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Inhibition of *Fusarium virguliforme* by Prokaryotes *In Vitro*

L. J. Xing and A. Westphal

Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN

ABSTRACT

Sudden death syndrome (SDS) of soybean, caused by *Fusarium virguliforme* in North America (synonym: *F. solani* f. sp. *glycines*), is a severe disease in the Midwest of the United States. An *in vitro* assay was developed for testing inhibition of the fungal pathogen by biological control agents. Biological control agents, isolated from commercial products, were added to wells in potato dextrose agar seeded with macroconidia of *F. virguliforme*. The assay was used to compare inhibition activity of four commercially formulated biological control agents, *Bacillus pumilus* GB34, *B. subtilis* GB03, *Streptomyces lydicus* WYEC108 and *S. griseoviridis* K61, against 12 isolates of the pathogen. *Bacillus subtilis* GB03 and *S. griseoviridis* K61 inhibited mycelial growth of some isolates. *Bacillus pumilus* GB34 and *S. lydicus* WYEC108 did not inhibit mycelial growth.

Additional Index Words: Actinovate Soluble™, biological control, *Fusarium solani* f. sp. *glycines*, *Glycine max*, Kodiak™, Mycostop G™, sudden death syndrome, Yield Shield™

Sudden death syndrome (SDS) of soybean, caused by *Fusarium virguliforme* in North America (Aoki et al. 2003; Covert et al., 2007), synonym: *F. solani* f. sp. *glycines* (Roy 1997), is one of the most severe diseases of soybean in the Midwest of the United States (Wrather et al. 2003). Managing the disease is difficult because the ecology of the pathogen is not fully understood. The use of resistant cultivars, crop sequence with non-hosts, a delay of planting date, and in some areas tillage that provides effective drainage mitigate disease severity (Roy et al. 1997; Grau et al. 2004).

Biological control agents (BCAs) belonging to *Bacillus* spp. and *Streptomyces* spp. have been formulated commercially (Emmert and Handelsman 1999). Kodiak™ (*Bacillus subtilis* GB03) and Mycostop G™ (*Streptomyces griseoviridis* K61) can suppress root diseases caused by *Fusarium* spp., *Phythium* spp., and *Rhizoctonia* spp. on different crops (Anonymous 2000; Brannen and Kenney 1997). Only a limited number of tests have evaluated BCAs against *F. virguliforme*. When testing potential fungal BCAs isolated from the rhizosphere and rhizoplane of only mildly SDS-exhibiting plants, *Fusarium solani* and *F. oxysporum* exhibited some disease suppressing activity in greenhouse and limited activity under field conditions but had no activity *in vitro* (Rupe et al. 1996). Scandiani et al. (2004) found that various *Streptomyces* spp. inhibited the South American SDS-pathogen on media plates when streaks of the *Streptomyces* spp. were overlaid with conidia suspensions of the fungal pathogen. In greenhouse tests, several *Pseudomonas* spp. protected soybean plants against SDS when soybean seeds were bacterized before seeding into infested soil (Cattelan et al. 2004). An *in vitro* assay for evaluating various prokaryote BCAs for their inhibition potential of *F. virguliforme* is currently not available.

Numerous reports describe different approaches used to test BCAs against plant pathogenic fungi or bacteria on solid media. In these methods, the plant pathogens and the BCAs are applied in colonized agar plugs, colonized agar layers, streaks, or as cell suspensions (Jones and Samac, 1996; Elson et al. 1994; Getha and Vikineswary 2002; Rupe et al. 1996; Walker et al. 1998). Cell suspensions of the plant pathogen may also be incorporated into the media before solidifying and then be challenged by applying a dilution or a streak of the single BCA onto the solidified seeded media (Moline et al. 1999; Földes et al. 2000). These methods depend on somewhat similar growth rates of the BCAs and the pathogens tested.

Fusarium virguliforme grows slowly on potato dextrose agar (PDA) (Roy et al. 1997). The fungus produces blue-pigmented three-septate (rarely four septate, occasionally 5-septate) macroconidia in slimy masses on effuse sporodochia in the center of the fungal culture, but it almost never produces

microconidia (Roy et al. 1997). Although reportedly useful for testing fast-growing fungi and BCAs with restricted colony spread, the above *in vitro* methods were of limited usefulness to investigate the inhibition potential of BCAs against *F. virguliforme* among fast growing prokaryotes (Xing and Westphal, unpublished).

