Trichoderma Isolates Inhibit *Fusarium virguliforme* Growth, Reduce Root Rot, and Induce Defense-Related Genes on Soybean Seedlings

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Abstract

Sudden death syndrome (SDS) caused by *Fusarium virguliforme* is among the most important diseases affecting soybean in the United States. The use of biological control agents (BCAs) such as *Trichoderma* spp. can be a valuable resource to suppress *F. virguliforme* populations. Therefore, this research focused on screening possible BCAs against *F. virguliforme* and evaluating mycoparasitism and the induction of systemic resistance as mechanisms underlying the antagonistic activity of selected BCAs against *F. virguliforme*. In total, 47 potential BCAs, including 41 *Trichoderma* isolates and 6 *Mortierella* isolates, were screened in a dual-plate assay. The most effective isolates belonged to the *Trichoderma harzianum* species and were able to inhibit *F. virguliforme* radial growth by up to 92%. Selected *Trichoderma* isolates were tested in the greenhouse and in a microplot study. They reduced root rot caused by *F. virguliforme* when the plants were

coinoculated with the pathogen and the BCA. The tested BCA's ability to reduce *F. virguliforme* growth may be related to several mechanisms of action, including mycoparasitism and induction of defense-related genes in plants, as revealed by monitoring the expression of defense-related genes in soybean. Our results highlight the potential of native *Trichoderma* isolates to inhibit *F. virguliforme* growth and reduce SDS severity, providing the basis for future implementation of biological control in soybean production. More efforts are needed to implement the use of these approaches in production fields, and to deepen the current knowledge on the biology of these highly antagonistic isolates.

Keywords: biological control, induced systemic resistance, mycoparasitism, seedling diseases, sudden death syndrome

Sudden death syndrome (SDS), caused by *Fusarium virguliforme* O'Donnell & T. Aoki, is one of the most destructive diseases of soybean (*Glycine max* (L.) Merr.). SDS ranked fourth among the most important diseases reducing soybean yield in the United States in 2006 (Wrather et al. 2010), with losses estimated at nearly 1.7 million metric tons in 2014 (Allen et al. 2017). First reported in Arkansas in 1971 (Roy et al. 1997), the disease has become widespread in soybean-growing regions in the United States (Hartman et al. 2015). SDS has been more severe in the Midwest and North-Central regions, where conditions of high soil humidity and cold weather favor disease development. *F. virguliforme* can cause root rot symptoms and poor root development in infected plants. The production of phytotoxins by the pathogen in infected roots and their translocation leads to foliar interveinal chlorosis and necrosis in the later reproductive stages of the plant, in addition to defoliation and pod abortion with severe infections (Hartman et al. 2016; Roy et al. 1997). Losses caused by *F. virguliforme* may vary depending on environmental conditions, abundance of primary inoculum, susceptibility of the cultivar, and growth stage at the time of infection. Losses can amount to up to

80% yield reduction under conditions favorable to disease development (Roy et al. 1997).

Current SDS management practices focus on the use of tolerant varieties, seed treatment with fungicides, and improving soil physical characteristics by tillage and the avoidance of soil compaction (Hartman et al. 2015; Kandel et al. 2016). However, SDS management continues to be challenging because the current approaches are not completely effective against *F. virguliforme*. An integrated approach is often needed to reduce disease severity (Hartman et al. 2015; Mueller et al. 2003; Srour et al. 2017; Zaworski 2014). Therefore, the exploitation of biological control agents (BCAs) can be a valuable tool for an efficient management of SDS.

