SUMMARY

Six isolates of *Bacillus* spp. (BM1, BM2, BM3, BM4, BM5 and BM6) were evaluated to determine their antagonistic activity towards *Fusarium moniliforme* in maize seeds. A preliminary survey detected *F. moniliforme* (*Giberella fujikuroi*) in all subsamples of maize seeds apparently free from disease. The fungus incidence in the seeds ranged from 26.7% to 61.4%. All *Bacillus* spp. isolates suppressed the growth of *F. moniliforme* in vitro and in maize seed trials. The highest disease control in seeds was given by isolate BM2 (79.6%) and the lowest by isolate BM5 (54.2%). In soil trials, the reduction in diseased plants ranged from 65% to 78% and no significant differences (p ≤ 0.05) were observed among *Bacillus* spp. isolates in controlling the pathogen. All *Bacillus* spp. isolates produced chitinase, but isolates BM2 and BM3 had the highest chitinase activity. These results indicate that *Bacillus* spp. isolates have antagonist activity towards *F. moniliforme* and have a potential to be used as a biocontrol agent. The possible role of chitinase secreted by *Bacillus* spp. isolates in the suppression of the fungal infection is discussed.

Key words: *Bacillus*, *Fusarium*, chitinase, biocontrol.

INTRODUCTION

*Fusarium moniliforme* (*Giberella fujikuroi*), commonly infects a wide range of crops and is a major pathogen of Gramineae, particularly in tropical and subtropical regions. On maize (*Zea mays* L.) the fungus causes seedling blight as well as root, stalk, ear and kernel rot (Galperin et al., 2003). Its pathogenicity is affected by biotic and abiotic conditions (Velluti et al., 2000). *F. moniliforme* overwinters as chlamydomspore-like structures and mycelium in plant debris or crop residues (Nyvall and Kommedahl, 1970) and as mycelium on seed. The infection may occur either at the seedling stage, and the fungus then grows systemically producing symptoms during the later stages of plant development, or later in the growing season. Common points of entry are roots and stalks at the base of leaf sheaths.

Biological control of soilborne plant pathogens is a powerful alternative management strategy. In addition to protecting seeds, biocontrol agents colonize the rhizoplane or rhizosphere when added as seed treatment, and may protect the underground parts of plants from attack (Ahmad and Baker, 1987).

Endophytic bacteria grow in different tissues of various symptomless plants and many genera have or may have potential for biocontrol including *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Erwinia*, *Streptomycyes*, *Serratia*, and *Xanthomonas* (Weller, 1988; Dowling and Thompson, 2000; Kawase et al., 2004; Hoster et al., 2005). *Bacillus* spp. have been tested on a wide variety of plants for ability to control diseases (Paulitz and Belanger, 2001), although relatively little is known about the exact mechanisms of control (Huang et al., 2005).

Many mechanisms of pathogen suppression by bacteria have been suggested: substrate competition and niche exclusion, production of siderophores and/or production of antibiotics and induced resistance. In addition, chitinases (EC 3.2.1.14) are present in a wide range of microorganisms such as bacteria and fungi. These enzymes are glycosyl hydrolases which catalyse the degradation of chitin. Application of chitin to soils or foliage increases numbers of chitinolytic bacteria, providing pathogen control (Mitchell and Alexander, 1961). *Aeromonas*, *Serratia*, *Mycobacterium*, *Vibrio*, *Streptomycyes*, and *Bacillus* produce chitinases (Cody et al., 1990; Jian-Gang et al., 2008) mainly for utilizing chitin as an energy source (Roberts and Selitrennikoff, 1988; Helisto et al., 2001; Hoell et al., 2005). Although strains of *Serratia marcesens*, *Enterobacter agglomerans*, *Stenotrophomonas matophilia*, *Bacillus chitinolyticus* and *Bacillus circulans* are reported to effectively control *Sclerotium rolfsii* and *Rhizoctonia solani* (Dowling and Thomson, 2000; Hoster et al., 2005; Jian-Gang et al., 2008), the data related to suppression of *Fusarium* wilts...
by chitinolytic bacteria are limited.

The objective of this study was to evaluate the effectiveness of Bacillus spp. isolates as biocontrol agents of Fusarium moniliforme in maize and to measure the production of chitinases by these isolates.

