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Evaluation of *Bacillus mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 for the control of anthracnose of cucurbits caused by *Glomerella cingulata* var. *orbiculare*

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ABSTRACT

Bacillus mycoides isolate BmJ (BmJ) and *Bacillus mojavensis* isolate 203-7 (203-7) were tested in the greenhouse for their ability to control *Glomerella cingulata* var. *orbiculare* the causal agent of anthracnose of cucumber by induced systemic acquired resistance (SAR). BmJ and 203-7 delayed disease onset and reduced total (43% and 56%) and live spore production (38% and 49%) per mm^2 of lesion area when used to induce SAR in cucumber. 203-7 also reduced lesion diameter. Induction by *G. cingulata* conidia resulted in delayed disease onset, reduction of number of lesions per leaf and lesion diameter. Assays of cucumber apoplastic proteins extracted 6 days after induction showed that BmJ increased β-glucanase activity by 135%, and 203-7 increased β-glucanase activity by 72% and peroxidase activity by 79% when compared to the water control. Acibenzolar-*S*-methyl induced the highest ($P = 0.05$) levels of chitinase (950%) and peroxidase (420%) activity compared to water controls. Field experiments (2004 and 2005) evaluated applications of BmJ and fungicides for the control of anthracnose in cucumber (var. 'General Lee') and cantaloupe (var. 'Athena'). BmJ was compared to full and half labeled rate alternate applications of azoxystrobin and chlorothalonil, and BmJ with half rate of azoxystrobin and chlorothalonil. BmJ applied seven days before inoculation reduced disease severity by 41% in cucumber in 2004 and by 24–21% in cantaloupe for both years compared to water controls which was statistically equal to the fungicide treatments. The full and half rate fungicide program provided 97–37% disease reduction compared to water controls. BmJ applied one week before inoculation significantly reduced AUDPC ($P = 0.05$) in cucumber compared to the water control in 2004 on cantaloupe for both years while the full and half rate fungicide program were equivalent and provided the lowest AUDPC. No yield reduction was noted as a result of the disease or treatment for either cantaloupe or cucumber.

1. Introduction

Bacillus-based biological controls have become important tools for control of pests and diseases affecting agricultural and horticultural production (Jacobsen et al., 2004; Jacobsen, 2006). Some researchers (Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003; Kloepper et al., 2004; Mahaffee and Backman, 1993; McSpadden-Gardner and Driks, 2004; Raupach and Kloepper, 1998, 2000; Turner and Backman, 1991; Wei et al.,1991, 1996) have focused on *Bacillus*-based plant growth-promoting rhizobacteria (PGPR) seed or root treatments while others have focused on foliar or post harvest disease control (Jacobsen, 2006). Foliar applications of *Bacillus mycoides* isolate BmJ (Bargabus et al., 2002) provided 38–91% control of Cercospora leaf spot (*Cercospora beticola* Sacc.) of sugar beet in a six year study using susceptible sugar beet varieties. Jacobsen et al. (2004) showed that BmJ provides control of Cercospora leaf spot equivalent to the standard fungicides on sugar beet varieties with moderate levels of resistance. Bargabus et al. (2002, 2003, 2004) and Bargabus-Larson and Jacobsen (2007) showed that *B. mycoides* isolate BmJ and *B.* (*pumilus*) *mojavensis* isolate 203-7 reduced Cercospora leaf spot and bacterial vascular necrosis (*Erwinia carotovora* pv. *betavasculorum*) of sugar beets by inducing systemic acquired resistance (SAR). Systemic acquired resistance has been demonstrated to control *Colletotrichum orbiculare* (Berk. & Mont.) Arx (*Colletotrichum lagenarium* (Pass.) Ellis & Halst) on cucumber (*Cucumis sativus* L.) by induction of lower leaves by foliar applications of conidial suspensions of *C. orbiculare* (Dean and Kuć, 1986; Kubota and Abiko, 2000). Applications of low concentrations of chemical inducers such as Actigard (acibenzolar-*S*-methyl), DCINA (dichloroisonicotinic acid), salicylic acid, and dibasic potassium phosphate Lopez and Lucas (2002) provided good control of *Colletotrichum gloeosporioides*, the causal agent of anthracnose of cashew (*Anacardium occidentale*) without phytotoxic effects. The ability of dibasic potassium phosphate to control *C. orbiculare* in cucumber was further investigated by Irving and Kuć (1990), and by Orober et al. (1999, 2002). Only Orober et al. (2002) reported hypersensitive-