The objective was to develop an *in vitro* test to evaluate the efficacy of potential prokaryote BCAs against *F. virguliforme.* This *in vitro* test was then used to compare effectiveness of four BCAs on mycelial growth of 12 different isolates of the pathogen. A preliminary report of this project has been published (Xing and Westphal 2005).

MATERIALS AND METHODS

Twelve isolates of *F. virguliforme* were obtained from collaborators in Indiana and Illinois and included the most widely tested virulent isolate i300 (Mont-1). New Indiana isolates, collected in 2003, were identified by morphological characteristics. The isolates represented a diverse collection of the pathogen from two distinct geographical areas (Table 1). The BCAs of four commercial products were used because of their reported effectiveness against soil-borne fungal pathogens, including *Fusarium* spp. *Bacillus pumilus* strain GB34 (Yield Shield™, Gustafson, Inc., Plano, TX, USA), *B. subtilis* strain GB03 (Kodiak™, Gustafson, Inc.), *Streptomyces lydicus* strain WYEC108 (Actinovate Soluble™, Natural Industries, Inc., Houston, TX, USA) and *S. griseoviridis* strain K61 (Mycostop G™, AgBio, Inc., Helsinki, Finland) were isolated on PDA and kept in 50% glycerol solution at -80 °C.

The four BCAs were cultured on PDA for one week. For *Bacillus pumilus* and *B. subtilis*, one loopful of bacteria was suspended in 5 ml of sterile double deionized water (ddH_2O). For *Streptomyces lydicus* and *S. griseoviridis*, several colonies from PDA culture were scraped off the media, suspended in 5 ml of sterile ddH_2O, vortexed (Vortex-Genie 2; Scientific Industries, Inc., Bohemia, NY, USA) for 2 minutes, and sonicated for 5 minutes at room temperature (20 ± 3 °C) (Branson 1200, Branson Ultrasonics Corporation, Danbury, CT; frequency preset: 50/60 HZ).

In experiment 1, two *F. virguliforme* isolates, AW108 and AW109, were cultured on PDA for two weeks. Macroconidia were harvested from the center of the colony with a sterile transfer loop and suspended in 9 ml of sterile ddH_2O to a concentration of 10^5 macroconidia per ml. Twenty microliters of this suspension were placed in the center of one 8.5-cm-diameter polystyrene Petri dish, 20 ml of cooled (45° C), sterile PDA added, and the plate swirled for 1 minute for uniform horizontal distribution of macroconidia of *F. virguliforme*. This resulted in a final concentration of 10^2 macroconidia per ml media. Nine wells were cut into the solidified media with a sterile 7-mm-diameter cork borer in a 5-cm concentrical pattern (8 wells) and at the center (one well). Fifty microliters of the suspensions of the BCA (ca.10^8 c.f.u./ml) were pipetted into the periphery wells of the plates and the center well received the same amount of sterile ddH_2O. Plates were sealed with parafilm and incubated in darkness at room temperature for 2-3 weeks. Each treatment was repeated three times with simultaneous incubation. The zones of inhibition of fungal growth were measured across the inoculation well in two perpendicular diameters for each of the three replications. The radius of the zone of inhibition was reported excluding the diameter of the inoculation well. In experiment 2, no wells were cut into the solid media and the BCAs were applied to the media surface with a sterile transfer loop from the pure culture at nine equidistant spots in the media surface. The plates were incubated at the same conditions as in experiment 1. The zones of inhibition of fungal growth around the inoculation spot were measured in two perpendicular diameters for each of the three replications. The average radius of the zone of inhibition of fungal growth was reported.

Because the well application in experiment 1 contained the BCAs in a discreet spot and thus allowed for proper recording of zones of inhibition, this method was chosen for testing the sensitivity of 12 isolates of *F. virguliforme*. In experiment 3, the 12 isolates of *F. virguliforme* (Table 1) were cultured on

Table 1. Isolates of *Fusarium virguliforme* used in the *in vitro* test.

