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Impact of time between field application of *Bacillus subtilis* strains SB01 and SB24 and inoculation with *Sclerotinia sclerotiorum* on the suppression of *Sclerotinia* stem rot in soybean

Jinxiu Zhang · Allen G. Xue · Malcolm J. Morrison · Yao Meng

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Abstract This study evaluated the impact of time between the application of cell suspensions or cell-free filtrates of *Bacillus subtilis* strains SB01 or SB24 on soybean plants under field conditions and inoculation with *Sclerotinia sclerotiorum* on their effectiveness for suppression of *S. sclerotiorum*. The results showed that the cell suspensions of two strains provided greater effectiveness than the cell-free filtrates, but the suppression effectiveness decreased as the time between application in the field and *S. sclerotiorum* inoculation increased. The *B. subtilis* cell suspensions applied on soybean leaves for up to 10 days under field conditions were able to provide a significant ($P<0.01$) reduction in disease severity by approximately 20–90% at 5 days after the *S. sclerotiorum* inoculation. When rated 15 days after *S. sclerotiorum* inoculation, plants treated with bacterial cells for ≤6 days reduced *Sclerotinia* stem rot severity by 15–70%. Most effectiveness was provided by the cell suspensions present on soybean leaves for <3 days under field conditions, which significantly ($P<0.01$) reduced disease severity by 40–70% over 15 days. In comparison, the cell-free filtrates remaining on leaves for <6 days significantly ($P<0.01$) reduced disease severity during the first 5 days after the inoculation, while the best cell-free filtrate treatments were those with ≤1-day intervals, which significantly ($P<0.01$) reduced disease severity by 10–40% during 15 days after the inoculation. The effectiveness of *B. subtilis* was reduced when it rained after application.

Keywords *Bacillus subtilis* · Biocontrol · Interval · *Sclerotinia sclerotiorum* · *Sclerotinia* stem rot · Soybean

J. Zhang (✉) · A. G. Xue · M. J. Morrison
Eastern Cereal and Oilseed Research Centre,
Agriculture and Agri-Food Canada,
960 Carling, Avenue,
Ottawa, ON K1A 0C6, Canada
e-mail: jinxiu.zhang@agr.gc.ca

Y. Meng
Scientific Research Division,
Heilongjiang Academy of Land Reclamation Science,
382 Anqing Road, Jiamusi,
Heilongjiang, People's Republic of China 154007

Introduction

Sclerotinia sclerotiorum [(Lib.) de Bary] has a wide host range, causing white mold diseases in many plant species in Canada (Boland and Hall 1994). Sclerotinia stem rot (SSR) is a major disease of soybean (*Glycine max* (L.) Merr.) in North America (Wrather and Koenning 2006). Yield loss in soybean has been estimated at 170–330 kg/ha for each 10% increment in diseased plants (Chun et al. 1987; Hoffman et al. 1998). It was reported that SSR caused soybean losses of 11,300 metric tons in Canada in 1994 (Wrather et al. 1997), and 1.63 million tons in the United States in 2004 (Wrather and Koenning 2006).

SSR is difficult to control because the pathogen has a wide host range and can persist in the soil for extended periods in resting sclerotia (Phillips 1989). Crop management practices such as using disease free seeds, early planting dates, soil tillage, and adjusting row width and population density contribute to a reduction of SSR severity, but the effectiveness of these practices can be limited (Steadman 1979; Mueller et al. 2002). Resistant cultivars remain the most economical long-term approach for controlling the disease. It has been reported that some soybean genotypes have partial field resistance to white mold by physiological or avoidance mechanisms (Grau et al. 1982; Tu 1985; Kim et al. 2000; Hoffman et al. 2002); however the expression of field resistance may be modified by inoculum potential and other field conditions (Mueller et al. 2002). The effectiveness of fungicides in controlling white mold has been inconsistent (Grau et al. 1994; Mueller et al. 2002), primarily due to difficulties in achieving good coverage and in coordinating the timing of application with the release of ascospores (Hunter et al. 1978; Steadman 1979). In addition, the continuous use of chemicals could affect non-target species, cause environmental damage, and may result in the development of fungicidal resistance in the pathogen (Hawthorne and Jarvis 1973; Mueller et al. 2002). Compared to chemical control, using microbial agents to control plant pathogens can be an environmentally-friendly component of an integrated management program (Mao et al. 1997). In the search for alternatives to fungicides, several microorganisms have been reported effective as potential biocontrol agents for managing white mold (Adams 1990; Whipps and Budge 1990). To date, the most extensively investigated fungus is *Coniothyrium minitans* Campbell (Thaning et al. 2001). It is incorporated into a commercial product for destroying sclerotia in soil (Gerlagh et al. 1999). The application of the bacterium *Bacillus subtilis* (Ehrenberg) Cohn to bean leaves was reported to reduce SSR incidence and severity (Boland 1997; Tu 1997) and its effectiveness appeared to be accumulative over a 2-year trial (Tu 1997).