MATERIALS AND METHODS

Macroscopically disease-free maize (Zea mays) seeds of cv. BR201 were divided into six subsamples and surveyed for Fusarium moniliforme incidence. Twenty five maize seeds were placed in a plastic germination box (10.5 × 10.5 cm) containing sterilized paper over 4% PDA (potato-dextrose-agar). The germination boxes were incubated for 8 days at 22±2°C, in a 12/12 h light/dark photoperiod. The percentage incidence of F. moniliforme on maize seed was evaluated using a stereomicroscope at 50X magnification (Dai et al., 1987).

Bacillus spp. isolates (BM1, BM2, BM3, BM4, BM5 and BM6) were selected by recovering and screening endophytic bacteria from apparently healthy maize plants, which were classified as nonpathogenic in tests previously conducted in greenhouse conditions. The isolates were grown and maintained on D2 medium (Kado and Heskett, 1970) in the collection of Embrapa Milho e Sorgo, Sete Lagoas, MG, Brazil.

Bacillus spp. isolates were selected in vitro for chitinolytic activity on chitin-agar medium (Hsu and Lockwood, 1975). Colloidal chitin, prepared from practical-grade chitin by the method of Roberts and Selitrennikoff (1988), was added to the agar medium as sole carbon and energy source. Chitinase production was determined by plating a 20 μl drop of a suspension of the isolate (10^7 CFU ml^-1) on 0.2% chitin agar medium and incubating the plates at 28°C. Colonies were regarded as chitinase-producing if they induced clear zones of chitin hydrolysis. For each strain there were 10 replicate plates.

Chitinase production by Bacillus spp. isolates was measured using individual 50 ml Erlenmayer flasks containing 80 ml of minimal salt medium amended with 2 mg ml^-1 colloidal chitin and inoculated with 3 ml of a suspension (10^7 CFU ml^-1) of each isolate grown at 28°C for 72 h in liquid medium containing (g/l): potato starch, 10.0; yeast extract, 3.0; (NH4)2 HPO4, 2.0; and KH2PO4, 2.0. Five replicate Erlenmeyer cultures were used for each isolate. The flasks were incubated on a rotary shaker at 180 rpm for 7 days at 28°C and the suspension from each flask was centrifuged at 30 min at 10,000g. The supernatant was filtered through sterile Millipore membranes, collected in sterile tubes and was used as a source of crude enzymes. Chitinase activity was assayed by measuring the release of N-acetyl-D-glucosamine (NAG), according to Tweddell et al. (1994). A reaction mixture containing 1 ml of the culture supernatant and 1 ml of colloidal chitin (2% wt/vol) in 45 mM sodium acetate buffer, pH 6.8, was incubated in a water bath for 1 h at 36°C. After incubation the reaction was terminated by heating in boiling water for 20 min and the mixture was centrifuged at 3,000g for 20 min. The concentration of NAG in the supernatant was determined as described by Reissig et al. (1955). One unit of chitinase activity (CHU) was defined as the amount of enzyme that liberated 1 μmol of NAG mg-1 protein per hour at 50°C. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as standard. The experiment was repeated twice.

In vitro tests to detect antagonistic activity against F. moniliforme were done in Petri dishes containing PDA medium. The same six Bacillus spp. isolates (10^7 CFU ml^-1) with chitinolytic activity were selected and streaked in the center of the Petri dishes. The agar disc method (Hinton and Bacon, 1995) was used to evaluate in vitro antifungal activity. One disc was cut from a F. moniliforme culture and placed on the outer margin of the Petri dish. Plates were incubated at 28°C for 8 days and observed for mycelial inhibition. Plates inoculated with F. moniliforme alone served as the control.

An experiment to assess disease in maize seeds inoculated with F. moniliforme and Bacillus spp. isolates was arranged in a completely randomized design using the F. moniliforme isolate FMS1 and 6 Bacillus spp. isolates. The replicates were 10 germination boxes containing 25 maize seeds for each treatment.