Species from the genus *Trichoderma* (teleomorph *Hypocrea*, Ascomycota, Dikarya) are among the most studied BCAs because of their antagonistic activity against a broad range of plant-pathogenic organisms (Verma et al. 2007; Weindling 1932). Also, *Trichoderma* spp. are ubiquitous, commonly found in the majority of soils. *Trichoderma* spp. are effective against phytopathogens through the use of multiple antagonistic mechanisms, including competition, production of antimicrobial compounds, and mycoparasitism (Chet et al. 1981; Hermosa et al. 2014; Verma et al. 2007). The ability of *Trichoderma* spp. to parasitize other fungi is one of the most prominent characteristics of this group. Different *Trichoderma* spp. are documented to parasitize a wide range of fungal hosts, including important plant pathogens such as *Rhizoctonia solani*, *Phytophthora ramorum*, *Pythium ultimum*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, and *Fusarium* spp. (Ali et al. 2009; Chet et al. 1981; Khalili et al. 2016; Kim and Knudsen 2008; Weindling 1932; Widmer 2014). *Trichoderma*'s mycoparasitic activity involves molecular recognition of the fungal host followed by direct attachment. This triggers the production of hydrolytic enzymes and secondary metabolites by *Trichoderma* which is necessary for penetrating and killing the fungal host (Howell 2003; Kubicek et al. 2011; Steindorff et al. 2012). Therefore, the secretion of cell-wall-degrading enzymes (CWDEs) such as chitinases, glucanases, and proteases is essential for the mycoparasitism to be successful (Kubicek et al. 2011). The induction of mycoparasitism-specific genes, including

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β -1,3-glucanase (*glu1*) α -1,3-glucanase (*agluc75*), chitinase 42KDa (*chit42*), and endochitinase 42 (*endo*), has been studied in different *Trichoderma*–fungal host interactions (Kubicek et al. 2011; Vieira et al. 2013).

Trichoderma spp. are also known to promote plant growth, and to trigger local and systemic resistance against many fungal, bacterial, and viral pathogens (Mendoza-Mendoza et al. 2018; Salas-Marina et al. 2014). *Trichoderma*–root colonization activates induced systemic resistance (ISR) by triggering jasmonate/ethylene (JA/ET) signaling cascades, which activates defense enzymes, including hydroperoxide lyases, peroxidases, and phenylalanine ammonia lyases (Druzhinina et al. 2011; Mukherjee et al. 2012). In addition, *Trichoderma* can trigger a salicylic acid (SA)-mediated systemic response (SAR) leading to the expression of pathogenesis-related (PR) proteins (e.g., β -1,3-endoglucanases, chitinase class I, and defensin precursor) that are responsible for lysing invading cells, reinforcing cell walls boundaries, or inducing hypersensitive reactions (Harman et al. 2004a; Yadav et al. 2015). Induced resistance usually persists for extended time periods and is often not specific, offering protection against a broad range of pathogens.

Despite the fact that a variety of microbial antagonists have been reported to control several pathogens on numerous crops, including soybean, studies involving the use of BCAs against *F. virguliforme* are still relatively limited. In 1996, Rupe et al. evaluated the in vitro antagonistic activity of 151 fungal isolates collected from the rhizosphere and the rhizoplane of healthy and moderately affected soybean plants against soybean pathogens. Almost 30% of the tested isolates inhibited the growth of *F. solani* and *F. oxysporum*. Nevertheless, the in vitro results were not consistent with data obtained under field conditions (Rupe et al. 1996). More recent in vitro studies have been performed using antagonistic bacteria such as *Bacillus pumilus*, *B. subtilis*, *Streptomyces lydicus*, *S. griseoviridis*, *Pseudomonas fluorescens*, and *Chryseobacterium vietnamense* against *F. virguliforme* (Pin Viso et al. 2016; Xing and Westphal 2007). Seed treatment with the nitrogen-fixing bacterium *Bradyrhizobium japonicum* were also reported to reduce SDS foliar symptoms (Huynh et al. 2016). Another biological seed treatment using saponins of *Chenopodium quinoa* showed positive results in suppressing SDS (Navi and Yang 2016). In contrast, a different study using resistant and susceptible soybean cultivars in different locations did not find significant SDS reduction by using saponin seed treatment (Kandel et al. 2019). The arbuscular mycorrhizal fungi *Rhizophagus irregularis* reduced root infection by *F. virguliforme* during in vitro experiments (Giachero et al. 2017); however, no further experiments were conducted. This research focused on the evaluation of possible BCAs against *F. virguliforme*. Our objectives included (i) performing an in vitro screen of potential native BCAs against *F. virguliforme* and selecting isolates antagonistic to the pathogen, (ii) evaluating the effectiveness of the selected BCA isolates in reducing SDS severity under greenhouse and semifield conditions, and (iii) evaluating mycoparasitism and the induction of systemic resistance as mechanisms of action underlying the antagonistic activity of the selected BCAs against *F. virguliforme*.