Although *B. subtilis* has been reported as a SSR biocontrol agent on other crops (Theoduloz et al. 2003; Zhang et al. 2005), no information is available on controlling SSR on soybean with *B. subtilis*. Since 2006, we have isolated 140 strains of *B. subtilis* from soybean roots in a long term crop rotation field on the Central Experimental Farm (CEF), Ottawa, Canada. We evaluated the antagonistic ability of *B. subtilis* strains SB01 and SB24 to suppress mycelial growth of *S. sclerotiorum* and the effectiveness of SB24 at different concentrations for control of SSR under the controlled conditions in soybean in the previous experiments (Zhang and Xue 2010). The persistence of SB01 and SB24 effectiveness to suppress *S. sclerotiorus* under the field conditions is closely related to application time of two bacterial strains in field. The objectives of this study were to investigate the persistence of effectiveness of the SB01 and SB24 to suppress *S. sclerotiorum* in soybean with the increase in time after they were exposed under field conditions

Materials and methods

Isolation of *S. sclerotiorum* and inoculum preparation

The strain of the *S. sclerotiorum* used for inoculation in this study was isolated from a sclerotium collected from a diseased soybean plant at the Central Experimental Farm (CEF), Ottawa, Canada in 2006. The sclerotium was surface sterilized in 70% (V/V) ethanol for 2 min, rinsed in sterile distilled water, and bisected. One of the two sclerotial halves was placed on potato dextrose agar (PDA) in a Petri plate with the freshly cut surface towards the agar. The inoculated plate was incubated for 4 days at 25°C in darkness. The strain, developed from a single mycelial plug cut from the sclerotial culture with a 6-mm-diameter cork-borer, was stored at 4°C for preparation of culture. Usually an ascospore suspension of *S. sclerotiorum* is used for inoculation in studies associated with the biology or etiology of this flower-associated disease. In this study, we are testing the ability of two bacterial strains to suppress the SSR disease. Therefore, the mycelial suspension rather than ascospores was used for plant inoculation using the inoculation technique developed by Chen and Wang (2005) to avoid the preparation procedure of ascospores from sclerotia. To make the mycelial suspension culture for plant inoculation, 20 mycelial plugs were added to 1 l of potato dextrose broth (PDB) in a 2000-ml flask and cultured at room temperature (21–23°C) for 5 days with shaking at

150 rpm. Before plant inoculation the mycelia were homogenized in the culture broth medium by blending for 30 s. The concentration was adjusted to approximately 4×10^5 CFUs/ml of mycelial suspension by a series of plating, and amended with 0.05% Tween 20 before inoculation.

Preparations of *B. subtilis* strains SB01 and SB24

B. subtilis strains SB01 and SB24 were used as the biocontrol agents in this study. These strains were isolated from soybean roots at the Central Experimental Farm (CEF), Ottawa, Canada in 2006 and evaluated for their inhibition to the mycelial growth and sclerotial production of *S. sclerotiorum* in previous trials (Fig. 1) (Zhang and Xue 2010). Both cells and cell-free filtrates of each strain were used in the trial because they showed the different effectiveness for the SSR suppression, respectively, in the previous study (Zhang and Xue 2010). The two bacterial strains were cultured in Luria-Bertani broth (LB, 10 g Bacto-tryptone, 5 g Baco-yeast extract, and 10 g NaCl in 1 l distilled water), and aliquots of the bacterial suspensions were kept at −80°C in 15% glycerol (V/V) for a long-term storage. To prepare cell suspension and cell-free culture filtrate, SB01 and SB24 were cultured in LB medium for 72 h at room temperature (21–23°C) in flasks shaken at 150 rpm, respectively. The broth culture in LB medium was adjusted to 5×10^8 CFU ml^{-1} with sterilized distilled water and centrifuged at 10,000 rpm. A cell-free culture was obtained by passing the supernatant through a sterile 0.22-μm filter, and the cell suspension at 5×10^8 CFU ml^{-1}, approximately 70% as vegetative cells and 30% as endospores, was regained by washing, re-centrifuging and suspending the cell pellet in sterilized distilled water with the original volume. These two cultures were used to test the efficacy of the biocontrol agent and the effect of time interval between biocontrol application and Sclerotinia inoculation on the SSR suppression.

