antagonist (10^8 ufc/ml) were used to inoculate florets on 150 wheat heads (30 heads per replicate, 5 replicates/treatments). The conidial suspensions of *F. graminearum* and the liquid bacterial cultures were prepared in the same way as was performed for the in vitro assays. At each application, the suspension was sprayed evenly on to the spikes in each plot using a polyethylene compressed air sprayer. Treatments were applied in late afternoon approximately 2 h before sunset to minimize potential UV degradation of antagonist cells. The primary control treatment consisted of plants sprayed with conidial mix of strains of *F. graminearum* FgC03.001, FgC03.002, FgC03.003, FgP03.001, FgP03.002, FgSJ03.001, FgSJ03.002, FgS03.001, FgS03.002, FgRN03.001, and FgRN03.006. A second control consisted of untreated plants.

When grains were at late milk stage, field assessment of FHB incidence and severity evaluation was performed on 30 heads per replicate (150 heads/treatments) (Stack and McMullen 1995). When grains reached full maturity, grain samples obtained from each replicate were evaluated for 100-kernel weight and DON content using a Ridascreen Fast DON (R-Biopharm, Darmstadt, Germany). Fisher's protected LSD test ($p \leq 0.05$) (SigmaStat Version 3.0) was used to determine if existed significant differences with control.

Effect of heat-treated cultures on *F. graminearum*

The effect of the temperature on antifungal activity produced by metabolites of the bacterial strains was also evaluated. Cell-free cultures of selected bacteria were held at temperature of 121 °C for 15 min at 1 atm and were tested for their activity on mycelia growth and spore germination of *F. graminearum in vitro* and for their ability to reduce FHB under field conditions as described above.

Strains identification

Bacterial strains showing the best performance in vitro and in field assays were identified by morphological and biochemical characteristics using BBL Crystal™ RGP ID Systems (Becton, Dickinson and Company).

For 16S rRNA gene sequencing analysis, the bacterial isolates were cultured at 28 °C for 2 days, and its genomic DNA was extracted from the colony using a FastDNA spin kit (MP Biomedicals, Santa Ana, CA, USA) using manufacturer's instructions. To amplify the 16S rRNA gene, polymerase chain reaction was performed with the primers 518F (5′-CCAGCA GCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATC TAATCC-3′). The PCR mixture contained 5 μL 10× PCR buffer, 5 μL dNTPs (2.5 mM), 2 μL of each primer (10 μM), 2 μL DNA template, and 2 U Taq DNA polymerase in a total volume of 50 μL. The PCR amplification process included initial step at 95 °C for 5 min, 30 cycles of 95 °C for 30s, 52 °C for 45 s and 72 °C for 90s, and a final extension at 72 °C for 10 min. Then, the purified PCR products were sequenced by Macrogen Inc., Korea. The 16 s rDNA sequences of the strains BM1, BS43, BSM2, and BSM0 were submitted to the GenBank database (accession numbers

Table 1 Antifungal activity from bacterial culture in vitro assay

Treatment	Growth rate (mm/h)	Growth rate reduction (%)	Spore germination (no. of spore)	Spore germination reduction (%)
Control	2.128	–	100	–
BM1	0.868*	63.3	20*	80
BS43	0.916*	61.7	28*	72
BSM0	1.032*	56	39.5*	60.5
BSM2	1.090*	55.3	46.5	53.5

Within a column, means followed by an asterisk are significantly different from the control (Fisher's LSD≤0. 05)

KP941576, KP941575; KP941573 and KP941574, respectively) and aligned with published sequences using BLAST program.

Results

Selection of antifungal endophyte bacteria

Of the 15 endophytic bacterial strains isolated from wheat grains, only four strains belonging to *Bacillus* spp. showed antagonist activity against 11 strains of *F. graminearum*. These four strains were identified as *Bacillus megaterium* (BM1) and *Bacillus subtilis* (BS43, BSM0 and BSM2). All the antagonists were able to reduce significantly *F. graminearum* growth ($p<0.05$), among which BM1 exerted the strongest inhibitory effect on fungal growth (63.3 %) (Table 1). Also, BM1, BS43, and BSM0 isolates reduced significantly ($p<0.05$) the spore germination of *F. graminearum* by more than 50 %, BM1 showing the highest inhibition (80 %) (Table 1). The micromorphology of the mycelia in the interaction zone exhibited empty, vacuolated, and swollen hyphae (Fig. 1).