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reaction like lesions on leaves previously treated with dibasic potassium phosphate. In comparison to phosphate and acibenzolar-*S*-methyl (Baysal et al., 2003; Brisset et al., 2000; Lemay et al., 2002; Maxson-Stein et al., 2002), *B. mycoides* isolate BmJ induces SAR without causing hypersensitive cell death in sugar beet (Bargabus et al., 2003) and other plants such as pepper, tomato, potato, geranium, and cucumber (Jacobsen, unpublished).

The objective of this research was to test *B. mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 for their ability to control anthracnose of cucumber and cantaloupe (*Cucumis melo* L.) caused by *Glomerella cingulata* var. *orbiculare*, the teleomorph of *Colletotrichum orbiculare* (Berk. & Mont.) Arx, and to further investigate the potential to integrate BmJ with low rates of fungicides in field studies.

2. Materials and methods

2.1. Bacterial cultures

Bacillus mycoides isolate BmJ (BmJ) was originally isolated from sugar beet leaves. *Bacillus mojavensis* isolate 203-7 (203-7) originally isolated from sugar beet seed embryos was included in the greenhouse experiments since it showed good induction of SAR in previous experiments (Bargabus et al., 2004). Both isolates were stored at −80 °C in 10% glycerol and 1% tryptic soy broth (TSB, EMD Chemicals Inc., Darmstadt, Germany). Bacteria were cultured in 3% TSB for 24 h at room temperature (22 °C) on an orbital shaker (Model OS-500, VWR International, West Chester, PA) at 250 rpm. Fresh cells were harvested by centrifugation for 20 min at 5000 rpm at 4 °C. The pellet was re-suspended in sterile-distilled water and pelleted twice by centrifugation for 20 min at 5000 rpm at 4 °C to assure that all fermentation beer was separated from the cells. The inoculum density was adjusted to 10^8 colony forming units (CFU)/ml with distilled water for greenhouse and field experiments.

2.2. Fungal culture

A Montana isolate of *G. cingulata* var. *orbiculare* (isolate MT-1) was maintained for long-term storage at −80 °C as described above. For the experiments the cultures were transferred to potato dextrose agar (PDA, EMD Chemicals Inc., Darmstadt, Germany) and incubated for 1 week at 28 °C in the dark. Cultures were scraped with a sterile glass rod to loosen conidia. The plates were subsequently flooded with sterile-distilled water and the conidia suspension was decanted and filtered through two layers of cheesecloth to remove mycelium and agar pieces. Inoculum density was adjusted to 10^5 conidia/ml for the greenhouse and field experiments.

2.3. Chemical inducer and fungicides

Acibenzolar-*S*-methyl (ASM) (Actigard 50WG Fungicide, Syngenta, Greensboro, NC) was used as the chemical inducer in the greenhouse experiments at a rate of 50 μg/ml and was applied using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) with applications made to run-off.

For the field experiments, the fungicide treatments consisted of alternate applications of azoxystrobin (Quadris, Syngenta, Greensboro, NC) at a rate of 1.08 or at 0.54 kg of product/ha and chlorothalonil (Bravo WeatherStik, Syngenta, Greensboro, NC) at 2.20 or 1.10 kg of product/ha. Fungicides and BmJ were applied with a CO_2 pressurized (207 kPa) sprayer outfitted with Teejet 8002VS nozzles (Spraying Systems Co., Wheaton, IL) with a total spray volume of 234 L/ha.