Plant materials, *B. subtilis* preparation treatment and *S. sclerotiorum* inoculation

To apply SB01 or SB24 strain on the field plants, a susceptible soybean cultivar, PS RR46 was planted in the field on May 18, 2008. The plants were sprayed in the field with a cell suspension or cell-free culture of *B. subtilis* strain SB01 or SB24. The plants were sprayed with the bacterial cultures on two dates: June 12, when the plants were at the V3 to V4 stages and on July 4 when the plants were at the V6 to V8 stages. Before applying the bacteria, the growing points of the plants were cut off to prevent the growth of new leaves during the experiment. The cell suspension or cell-free culture from both strains were amended with 0.05% (V/V) Tween 20 and sprayed on the plants in field with a hand-pump sprayer at a rate of 5 (date 1) and 10 (date 2) ml per plant. The previous experiments indicated that plants treated with *S. sclerotiorum* mycelial suspension under the field conditions did not provide appropriate disease pressure for evaluation of SB01 or SB24 effectiveness (data not shown) because of high temperature and low leaf wetness without a misting system under field conditions. Therefore, plants treated with SB01 or SB24 were dug from the field, transplanted into trays with roots carrying intact soil and moved into the growth room for inoculation with *S. sclerotiorum* at 8 time intervals after bacterial application: 0 (plants were transplanted and inoculated immediately after

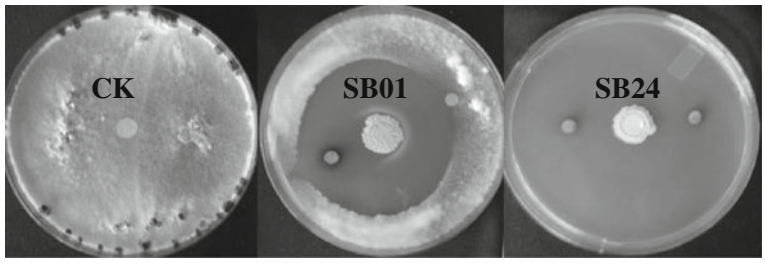

Fig. 1 Inhibition of mycelial growth and sclerotial production of *Sclerotinia sclerotiorum* in soybean by the cell suspensions of *Bacillus subtilis* strains SB01 and SB24. Both strains completely suppressed sclerotial production of the pathogen after 10-day dual culture. SB01 inhibited the partial mycelial growth on PDA, while SB24 completely stopped mycelial growth

bacterial application); 1 (plants were transplanted and inoculated 1 day after being bacterial application), 2, 3, 4, 5, 6 and 10 days after application (DAA) with the bacterial preparations. The plants were inoculated with a homogenized mycelial suspension of *S. sclerotiorum* at a dose of 5–15 ml per plant. Trays containing inoculated plants were arranged in a completely randomized design in a growth room, with a 16-h photoperiod set at 20/18°C (day/night) and a relative humidity of 100% provided by an ultrasonic humidifier operated intermittently. Plants treated with just the bacteria and not inoculated with *S. sclerotiorum* served as negative controls, while plants not treated with bacteria but infected with *S. sclerotiorum* were positive controls. Disease severity was rated at 5 and 15 days after inoculation (DAI) with *S. Sclerotiorum*. Each time the severity of disease was rated on a 0–5 scale where: 0 = no disease; 1 = a few necrosis or lesions on leaves with total diseased area of <5%; 2 = lesions on leaves with total diseased area of 5–10%; 3 = many lesions with total diseased area of 11 to 30% on leaves and stem rot occurs on stem tops; 4 = extensive rot or lesions on leaves with total diseased area of 30–50%, and stem tip is rotted; and 5 = extensive rot on leaves with total diseased area of ≥50% and rot on stem tip progresses down stems or plant dying. In the two-date experiments, each treatment had 4 replicates, and an average severity on 18 plants was obtained for each replicate.