Field trial of antagonists against FHB and DON production

The isolates BM1, BS43, BSM0, and BSM2 that evidenced the highest biocontrol activity against *F. graminearum* in vitro were selected to evaluate their ability to reduce FHB in wheat under field conditions. Wheat treated with every antagonist had significantly reduced FHB incidence compared with the positive control (mixture of 11 *F. graminearum* strains, $p<0.05$). Treatment with the antagonist BM1 was the most effective, reducing the FHB incidence and severity by 93 and 54 %, respectively. On the other hand, BSM2 and BSM0 only reduced the incidence but not the severity of FHB.

The average of DON amount produced in the spikes inoculated with *F. graminearum* was 6.8 mg/Kg. All strains assayed showed a significant reduction of DON content in wheat grain, ranging from 50 to 90 % reduction. BM1 and BS43 significantly reduced DON content compared with the positive control ($p<0.05$). In order to evaluate the FHB control, only the strain BSMO shows a significant decrease in 100-kernel weight when compared to the controls ($p<0.05$) (Table 2).

Effect of heat-treated cultures on *F. graminearum*

Data showed that the antifungal activity of culture filtrate of all bacteria was relatively thermostable even after the sample

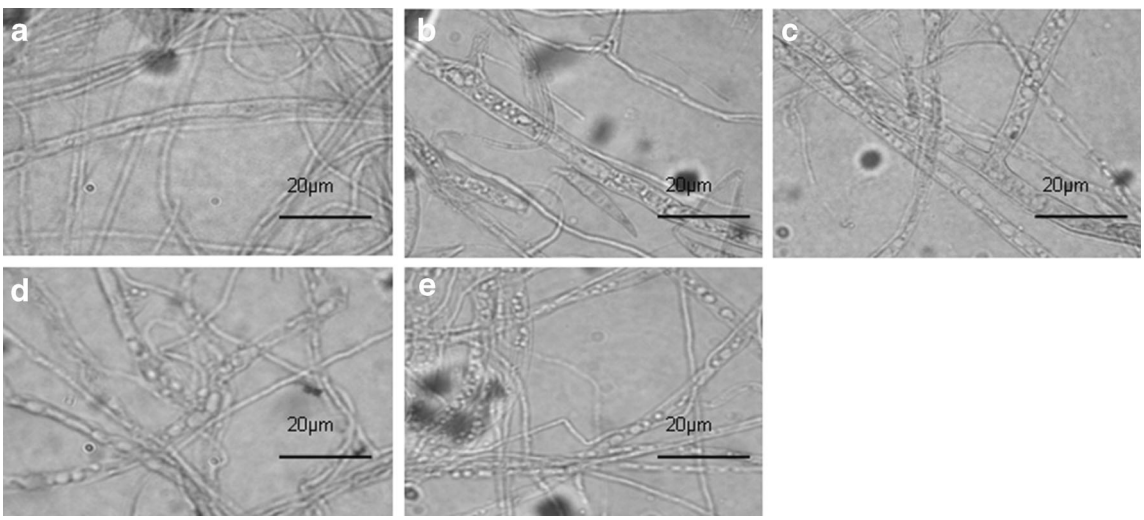

Fig. 1 Morphological effects of antagonist bacteria on *F. graminearum* (**a**) normal hyphae of *F.graminearum*, (**b**) interaction with BM1, (**c**) interaction with BS43, (**d**) interaction with BSMO, (**e**) interaction with BSM2. *Scale bar*=20 μm