Materials and Methods

The isolate of *F. virguliforme* and all the potential BCA isolates used in this study were obtained from a fungal collection maintained in the Department of Plant, Soil, and Agricultural Systems at Southern Illinois University Carbondale (SIUC) (Warner 2016). The potential BCA isolates were originally isolated from seedlings collected from soybean fields in the U.S. Midwest and they were identified using morphological characteristics, as well as molecularly using the internal transcribed spacer (ITS) and the *translation elongation factor* gene (Warner 2016). The soybean cultivar Spencer was used in the greenhouse experiments. In order to test the BCAs under more field-like conditions, the commercial cultivar AG4632 (Monsanto Co., St. Louis, MO, U.S.A.) was used in the microplot trial.

In vitro screening of potential BCAs against *F. virguliforme*. The antagonistic activity of 47 potential BCAs against *F. virguliforme*

was evaluated in vitro using a dual culturing method (Rahman et al. 2009). Briefly, mycelial plugs (6 mm in diameter) from *F. virguliforme* (7-day-old culture) and each BCA isolate (3-day-old cultures) were transferred in parallel to opposite sides of a potato dextrose agar (PDA) plate at a distance of 1 cm from the outer edge of the plate. *F. virguliforme* plugs were transferred 3 days in advance due to its slower growth rate in comparison with the growth rate of the BCAs under the conditions of the assay. The experimental design consisted of a completely randomized design with three replicates. Only *F. virguliforme* was grown in the control plates. The plates were incubated at $25 \pm 1^\circ\text{C}$ with a 16-h photoperiod, and *F. virguliforme* radial growth was measured after 7 days of confrontation between the two organisms. The percentage of radial growth inhibition (PRGI) was calculated using the following formula: $\text{PRGI} = [(C - T)/C] \times 100$, where C = radial growth in the control and T = radial growth in the treatment (Rahman et al. 2009). The interactions between the BCAs and *F. virguliforme* were also evaluated and data regarding the formation of inhibition zones or overgrowth were recorded. PRGI values were analyzed using one-way analysis of variance (ANOVA), and treatment means were clustered using the Scott-Knott ($P < 0.05$) algorithm in R, version 3.5.2 (Jeliahovschi et al. 2014).

***F. virguliforme* inoculum and BCAs seed treatment.** The *F. virguliforme* strain Mont-1 (NRRL 22292) was used in these experiments due to its wide use as a reference strain in the scientific community (Li et al. 2009). The inoculum was produced as described by Hashmi et al. (2005). Briefly, sorghum grain (50 g) was soaked in distilled water in a 500-ml Erlenmeyer flask for 48 h. Excess water was removed, and the sorghum was autoclaved for 60 min at 121°C . The autoclaving was repeated twice at 24-h intervals. Subsequently, each flask was inoculated with five mycelial plugs (6 mm each) from a 14-day-old *F. virguliforme* culture growing on PDA. The inoculated flasks were incubated in complete darkness for 2 weeks at room temperature. The inoculum was air dried, collected, and stored at 4°C until use.

For the seed treatment with BCAs, soybean seed were first surface sterilized by incubating in a 1% sodium hypochlorite solution for 3 min and then washing twice with sterile distilled water. A spore suspension of each BCA (5×10^6 spore/ml) was prepared from 5-day-old colonies growing on PDA. A slurry was prepared by mixing 20 ml of the spore suspension with 20 ml of 1% carboxymethyl cellulose (CMC) (Acros Organics, Fair Lawn, NJ, U.S.A.). The seed were soaked in the BCA-CMC mix for 30 min, with occasional stirring. For the control, seed were treated only with CMC and water. The seed were air dried overnight under aseptic conditions before sowing.