Data analysis

Analysis of variance (ANOVA) was completed using the general linear model procedure (PROC GLM) in SAS (SAS Institute, Cary, NC). Means were compared with Fisher's protected least significant difference test at $P \leq 0.05$ in each date.

Results

There was a significant interaction ($P<0.001$) between the date of application and the time interval between the bacterial application and fungal inoculation, therefore the dates were analyzed separately. For date 1, the effectiveness of disease suppression was affected by the time intervals between the SB01 or SB24 cell suspension and cell-free filtrate application in field and the *S. sclerotiorum* inoculation in the growth room. At 5 DAI, plants treated with SB01 cell suspension in the field showed a significant reduction in SSR severity when the duration between application and inoculation was ≤3 DAA (Fig. 2a) while plants treated with SB24 showed a significant reduction in disease severity during the first 5 DAA (Fig. 2b). When plants were inoculated within 2 DAA with either strain, SSR severity was reduced by more than 85% at 5 DAI and by approximately 40–60% at 15 DAI. Plants inoculated with *S. sclerotiorum* at an interval >2 DAA were dead at 15 DAI (Fig. 2a and b).

Unlike the disease progress patterns in date 1, in date 2 SSR progressed more slowly for all of the time interval treatments for cell suspensions of both strains of the bacteria (Fig. 2c and Fig. d). The reduction in SSR severity for both strains (Fig. 2c and d) ranged from approximately 20–90% at 5 DAI. By 15 DAI, plants that were inoculated ≤5 DAA with the SB01 cell suspension (Fig. 2c) and ≤6-DAA with the SB24 cell suspension (Fig. 2d) still had significantly reduced SSR disease severity by 15–70%. Cell suspensions of both strains showed better efficacy in date 2 than in date 1.

Effectiveness of SSR suppression by the cell suspension decreased with the time from bacteria application to disease inoculation. This pattern was most evident in the time interval-response data collected at 15 DAI in date 2 (Fig. 2c and d), in which the time interval was positively correlated with disease severity increase ($r_{SB01}=0.91$, $r_{SB24}=0.94$, $P<0.01$) and a linear model fitted the relationship of the time interval with increase in disease severity (Fig. 3).

The efficacy of the SB01 and SB24 cell suspensions for suppression of soybean SSR was greatly affected by precipitation after application. In date 1, there was no rainfall in field during the first 2 DAA (Fig. 4) with the bacterial cell suspensions. Plants transplanted and inoculated during these 2 days had lower disease severity ($P<0.01$) than those from other time intervals when rated at 5 and 15 DAI with *S. sclerotiorum* (Fig. 2a and b). On the third DAA there was 28-mm of rain (Fig. 4) followed by four >5-mm days of rain. Therefore, those plants with >2 DAA with the bacteria had a much higher white mold severity at the two ratings (Fig. 2a and b). In date 2 it did not rain for the first 5 DAA (Fig. 4), and disease severity ratings in the first 5-DAA were low at the two ratings (Fig. 2c and d). In comparison, the several >2-