Isolate	Origin	Source
AW109	Francesville, IN	S. Abney (2000, root)
AW108	Lafayette, IN	S. Abney (2001, root)
i45	Illinois	G. Hartman (1994, root)
i94	Illinois	S. Li (1997, root)
i522	Illinois	S. Li (2002, root)
i528	Illinois	S. Li (2003, root)
i300 (Mont-1)	Illinois	P. Stevens (1993, root)
LJ203	Rushville, IN	L.J.Xing (2003, root)
LJ204	Delphi, IN	L.J.Xing (2003, root)
LJ205	Delphi, IN	L.J.Xing (2003, root)
LJ206	Delphi, IN	L.J.Xing (2003, root)
LJ208	Tipton Co., IN	L.J.Xing (2003, root)

PDA for one month. Macroconidia suspensions of each isolate were prepared as in the first two tests. Macroconidia were harvested with sterile ddH_2O and diluted tenfold into PDA at 45 °C to obtain the ten-fold higher final concentration than in experiment 1 of 1.04×10^3 macroconidia per ml media. Higher concentrations were chosen to ensure a uniform lawn of *F. virguliforme*. Eight milliliters of this conidia suspension were dispensed into 5-cm-diameter Petri dishes. After solidifying, 7-mm wells were cut with a sterile cork borer in the center of each media-plate. Eighty microliters of suspensions of BCAs (8×10^4 to 8×10^6 c.f.u./ml) were pipetted into the wells of the 5-cm plates. Control plates for each fungal isolate received the same amount of sterile ddH_2O. The plates were sealed with parafilm and incubated in darkness at room temperature for 2-3 weeks. The zone of inhibition of fungal growth was measured as in experiment 1. Each treatment had three replicates that were incubated at the same time.

Data analysis. A *t*-test was used for each BCA to determine whether a significant zone of inhibition developed around its inoculated center compared to the water control. For experiments 1 and 3 (media well-applied tests) for each replicate plate**,** the diameter of the wells was subtracted from that of the zone of inhibition of fungal growth and divided by two to calculate the radius of the zone of inhibition of growth of *F. virguliforme* (experiment 1 and 3). For experiment 2 (the media surface applied test) the average radius of the zone of inhibition was used in the statistical analysis.

RESULTS AND DISCUSSION

An efficient method to test prokaryote biocontrol organisms against the slow-growing *F. virguliforme* was developed. The method of cutting wells into media seeded with *F. virguliforme* was more versatile than the surface application method and is the superior method based on these experiments. The wells restricted growth of fast growing prokaryotes sufficiently to allow for observation of potential fungal inhibition (Fig. 1). The antifungal activity present during incubation was clearly visible and probably due to metabolites released from the prokaryote BCAs into the culture media. The proposed technique was a modification of the method used by Moline et al. (1999) and Földes et al. (2000). *Bacillus subtilis* GB03 inhibited growth of twelve and *S. griseoviridis* K61 inhibited growth of nine isolates of *F. virguliforme* compared to the water control at the cell density tested (Table 2 and 3), while *B. pumilus* and *S. lydicus* did not inhibit mycelial growth (data not shown). Inhibition by *B. subtilis* was less consistent and not observed when AW108 was seeded at higher concentrations into the media (Table 3). The difference observed in sensitivity of AW108 between the two tests exemplified the rigor of the final test method because zones of inhibition were measured at the higher macroconidia concentrations. Most of the isolates of *F. virguliforme* from different regions within Indiana and Illinois, including the commonly studied Mont-1 isolate, were inhibited under these stringent conditions (Table 3), suggesting that antibiosis to this fungal pathogen exists and has potential for biological control of the disease.

In previous studies, *Bacillus subtilis* inhibited *Fusarium oxysporum in vitro* (Földes et al., 2000). *Streptomyces* spp. has been used in the research on biological control of soil-borne diseases, e.g., root rot (Xiao et al. 2002), damping-off (Jones and Samac 1996), and Fusarium wilt (Getha and Vikineswary

Table 2. Zone of growth inhibition of *Fusarium virguliforme* by *Bacillus subtilis* GB03 and *Streptomyces griseoviridis* K61 on PDA media†

	Zone of Inhibition (mm)	
	AW108	AW109
Test Prokaryote	Mean ± SE	Mean ± SE
Experiment 1‡		
Bacillus subtilis GB03	3.0 ± 0.5 *	4.5 ± 0.2 **
Streptomyces griseoviridis K61	2.4 ± 0.5 *	1.8 ± 0.9 n.s.
Experiment 2¶		
Bacillus subtilis GB03	5.1 ± 0.6 *	4.7 ± 0.2 **
Streptomyces griseoviridis K61	4.8 ± 0.5 **	5.3 ± 0.9 *

† Data for only two of the four tested prokaryotes are shown since no inhibition was observed in the other prokaryotes tested. Numbers followed by ** and * are significantly different from zero (=no inhibition) at P=0.01 and P=0.05, respectively when tested with *t*-test (one-tailed *t*); n.s.: non significant.