FMS1 was isolated from maize cultured on PDA solid media for 10 days at 22±2°C and maintained in 15% glycerol at -80°C in the collection of Embrapa Milho e Sorgo. Inocula consisted of both conidia and mycelium together obtained by flooding a Petri dish with sterile distilled water. The conidial concentrations averaged from 10^9 to 10^10 conidia ml^-1 and were brought to a standard concentration of 10^9 conidia ml^-1. Bacterial inoculum suspensions were adjusted to 10^7 CFU ml^-1.

Seeds of maize cv. BR 201, were disinfected prior to treatment by dipping them in a solution of commercial bleach of (5.15% NaClO), containing two drops of Tween 20 for 5 min on an orbital shaker, followed by five rinses in sterile distilled water under agitation. Samples of 25 disinfected seeds were placed in a plastic germination box containing sterilized filter paper over 4% PDA. This medium was used to promote fungal development and to facilitate evaluation of the treatments. The boxes were arranged in a completely randomized design with the following treatments: surface-disinfected seed + F. moniliforme isolate, surface-disinfected seed + F. moniliforme isolate + Bacillus spp. isolates. The positive controls were boxes with surface-disinfected seeds inoculated with F. moniliforme and the negative controls consisted of boxes containing only disinfected seeds. The germination boxes were incubated for 8 days at 22±2°C, in a 12/12 light/dark photoperiod.
Evaluation of fungal infections in the seeds was made under a stereomicroscope. The trial was repeated twice.

Effects of Bacillus isolates on Fusarium disease development in maize plants were tested in a potting soil bioassay in a greenhouse, using pots containing 10 kg of sterilized (120°C for 3 h), sandy-loam soil arranged in a completely randomized design experiment with the following treatments: 6 Bacillus sp. antagonist isolates, the F. moniliforme isolate FMS1 and 6 replicates for each treatment. Each replicate consisted of three plants. Positive controls included seeds not inoculated with Bacillus spp. planted in soil inoculated with the pathogen. Negative controls consisted of seeds not pot-inoculated with Bacillus spp. planted in soil not inoculated with the pathogen. Five pots each sown with five surface-sterilized maize seeds were used per treatment.

Soil inoculum consisted of propagules of F. moniliforme on talc powder produced by the method of Locke and Coulhoun (1974). Sterilized soil and talc powder inoculum were mixed to obtain final pathogen densities of 1 x 10^6 propagules g soil^-1.

Antagonistic Bacillus spp. isolate inocula were grown for 24 h at 28°C on KB-agar plates, and suspensions were prepared in sterile 10 mM MgSO_4. The inoculum was adjusted to 1 x 10^6 CFU ml^-1 before seed inoculation.

Seeds were inoculated by placing surface-disinfected maize seeds in beakers containing each bacterial inoculum and shaking on an orbital shaker at 100 rpm and 28°C for 24 h followed by air-drying. Non-inoculated seeds that were shaken under the same conditions but soaked in sterile deionized water served as negative controls.

For each treatment five seeds were sown in plastic pots containing 10 kg of a 1:1 mixture of steamed sandy-loam soil inoculated with F. moniliforme strain FMS1 (1 x 10^6 propagules g soil^-1). Ten days after sowing the seedlings were thinned to three per pot. Disease was monitored for up to 40 days and measured as the percentage of seedlings showing symptoms of Fusarium disease (yellowing, dropping of leaves or vascular discolouration). Disease incidence was confirmed by plating slices cut from the lower stem and roots from diseased seedlings, surface-disinfected in 1% sodium hypochlorite, on Komada Fusarium-selective medium (Komada, 1975). The experiment was repeated twice. All data were analysed using SAS (SAS Institute Inc., USA). Data were arcsine-square-root transformed prior to analysis. Means were compared by Duncan’s multiple-range test. Statistical significance was determined at P<0.05.

RESULTS AND DISCUSSION

In preliminary experiments, maize seeds were surveyed for F. moniliforme and its presence was confirmed in all subsamples apparently free from disease. Fungal incidence ranged from 26.7% to 61.4% (Table 1). F. moniliforme may remain undetected in kernels until germination, when it infects the emerging seedlings. Yields are reduced in symptomless infected plants (Galperin et al., 2001). Bacon et al. (2001) claimed that endophytic infections transmitted through systemically infected seeds are not controlled by fungicide seed dressings. Our data indicate that symptomless seeds may in fact be latently infected and are an important source of inoculum.