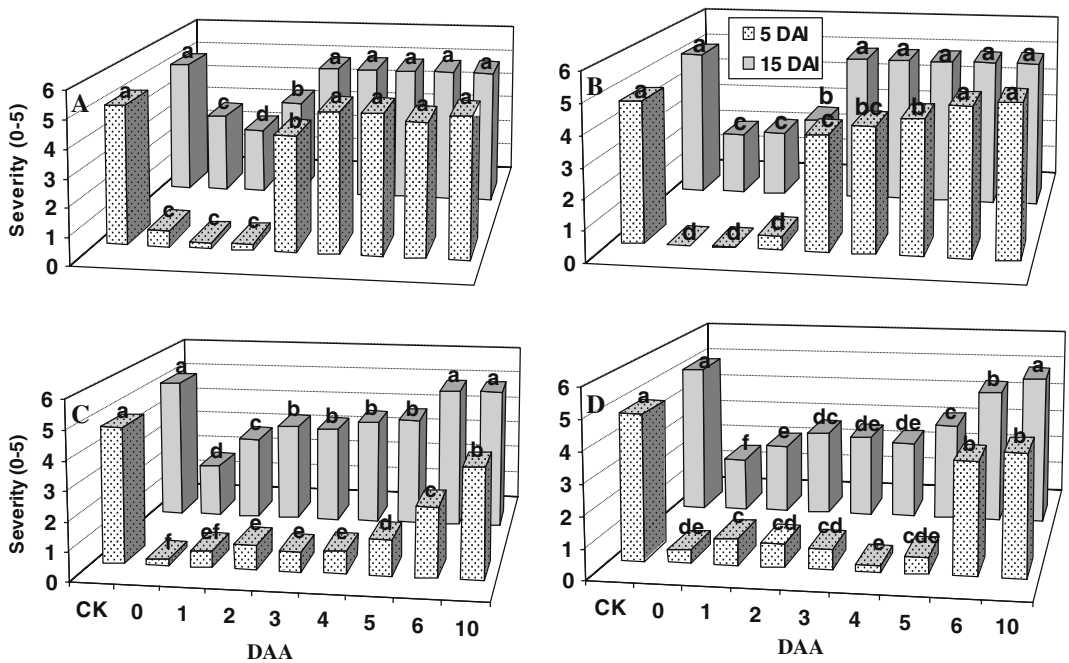

Fig. 2 Effects of the time intervals between field applications with the cell suspensions of *Bacillus subtilis* strains SB01 and SB24, and growth room inoculation with *Sclerotinia sclerotiorum* on the efficacy of soybean white mold control. **a** and **b** represent the cell suspension treatments with SB01 and SB24 in date 1, respectively; and **c** and **d** represent the cell suspension treatments with SB01 and SB24 in date 2, respectively. DAI = Days after the inoculation with *S. sclerotiorum*. DAA = Days after application of the bacteria. CK = plants inoculated with *S. sclerotiorum* alone. DAA 0 indicates that plants were treated with the SB01 or SB24 cell suspensions in field, and then immediately inoculated with *S. sclerotiorum* in the growth room. Time interval treatments with the different letters in rows were significantly different from each other ($P>0.05$)

mm rainfalls (Fig. 4) occurring after 5 DAA of the bacterial treatment in date 2 led to higher disease severity in the 6- and 10-day interval treatments with the SB01 (Fig. 2c) and SB24 (Fig. 2d) cell suspensions.

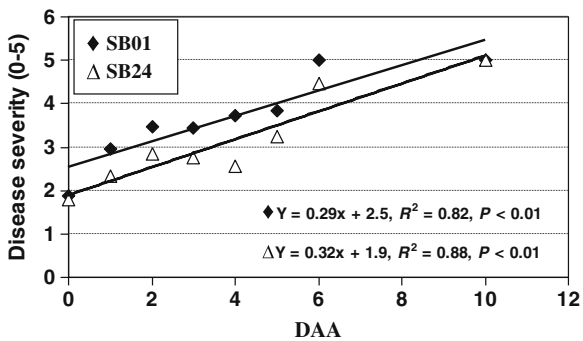

Fig. 3 Regression of *Sclerotinia* stem rot severity caused by *Sclerotinia sclerotiorum* at day 15 with the time intervals between the applications of SB01 and SB24 cell suspensions and the inoculation with *S. sclerotiorum*. DAA = Days after application of the bacteria. DAA 0 indicated that plants were treated with the bacterial cell suspensions in field and then immediately transplanted and inoculated with *S. sclerotiorum* in the growth room

In general, white mold disease developed faster in the plants treated with the SB01 and SB24 cell-free filtrates (Fig. 5) than that in those treated with the cell suspensions regardless of the time interval between application and inoculation. In date 1, the ≤3- DAA treatments with SB01 cell-free filtrate (Fig. 5a) and those ≤4-DAA treatments with the SB24 cell-free

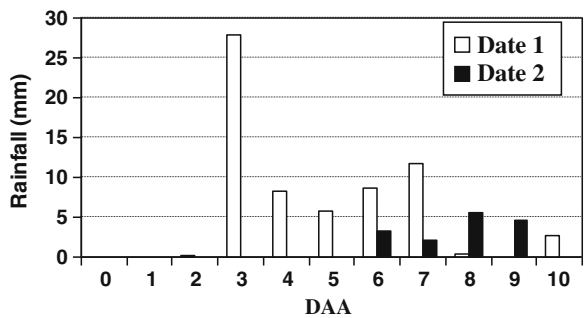

Fig. 4 Raining events and rainfalls during the two-date experimental periods. DAA = Days after application of the bacteria. DAA 0 indicated that plants were treated with the bacterial cell suspensions in field and immediately transplanted and inoculated with *S. sclerotiorum* in the growth room