‡ Prokaryote suspensions (ca. 10^8 /ml) were pipetted into the wells. The distance of inhibition from the rim of the well to the zone of normal fungal growth is reported.

¶ Test prokaryotes were applied to the surface of the agar plate with a loop. The radius of the zone of inhibition around the inoculation point of the prokaryote is reported.

Table 3. Zone of growth inhibition [mm] of *Fusarium virguliforme* in an *in vitro* test (mean ± SE)†

Fusarium virguliforme	*Bacillus subtilis* GB03	*Streptomyces griseoviridis* K61
AW109	7.25 ± 0.63 **	6.00 ± 1.38 *
AW108	0.00 ± 0.00 n.a.	0.67 ± 0.67 n.s.
i45	6.25 ± 0.29 **	5.33 ± 0.36 **
i94	11.00 ± 0.29 **	0.00 ± 0.00 n.a.
i522	7.50 ± 0.29 **	5.67 ± 0.42 **
i528	9.33 ± 0.51 **	3.42 ± 1.72 n.s.
i300 (Mont-1)	6.58 ± 1.28 *	9.58 ± 0.44 **
LJ203	10.08 ± 0.30 **	3.58 ± 1.21 *
LJ204	10.50 ± 0.00 **	3.08 ± 0.88 *
LJ205	11.00 ± 0.52 **	3.33 ± 2.03 n.s.
LJ206	6.33 ± 0.88 **	4.33 ± 0.30 **
LJ208	7.67 ± 0.60 **	3.75 ± 1.01 *

† Data for only two of the four tested prokatyotes are shown since no inhibition was observed in the other prokaryotes tested. Numbers followed by ** and * are significantly different from zero (=no inhibition) at P=0.01 and P=0.05, respectively when tested with t-test (one-tailed t); n.s.: non significant; n.a.: not applicable.

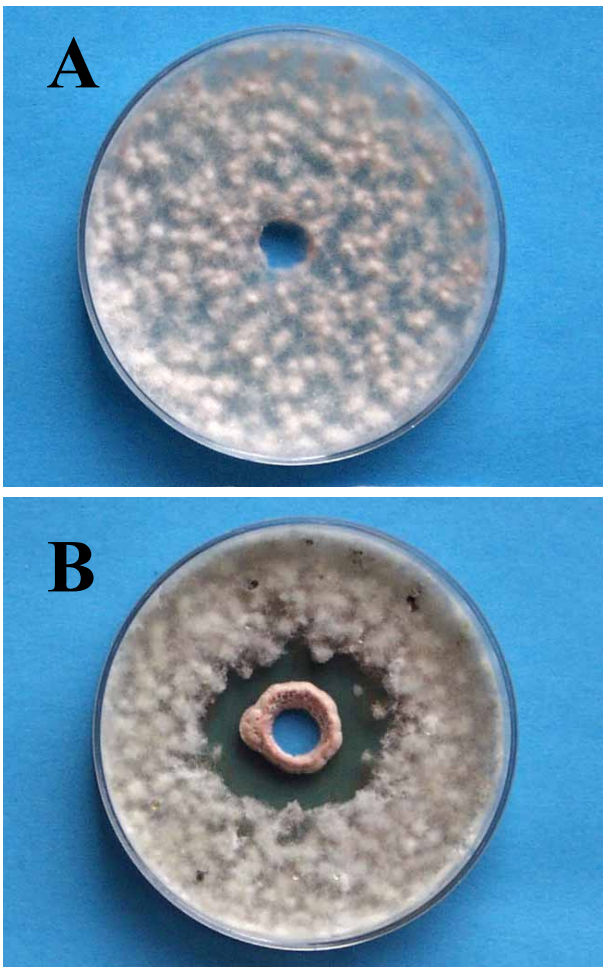

Fig. 1. Typical culture plates at reading of zone of inhibition of *Fusarium virguliforme*, isolate Mont-1: (A) Sterile water added in a well cut in the center of a *F. virguliforme* seeded PDA plate, no inhibition detected, and (B) *Streptomyces griseoviridis* K61 added to the center of a plate, zone of growth inhibition visible around the well in PDA media seeded with *F. virguliforme* macroconidia. The zone was measured two perpendicular directions via the well, inhibition was expressed as the distance between the rim of the application well and the area with unrestricted fungal growth by subtracting the well diameter from the overall diameter and dividing this number by 2.