Table 2 Reduction of FHB by bacterial antagonists under field conditions

Treatment	Incidence (%)	Severity (%)	DON (mg/kg)	Weight of 100 grains (g)
F. graminearum (F.g)	55.4	87.2	6.8	3.06
No treatment	6.7*	86.1	<0.2*	2.92
BM1 + F.g	4.2*	40	0.7*	3.12
BS43 + F.g	12.2*	58.9	0.7*	2.99
BSM0 + F.g	23.3*	83.1	3.4	2.33*
BSM2 + F.g	34.7*	92.4	2.0	3.04

Within a column, means followed by an asterisk are significantly different from the *F. graminearum* control (Fisher's LSD≤0. 05) F.g: conidial mix of 11 strains of *F. graminearum* (10^5 conidia/ml)

had been held at 121 °C for 15 min (Fig. 2). The BM1 thermostable metabolites were the most effective in reducing the mycelial growth of *F. graminearum* (62.7 %; $p<0.05$), whereas BS43, BSM0, and BSM2 exerted substantial fungal growth reduction by more than 50 %. However, the inhibitory effect of all antagonist filtrates on spore germination was reduced after temperature treatment, with a maximum inhibition of 34 % ($p>0.05$).

In the field trial, all thermostable metabolites significantly reduced FHB incidence compared with the control ($p<0.05$). The treatment with BM1 was the most effective, reducing the FHB incidence and severity by 84.3 and 45.1 %, respectively, and the production of DON by 60.3 %. By the other hand, two of the thermostable metabolites (BM1 and BS43) showed a significant reduction in DON content compared with the positive control ($p<0.05$). Only the thermostable metabolites from BSMO showed a significant decrease in 100-kernel weight when compared to the controls ($p<0.05$).

Discussion

Endophytic bacteria colonize the similar niche to that of plant pathogens favoring them as possible candidates for biocontrol. For this reason, the selection of native endophytic bacteria from wheat grain constitutes an advantage since strains are already adapted to the site where they will be applied. Similarly, some endophytic bacteria isolated from wheat, maize, and rice were able to inhibit the growth of several phytopathogenic fungi, including *F. graminearum* (Chen et al. 1995; Hinton and Bacon 1995; Hallman et al. 1997; Ait Barka et al. 2002; Palazzini et al. 2007; Zhao et al. 2010; Soria et al. 2012). However, despite the numerous reports in vitro and greenhouse studies, only limited field reports have shown that endophytes inoculated in plants confer disease control (Hallman et al. 1997). In this study, it could be demonstrated that endophytic wheat bacteria produce broad and strong antifungal activities against *F. graminearum* in vivo and in vitro.

The screening of endophytic wheat bacteria for putative antagonistic activity revealed that four isolates could inhibit both growth and spore germination of the eleven strains of *F. graminearum*. The inhibition halo of fungal colonies by the bacteria inocula could be attributed to diffusible antifungal metabolites produced by endophytic bacteria in agar culture. Beside, noticeable morphological changes were found in the hyphae of *F. graminearum* in the presence of the antagonistic bacteria. These results are consistent with those obtained by Chan et al. (2003), Lian et al. (2008), Perondi et al. (1996), Nourozian et al. (2006), Soria et al. (2012), and Zaho et al. (2014).

In vitro assays and trials in greenhouse and under field conditions showed that some bacteria belonging to the genera *Bacillus* and *Pseudomonas* were able to reduce *F. graminearum* growth and FHB disease (Khan et al. 2001;