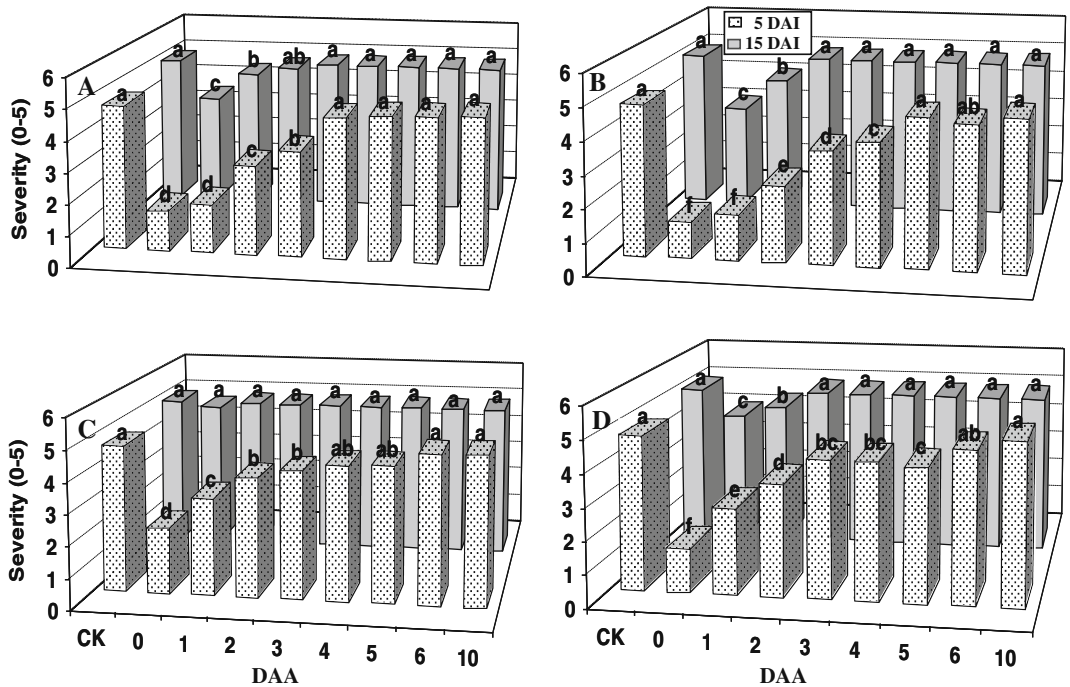

Fig. 5 Effects of the time intervals between the field applications of the cell-free filtrates of *Bacillus subtilis* strains SB01 and SB24, and the growth room inoculation of *Sclerotinia sclerotiorum* on the effectiveness of *B. subtilis*. **a** and **b** represent the cell-free filtrate treatments with SB01 and SB24 in date 1, respectively; and **c** and **d** represent the cell-free filtrate treatments with SB01 and SB24 in date 2, respectively. The disease severity was rated at 5 and 15 days after the inoculation (DAI) with *S. sclerotiorum*. DAA = Days after application of the bacteria. CK = plants inoculated with *S. sclerotiorum* alone. DAA 0 indicates that plants were treated with the cell-free filtrates of the SB01 or SB24 strains in field, and then immediately transplanted and inoculated with *S. sclerotiorum* in the growth room. Time interval treatments with the different letters in rows were significantly different from each other ($P>0.01$)

filtrate (Fig. 5b) significantly reduced white mold severity during the first 5 DAI. By 15 DAI, only plants infected with *S. sclerotiorum* ≤1-DAA with either strain of bacteria resulted in a significant reduction in white mold severity (approximately 10–40%), while the plants from the other time interval treatments were dead. Similar disease development patterns in date 1 were observed in date 2 for both strain cell-free filtrates. Plants in the time interval treatments with both strain cell-free filtrates in date 2 significantly reduced disease severity during the first 5 DAA (Fig. 5c and d), but all plants with the SB01 filtrate (Fig. 5c) were dead by 15 DAI and only ≤1-DAA treatments with the SB24 filtrate (Fig. 5d), despite developing high disease severity, significantly ($P<0.05$) suppressed disease severity by 18–23% compared to the untreated control (CK). The results showed that the cell suspension treatments (Fig. 2) exhibited better effectiveness for the control of soybean SSR development than the cell-free filtrate treatments (Fig. 5).