Performance of BCAs under greenhouse conditions. Four *Trichoderma harzianum* isolates (KMISO2-2-19A, WMNSO2-4-8, WNESO2-5-14, and KMISO2-2-15B), one *Mortierella elongata* isolate (WMISO2-3-25), and one *T. asperellum* isolate (WKSSO2-4-18) were tested. Each isolate was tested individually. Each experimental unit consisted of one plastic cone (3.8 cm in diameter and 14 cm in depth) containing three soybean plants. For the inoculation with the pathogen, the plastic cones were half-filled with Fafard Potting Mix (peat, perlite, and vermiculite mix). The *F. virguliforme*-inoculated sorghum (3 g) was applied on top of the potting mix as a uniform layer that was subsequently covered with an additional 2.5-cm layer of the potting mix. BCA-treated soybean seed were then sowed and covered with a thin layer of the potting mix. The control was inoculated with *F. virguliforme* but the seed were coated only with water and CMC. The experiment was conducted in a completely randomized design with seven replicates. The plants received a 16-h photoperiod using supplementary lights and were watered as needed. Twenty-one days after planting, the plants were carefully uprooted from the plastic cones and measurements of shoot and root lengths were taken using a ruler. Shoots were measured from the hypocotyl to the terminal bud, and roots were measured from the hypocotyl to the end of the primary root. Data were analyzed using one-way ANOVA and treatment means compared using Tukey's honest significance test ($P < 0.05$). Each treatment was also compared with the control using Dunnett's *t* test ($P < 0.05$) in R, version 3.5.2.

Quantification of *F. virguliforme* in roots. Plant roots were washed, dried, and stored at -20°C until DNA extraction for *F. virguliforme* DNA quantification using quantitative PCR (qPCR). The roots were ground in liquid nitrogen using a mortar and pestle. Approximately 150 mg of finely ground tissue was used for DNA extraction per sample. The DNA was extracted using the cetyltrimethylammonium bromide method developed by Saghai-Marouf et al. (1984), with minor modifications. The DNA concentration was measured using a NanoPhotometer P330 (IMPLEN, Schatzbogen, Germany), and adjusted to 60 ng/ μl . The absolute quantification of *F. virguliforme* was based on the specific primer pair forward 5'-GTCAAAATCAGTGTAGGGTAGGT-3' and reverse 5'-ACGGGTCGAGACCAGATTT-3', and the ZEN Double-Quenched probe coupled with Fam 5'-TCCGCAAAAATCAGGG CATCCAC-3' (Kandel et al. 2015). Each reaction was carried out in a 20- μl total volume with a final concentration of 1 U of GoTaq Polymerase (Promega Corp., Madison, WI, U.S.A.), 1 $\times$ reaction buffer, 2 mM MgCl_2 , 0.2 mM dNTP mix, 250 nM each primer, bovine serum albumin at 0.6 $\mu\text{g}/\text{ml}$, and 80 ng of sample DNA. The thermal profile for the reactions consisted of 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The concentrations of *F. virguliforme* DNA in each sample were calculated from the quantification cycle (C_q) measurement values using a standard curve generated from a serial dilution of pure genomic DNA of *F. virguliforme*.

Quantification of *Trichoderma* spp. in roots. Specific primers for the genus *Trichoderma* were developed based on the ITS1 region using the Primer-BLAST tool (Ye et al. 2012). The specificity of the resulting primers was tested in silico using the BLAST tool and further confirmed in the qPCR. All pairs of primers that amplified nontarget organisms were excluded. The selected primer pair Tri93F 5'-TTTCAACCCTCGAACCCTC-3' and Tri93R 5'-GCGAGAACCGCCACTGTATT-3' was used to quantify the absolute amount of DNA from *Trichoderma* isolates in soybean roots using a SYBR Green based qPCR assay. The qPCR was carried out in a 20- μl total reaction volume using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.), 500 nM each primer, and 80 ng of sample DNA using the Bio-Rad real-time thermal cycler CFX96. The thermal profile for the reactions consisted of 95°C for 3 min, 32 cycles of 95°C for 5 s and 60°C for 30 s, followed by a melt curve from 65 to 95°C with 0.5°C increments at 5 s per step. The concentrations of *Trichoderma* DNA in each sample were calculated from the C_q measurement values using a standard curve generated from a serial dilution of pure *Trichoderma* DNA.