2.4. Greenhouse experiments: Plant culture, treatments and inoculation

Cucumber plants (*C. sativus* L. variety 'General Lee') were grown in 10 × 10 × 10 cm plastic pots filled with equal parts (by volume) of PGC Soil Mix (1/3 loam soil, 1/3 washed concrete sand, 1/3 Canadian Sphagnum peat moss plus AquaGro 2000 G [Aquatrols, Paulsboro, NJ] wetting agent, aerated steam pasteurized at 80 °C for 45 min) and Sunshine Mix #1 (Sun Gro Horticulture Inc., Bellevue, WA). Plants were grown for 3 weeks or until the first true leaf was fully expanded under greenhouse conditions at 24 ± 2 °C day and 18 ± 2 °C night temperature with a 16 h photoperiod. Supplemental lighting was provided by SON AGRO 430 WATT HPS lights (Philips Lighting Company, Somerset, NJ). To maintain vigorous growth, plants were watered daily and fertilized twice a week with Peters Professional 20–20–20 General Purpose (The Scotts Company, Marysville, OH) at a rate of 200 μg/ml nitrogen.

The first true leaves of 10 cucumber plants per replicate were induced with either distilled water, BmJ, 203-7, or MT-1.

The bacterial suspensions at 1 × 10^8 cfu/ml as well as the sterile-distilled water were sprayed using a Crown aerosol sprayer (Aervoe Industries Inc., Gardnerville, NV) onto the first true leaves until run-off while covering the rest of the plant with a plastic bag. For the pathogen induced resistance treatment, the first true leaf was inoculated with 10 evenly spaced 10 μl drops of MT-1 conidia suspension adjusted to 10^5 conidia/ml. Plants were placed in a dew-chamber at 24 ± 2 °C and 100% relative humidity (rh) for 48 h, and then transferred to the greenhouse.

Five days after the induction, the second true leaves of all plants were challenge inoculated with the anthracnose pathogen as described above and placed in a dew-chamber for 48 h at 24 ± 2 °C under 100% rh. The experimental design was a randomized complete block with 10 replications per treatment. The experiment was repeated three times.

Plants were examined daily for disease development. The number of lesions was counted 10 days post inoculation and the lesion diameter was recorded in millimeters by making two perpendicular measurements and taking their average.

Two weeks post inoculation plants were placed in a dew-chamber for 48 h at 24 ± 2 °C under 100% rh to induce sporulation. After removal from the dew-chamber, two lesions were removed from each cucumber leaf and placed into a test tube filled with 10 ml of sterile-distilled water. The samples were vortexed (Fisher Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) for 10 s at highest speed and the lesion bearing tissue was removed from the water. Number of conidia per ml was estimated using a hemacytometer (Hausser Scientific, Horsham, PA). The number of conidia per ml and the lesion area were used to calculate the conidia per square millimeter of lesion area. Samples of this conidial solution were also plated onto PDA supplemented with 50 μg/ml tetracycline hydrochloride (OmniPur®, EMD Chemicals Inc., Darmstadt, Germany) with a spiral plater (model D, Spiral Biotech Inc., Norwood, MA). Developing colonies were counted after 4 days to estimate the number of viable conidia per lesion area.

For all described experiments, collected data were analyzed statistically by conducting an analysis of variance (Madden et al., 1982) using the general linear model procedure of the SAS program (SAS system, Version 9.00, SAS Institute Inc., Cary, NC). The treatment means were separated using Fisher's protected least significant difference test at $P = 0.05$.

2.5. Extraction of apoplastic fluid

The first true leaf of three- to four-weeks-old cucumber plants were induced, in replicates of five, with distilled water, BmJ, 203-7, ASM and *G. orbiculare* as described above. Plants were placed