Discussion

In this study, cell suspensions and cell-free filtrates of *B. subtilis* strains SB01 and SB24 were evaluated for their control of soybean white mold based on the time interval between the application of the biocontrol agents under field conditions and the inoculation with *S. sclerotiorum* in the growth room. The results showed that the cell suspensions and cell-free filtrates both provided suppression of soybean SSR even after they had been on the plant up to 10 days but the best efficacy was given by those treatments of the cell suspension applied on soybean plants in the field for <3 days. The effectiveness of SSR suppression decreased with the extension of time interval between the application of the bacteria and inoculation with *S. sclerotiorum*. There appeared to be no significant difference in SSR suppression between the two strains of bacteria, leading to the conclusion that the mechanism of fungal control is similar for

these strains. Several strains of *B. subtilis* have been registered in the United States for controlling major diseases in cherry, cucurbits, grapes, leaf vegetables, peppers, peanuts, potatoes, tomatoes and walnut (http://www.epa.gov/pesticides/biopesticides). Early field studies on the foliar application of *B. subtilis* showed a reduction in SSR incidence and severity in bean (Boland, 1997; Tu, 1997) and its effectiveness appeared to be cumulative over the 2-year trial (Tu, 1997). The current study adds new information on effectiveness of biocontrol of Sclerotinia stem rot in soybean using *B. subtilis* strains.

Bacillus species have been reported to produce a large number of peptide antibiotics such as subtilin, bacilysin, mycobacillin and iturin A as well as antifungal secondary metabolites representing at least 25 different basic chemical structures (Yoshida et al. 2000; Mutaz and Hasnain 2006). It was found that there was a large variation in the amount and variety of antifungal substances produced among strains of the *B. subtilis* species (Pinchuk et al. 2002). The suppression of SSR on soybean plants by cell-free filtrates of SB01 and SB24 suggests that the two bacterial strains might produce antifungal substances which were present in the media applied to the plants. Therefore, future work needs to determine the antifungal substances that *B. subtilis* strains SB01 and SB24 produce.

Our results showed that effectiveness for the suppression of SSR decreased with the time interval between the application of the bacteria in field and the *S, sclerotiorum* inoculation. In the field SSR epidemics usually start with the infection of soybean flower petals by ascospores (Abawi et al. 1975; Bardin and Huang 2001). To maximize the effectiveness of the SB01 and SB24 cell suspensions in field, an ideal application time for the application of bacterial cell suspensions on plants would be prior to the time when ascospores are released and land on flower petals. Therefore, a monitoring system to detect ascospore release would help determine the appropriate application time of the cell suspensions in field. We found that the bacteria were easily washed from the plants by rain and this reduced white mold suppression. This result suggests that cell suspensions may need to be applied on more than one occasions to successfully control SSR and that monitoring programs for the correction timing of the application of the bacteria need to account for rainfall.

The mechanisms of disease control by *B. subtilis* are still under study. It was proved that the colonization of plants by *B. subtilis* could influence expression of plant resistance genes. For example, Ongena et al. (2005) reported that induced systemic resistance by treatment with either vegetative cells or endospores of *B. subtilis* led to a significant reduction in anthracnose incidence, caused by *Colletotrichum lagenarium* in cucumber plants. An additional study is needed to confirm whether or not this mechanism is responsible for Sclerotinia stem rot suppression in soybean as in the current study. In addition, previous studies also showed that *B. subtilis* cells produced antibiotics on plant surfaces after they were applied on plants (Tu, 1997; Theoduloz et al. 2003), thus maintaining the disease control effectiveness. That might partially explain the fact that the cell suspension provided a greater effectiveness for the control of soybean SSR than the cell-free filtrates in the present study. The effectiveness of the SSR suppression decreased faster over time in the cell-free filtrate treatments than in the treatments with cell suspensions during 15 days. This may result from running-off or decomposition of effective antifungal substances such as the peptide antibiotics (Mutaz and Hasnain 2006) in cell-free filtrates over time. However, cell suspensions may continuously produce antifungal substances on plant surfaces, maintaining better suppression efficacy during 15 days. Therefore, the cell suspension would be preferable in the development of commercial formulations in future work.

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