2002). Mycostop G is temporarily registered for the control of damping-off, root and stem rot, and wilt diseases of greenhouse ornamentals and vegetables caused by *Fusarium* spp. (Anonymous 2000). In these studies, the *in vivo* suppression activity against soil-borne pathogens co-occurred with the *in vitro* production of antimicrobial compounds and the inhibition of pathogen growth. *In vivo* studies are still outstanding for *F. virguliforme* and the test BCAs presented in this current report.

We hypothesize that producing antifungal metabolites is one of the modes of actions of the two inhibitory BCAs, because antimicrobial molecules have been identified for both *B. subtilis* and *S. griseoviridis* (original report Delcambe and Devignat 1957, referenced in Maget-Dana and Peypoux 1994; Raatikainen 1991). Hyphae detected in the zones of inhibition were distorted, supporting the hypothetical causal role of antifungal compounds in the inhibition (Fig. 2). Apparently, sensitivity to antifungal compounds differs among different isolates of *F. virguliforme* because fewer fungal isolates were inhibited by *S. griseoviridis* than by *B. subtilis* (Table 3). The lack of inhibition by *B. pumilus* GB34 and *S. lydicus* WYEC108 does not discount them for biological control purposes since other modes of actions are known and may be involved in biological control of plant diseases (Kloepper et al. 2004). The presented method will be helpful to identify prokaryotes that can inhibit *F. virguliforme*.

We propose to confirm inhibition potential of BCAs by testing them against a number of *F. virguliforme* isolates to avoid overestimation of inhibition potential when only a single *F. virguliforme* isolate is sensitive

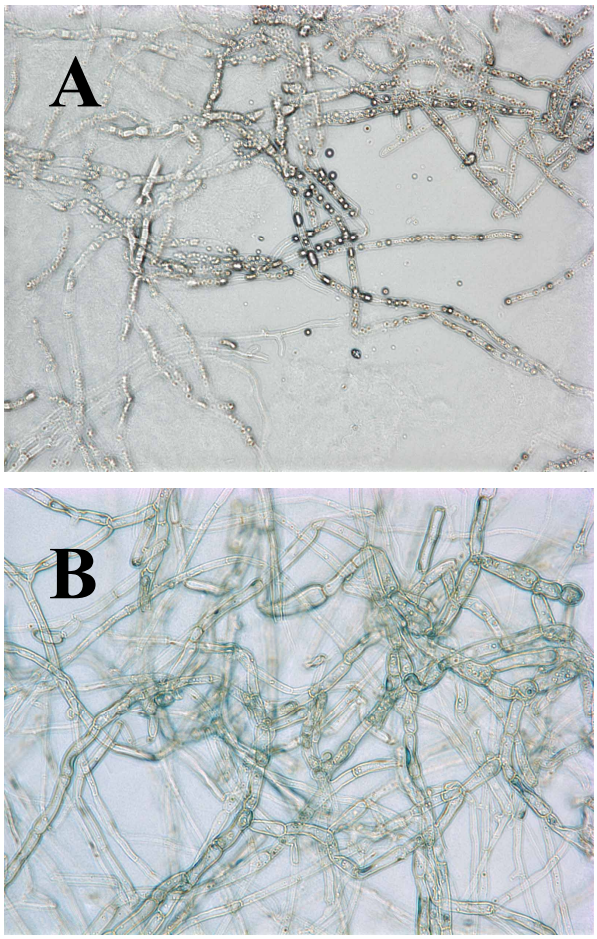

Fig. 2. Micrographs of mycelium of *Fusarium virguliforme*: (A) inside the zone of inhibition around colonies of *Streptomyces griseoviridis* K61; note the coagulation within the distorted and seemingly disrupted hyphal bodies. (B) Outside of the zone of inhibition, growing normally.

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