BCAs performance under semifield conditions. Three BCAs—*T. harzianum* (WMICO1-2-26), *T. harzianum* (KMISO2-2-19A), and *T. asperellum* (WKSSO2-4-18)—were tested. For soil inoculation with BCAs, the preparation of BCA inoculum followed the same methodology as described for the *F. virguliforme* sorghum inoculum. The microplot area was prepared by digging holes in the ground to fit 55-cm clay pots. The pots were then filled with steamed soil (2:1 clay/sand) adding *F. virguliforme* (12 g) and BCA (12 g) sorghum inocula to the respective treatments following the layered method, as previously described. For the BCA soil treatments, *F. virguliforme* inoculum was added first and covered with a soil layer before the BCA inoculum was added. A factorial design was used with five replicates, and each experimental unit consisted of a clay pot with six soybean plants. The control consisted of soil inoculated only with *F. virguliforme* and no BCA inoculum. The plants were watered uniformly and examined for SDS foliar symptoms daily. Disease severity was recorded 30 days after planting using a 0-to-100 scale based on the percentage of leaf area showing chlorosis and necrosis. Also, 30 days after planting, two plants per pot were randomly selected and carefully pulled from the soil for DNA extraction. The roots were washed with tap water, rated for root rot, and stored at -20°C until DNA extraction and quantification of *F. virguliforme* and *Trichoderma* DNA following the methodology described above. Root rot was rated using a 1-to-5 scale (1 = healthy roots and 5 = plant death) proposed by Gray and Achenbach (1996). Data were analyzed using a generalized linear mixed-model procedure using PROC

GLIMMIX in SAS 9.4 (SAS institute, Cary, NC, U.S.A.). The inoculation method and treatments were considered fixed effects, whereas replication was considered a random effect. Means were separated using lsmeans ($P < 0.05$).

Evaluation of *Trichoderma* mycoparasitism genes induction upon interaction with *F. virguliforme*. The isolate *T. harzianum* (KMISO2-2-19A) was used in this experiment. The induction of mycoparasitism-related genes in the BCA was evaluated using quantitative reverse-transcription PCR (RT-PCR). The selected genes were β -1,6-glucan synthase (*b16gsyn*), α -1,3-glucanase (*agluc75*), chitinase 42 kDa (*chit42*), chitinase 33 (*chit*), and endochitinase 42 (*endo*). The gene α -tubulin was used as a reference gene. The primer sequences, accession numbers, and references for all genes used are available in the supplementary material (Supplementary Table S2). A dual-plate assay was performed between *T. harzianum* KMISO2-2-19A and *F. virguliforme*. Mycelium plugs (6 mm) of the pathogen and the BCA were placed equidistantly in a Petri plate containing minimal media supplemented with 2% glucose and overlaid with cellophane (Steindorff et al. 2012). The plates were incubated at $25 \pm 1^{\circ}\text{C}$ under darkness. Mycelia of the BCA were harvested at three time points—prior to mycelia contact (T1), at contact (T2), and after contact (overgrowth) (T3)—as described by Atanasova et al. (2013). The control consisted of *T. harzianum* confronted with itself. Three biological replicates were used for each treatment. Total RNA was extracted using the Invitrogen TRIzol Plus RNA Purification System (Thermo Fisher Scientific, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. RNA samples were subjected to DNA removal using the RQ1 RNAase-Free DNAase kit (Promega Corp.). In total, 500 ng of RNA was converted into cDNA by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). RT-PCR was performed using a Bio-Rad real-time thermal cycler CFX96 and the iQ SYBR Green Supermix with the following thermal profiling: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Two technical replicates were used for each sample. Relative gene expression of each target gene was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001), where the *T. harzianum* confronted with itself was used for normalization and α -tubulin as the reference gene.

Observation of *Trichoderma* mycoparasitism against *F. virguliforme* using microscopy. A dual-plate assay similar to the one described above was used to observe the physical interactions between *T. harzianum* KMISO2-2-19A and *F. virguliforme*. Cellophane pieces (1.5 by 1.0 cm) were excised from the interaction zone after contact (overgrowth) and observed using light microscopy and scanning electron microscopy (SEM). For light microscopy, the cellophane piece was placed on a glass slide and observed under an EXI-410 inverted microscope (Accu Scope, Commack, NY, U.S.A.). For SEM, the samples were taken to the SIUC Integrated Microscopy center. The samples were dehydrated in 100% ethanol for 1 h, dried with carbon dioxide using a samdri-790 critical point dryer (Tousimis, Rockville, MD, U.S.A.), and mounted in aluminum stubs with double-sided tape. A 60-Å gold/palladium layer was deposited on the samples by magnetron sputtering using a Denton DESK II (Denton Vacuum, Moorestown, NJ, U.S.A.) and visualized in a Quanta FEG 450 scanning electron microscope with energy-dispersive detectors (Field Electron and Ion Company, Hillsboro, OR, U.S.A.). The SEM images were colored using the MountainsSEM software.