The chitinase activity of a wide range of bacterial isolates has been investigated by determining the size of a clear zone produced on chitin agar plates. This method is widely accepted as a standard test for chitinase activity. Chitinase was produced in all Bacillus spp. isolates studied (Table 2). Production did not differ significantly (p≤0.05) among the isolates (Table 2). Bacillus species

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<th>Table 1. Percentage of Fusarium moniliforme incidence in maize seed samples (survey trial).</th>
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*Within column, values followed by the same letter do not differ significantly at P≤0.05 according to Duncan’s multiple range test.

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<th>Table 2. Chitinolysis, total protein and chitinase activity (CHU) from Bacillus spp. isolates. Values are means of two experiments containing ten replications for each treatment.</th>
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<tr>
<td>Bacillus spp. isolates</td>
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*Clear zone of chitinolysis around colonies on chitin-agar medium. Within column, values followed by the same letter do not differ significantly at P≤0.05 according to Duncan’s multiple range test.
have been shown to produce a high level of chitinolytic enzymes (Cody et al., 1990; Champen et al., 1999). A high correlation between chitinolysis and production of bioactive compounds has been reported (Chen et al., 1991; Pisano et al., 1992).

Total protein and chitinase activity (CHU) varied among the isolates. Isolates BM2 and BM3 showed highest total protein and CHU activity. The chitinolytic activity of Bacillus spp. isolates on inhibition of conidial germination and germtube growth may be directly correlated with disease suppression as reported by Zhang et al. (2001). To demonstrate that antifungal activity is caused by enzymes such as chitinase, Hoster et al. (2005) inactivated the proteins using heat treatment or proteases and observed a strong reduction or loss of the antifungal properties. Our in vitro tests of the antagonistic activity of Bacillus spp. isolates showed that all Bacillus spp. isolates suppressed growth of F. moniliforme on plates. In the inhibition zone of the six isolates there was no physical contact between isolates and the pathogen suggesting that the Bacillus isolates produced antifungal metabolities. Chitinases detected in the previous trials may be involved in fungal growth inhibition by hydrolyzing the chitin in the fungal cell wall. These activities were also reported by Matsuda et al. (2001) and Hoster et al. (2005). Chitinase causes the swelling of fungal mycelium and the formation of vacuoles. This is sometimes accompanied by the degradation of hyphal cell walls and the release of intracellular components into the medium (Melentev et al., 2001). The capacity of Bacillus spp. isolates to grow actively on media containing chitin or fungal biomass indicate their mycolytic potential of these strains, which determines their antagonistic activity against phytopathogenic fungi.

In the maize seed trials where antagonistic activity of the Bacillus spp. isolates was evaluated, all isolates significantly reduced disease incidence. No significant difference in biocontrol activity of the F. moniliforme in the maize seeds was observed among the isolates BM1, BM2, BM3 and BM4. Highest disease reduction was obtained with the Bacillus isolate BM2 (79.6%) and the lowest with isolate BM5 (54.2%), compared to the control.

In soil treated with the pathogen, disease was effectively controlled by seed inoculation with Bacillus spp. isolates. No significant differences were observed in the efficacy of the Bacillus isolates in controlling the pathogen (Fig. 1). The control treatment (pathogen only) showed 98% Fusarium disease, whereas the biocontrol treatments had only 22% to 35% diseased plants.

These data confirm the results observed in the in vitro trial that showed the antagonistic activity of Bacillus spp. isolates to F. moniliforme. Pleban et al. (1997) found that the crude extracellular chitinase of an endophytic bacterium B. cereus 65, decreased spore germination of F. oxysporum and reduced disease incidence by 70%, whereas B. cereus YQ308 inhibited the growth F. oxysporum, F. solani and Pythium ultimum (Chang et al., 2003).

The expression of antagonism by a microorganism towards a pathogen in culture medium cannot generally be taken as evidence of control in situ (Hoitink and Boechn, 1999). However, results obtained in the present study showed that Bacillus isolates had the same antagonistic activities to F. moniliforme in culture medium as on maize seeds in soil.

This work indicates that Bacillus spp. isolated from maize have potential as biocontrol agents. The isolates clearly produce chitinases with antifungal properties and suppress the activity of F. moniliforme.

REFERENCES


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