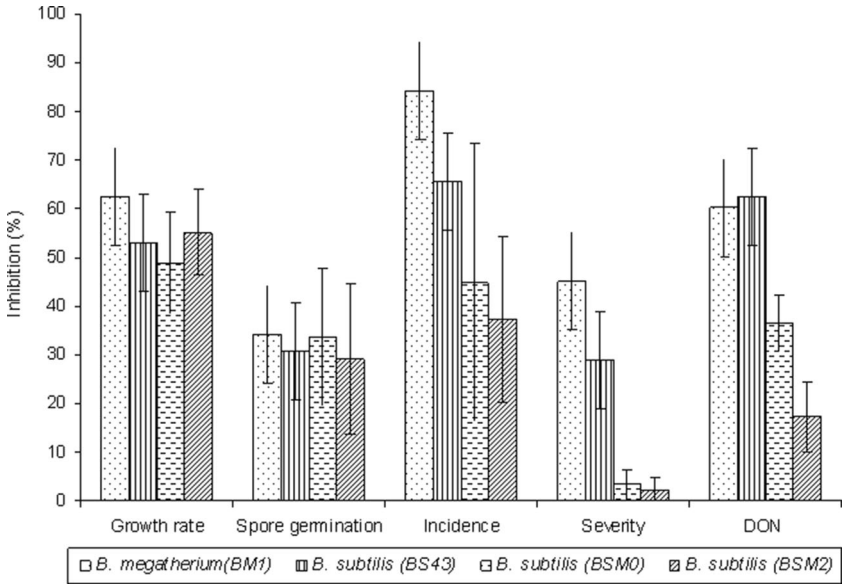

Fig. 2 Antagonic effect of the heat-treated cultures on *Fusarium graminearum* in vitro (growth rate and spore germination) and under field conditions (FHB incidence, FBH severity, and DON production)

Schisler et al. 2002, 2006; da Luz et al. 2003; Palazzini et al. 2007; Zhao et al. 2010, 2014; Shi et al. 2014) as it was shown in this study using *B. subtilis* and *B. megaterium*.

In the field assay, the endophytic bacteria selected reduced disease incidence and severity by up to 93 and 54 %, respectively. The reduction in FHB under field conditions was similar to those obtained by other authors who evaluated the antagonist activity of different bacteria (*Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Cryptococcus*) (Schisler et al. 2002; da Luz et al. 2003; Khan et al. 2004; Palazzini et al. 2007; Zhao et al. 2014).

DON production directly affects the quality and safety of grains, and is of particular concern to food safety regulation agencies. It is important that any potential biocontrol agent for FHB must have the ability to decrease fungal growth as well as DON production. Therefore, reduction of DON should be considered as one of the most important evaluation parameters in control of FHB (He et al. 2009). In this study, all antagonists assayed showed a significant reduction of DON content in wheat grain, ranging from 50 to 90 %. Similar results have been observed by Zhao et al. (2014) who showed that *B. subtilis* SG6 were able to reduce by 69.1 % the DON production under field conditions. Palazzini et al. 2007 showed that *Bacillus* strains isolated from wheat anthers reduce 60–100 % DON content in spikes.

It is well documented that *Bacillus* spp. are potentially able to synthesize a wide range of metabolites with antifungal activity when applied as a biological control agent to several plant diseases. *B. subtilis*, the most studied species, has a strong antifungal activity since it can produce fungicidal or fungistatic peptides synthesized non-ribosomally via a multienzyme process (Stein 2005). Moreover, some cyclic lipopeptides such as iturin, surfactins, and fengycins have been identified as the prominent compounds in *B. subtilis* strains acting against *F. graminearum* (Chan et al. 2003; Hou et al. 2006; Wang et al. 2007; Dunlap et al. 2011; Crane et al. 2013; Zhao et al. 2014). Possibly, the fengycins affect the cell membrane of *F. graminearum* to alter its permeability, resulting in release of cell contents (Ramarathnam et al. 2007; Romanenko et al. 2008).

Besides, *Bacillus* spp. can produce a broad spectrum of non-peptidic antimicrobial compounds such as polyketides, aminosugar, and phospholipid (Stein 2005; Zhao et al. 2014). In addition, bacterial endophytes might enhance plant resistance against pathogens (da Luz et al. 2003).

The antifungal activity of the culture filtrate of all bacteria against *F. graminearum* was relatively thermostable even after the sample had been heated at 121 °C for 15 min, similarly to the results obtained by Chan et al. (2003) and Zhao et al. (2010). They found that the antifungal activity of culture filtrates of *B. subtilis* and *B. vallismortis* were relatively thermostable with more than 50 % of this activity being retained even after the sample had been heated at 121 °C for 30 min.

As known, the effectiveness of biological control in the field tests mainly depends on the environmental conditions. The effect of the strains or their culture supernatants on FHB under conditions to be used in the practice has to be investigated before final assessment of their potential can be made.

Conflicts of interest None.

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