Evaluation of defense related genes induced by *Trichoderma* spp. The *Trichoderma* isolates *T. harzianum* (KMISO2-2-19A) and *T. asperellum* (WKSSO2-4-18) were used to evaluate the induction of plant defense-related genes. The expression level of six soybean defense-related genes— β -1,3-endoglucanase (*PR2*), chitinase class I (*PR3*), defensin precursor (*PR12*), allene oxide synthase (*AOS*), chalcone synthase (*CHS*), and basic peroxidase (*IPER*)—was evaluated using quantitative RT-PCR. The soybean actin was used as the reference gene. The primer sequences, accession number, and references for all genes are shown in Supplementary Table S2. This experiment was conducted following the methodology described in the greenhouse trial section. The experiment was set up in a completely randomized design with three replicates. Each experimental unit was composed of 10 plants. Root samples from each treatment were collected 2 weeks after

sowing. The roots were carefully washed and ground using liquid nitrogen. Approximately, 0.1 g of tissue was used for mRNA isolation using the TRIzol method (Thermo Fisher Scientific, Carlsbad, CA, U.S.A.) per the manufacturer's instructions. DNase treatment and cDNA synthesis were carried out as described above in the mycoparasitism assay section. RT-PCR was performed in a Bio-Rad real-time thermal cycler CFX96 using the iQ SYBR Green Supermix with the following thermal profiling: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression of each target gene was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001), where the noninoculated plants were used for normalization and *actin* as the reference gene.

Results

Inhibition of *F. virguliforme* growth by BCAs using in vitro screening. The in vitro antagonistic activity of 47 potential BCAs against *F. virguliforme* was evaluated following the dual-culture

method. Radial growth inhibition (PRGI) of *F. virguliforme* caused by the potential BCAs ranged from 0 to 92% (Fig. 1; Supplementary Table S1). Among the potential BCAs tested were isolates from nine *Trichoderma* spp. (*T. harzianum*, *T. hamatum*, *T. koningiopsis*, *T. asperellum*, *T. virens*, *T. gamsii*, *T. viridescens*, *T. spirale*, and *T. brevicompactum*) and two *Mortierella* spp. (*M. elongata* and *M. alpina*) (Warner 2016). From the total BCAs tested, 32 isolates inhibited the growth of the pathogen by more than 40%, indicating that the majority of the isolates exhibited a high antagonistic activity against *F. virguliforme*. The most extensive radial growth inhibition of *F. virguliforme* was detected with *T. harzianum* isolates WNESO2-5-14 (91.89%), WMISO2-2-15 (88.46%), WMISO2-2-10 (81.82%), KMISO2-2-19A (79.42%), WMISO2-2-12 (78.78%), and WMNSO2-4-8 (77.61%). It is to be noted that other tested *T. harzianum* isolates were less effective, causing inhibition values ranging from 40 to 45%. Isolates of *T. asperellum*, *T. spirale*, *T. virens*, *T. koningiopsis*, *T. gamsii*, and *T. viridescens* also reduced *F. virguliforme* radial

Fig. 1. Dual-plate assays for screening of potential biocontrol isolates against *Fusarium virguliforme* Mont-1. Bars represent mean ($\pm$ standard error) percentage of radial growth inhibition (PRGI%) of *F. virguliforme* caused by 47 potential biological control agents. Means connected by the same vertical bar are not significantly different by Scott-Knott ($P < 0.05$). Dark gray bars represent biocontrol isolates that formed an inhibition zone when confronted with *F. virguliforme*, while light gray bars represent overgrowth.

growth from 50 to 76% (Fig. 1; Supplementary Table S1). The tested *M. elongata*, *M. alpina*, *T. hamatum*, and *T. brevicompactum* isolates did not cause substantial reductions in *F. virguliforme* growth, with observed PRGI values ranging from 0 to 38%. Nevertheless, *M. elongata* WMISO2-3-25 resulted in a 50% reduction of *F. virguliforme* growth (Fig. 1). Other aspects of the interactions between the BCAs and the pathogen during the dual-plate assays were also noted. Most of the *T. harzianum* BCAs overgrew the *F. virguliforme* culture and exhibited profuse sporulation, indicating that they can be highly competitive for space and nutrients (Fig. 2). Growth patterns potentially indicative of antibiosis were more commonly observed with BCA isolates from other species. Often, inhibition zones were observed upon challenging *F. virguliforme* with those BCAs (Fig. 2).

Trichoderma protection against *F. virguliforme* under greenhouse conditions. A greenhouse experiment was conducted to evaluate the efficacy of six antagonistic BCA isolates selected from the dual-plate assay with strong antagonistic activity against *F. virguliforme*. Soybean plants were grown in potting mix infested with *F. virguliforme*. The fungus colonized the roots and caused root rot and vascular discoloration of the roots and stems 21 days after germination. The seed treatment with *T. harzianum* KMISO2-2-19A had significantly higher shoot length compared with the control ($F_{6,40} = 3.625$, $P = 0.0058$) (Fig. 3A). Soybean seed treated with the tested *Trichoderma* isolates had significantly higher root lengths ($F_{6,40} = 5.603$, $P = 0.0003$) compared with the plants inoculated only with *F. virguliforme*. The treatment with *T. harzianum* KMISO2-2-19A resulted in a 74% increase in root length compared with plants inoculated exclusively with *F. virguliforme*. The other isolates resulted in an increase in root length ranging from 40 to 57% (Fig. 3B). These results highlight the ability of the tested BCA isolates to protect the soybean root system against *F. virguliforme*. In fact, significant reductions in the amount of *F. virguliforme* in the roots, measured as nanograms of *F. virguliforme* DNA per 80 ng of total DNA, were observed in the *Trichoderma* treatments when compared with the control ($F_{6,40} = 9.633$, $P = 0.0001$) (Fig. 3C). Most of the tested BCA isolates reduced the amount of *F. virguliforme* present in the roots by more than 50%, with the exception of *T. harzianum* WNISO2-5-14, which reduced the detected *F. virguliforme* DNA by 33% (Fig. 3C).

Trichoderma isolates reduced root rot caused by *F. virguliforme* on soybean seedlings under semfield conditions. The performance of the potential BCAs against *F. virguliforme* was evaluated in a semfield environment by conducting a microplot study. Two different methods of BCA inoculation were used: seed treatment and soil inoculation. Three BCAs were tested: *T. harzianum* WMICO1-2-26, *T. harzianum* KMISO2-2-19A, and *T. asperellum* WKSSO2-4-18. Because all of the BCAs performed similarly in the greenhouse assay, we considered results from ongoing research in our laboratory to choose *T. harzianum* KMISO2-2-19A and *T. asperellum* WKSSO2-4-18 to be tested in the microplot based on their antagonism against other important soybean pathogens (data not shown). The isolate *T. harzianum* WMICO1-2-26 was additionally included in this study due to its broad spectrum of action against plant pathogens and potential effect on plant growth promotion (ongoing research, unpublished data). All three isolates tested showed significant reduction in root rot ($P = 0.0003$) caused by *F. virguliforme* on soybean seedlings (Table 1), confirming that these isolates offer a degree of protection against the SDS pathogen. *T. harzianum* KMISO2-2-19A and WMICO1-2-26 significantly reduced disease severity ($P = 0.0394$) compared with the *F. virguliforme* control (Table 1). Moreover, although not statistically significant, *T. asperellum* WKSSO2-4-18 showed a tendency to reduce the severity of SDS foliar symptoms (Table 1). The positive effect of the BCAs on reducing root rot and SDS foliar symptoms were observed both when BCAs were applied as seed treatment or as inoculum directly added to the soil. The effect of the inoculation method was not significantly different for the assessed variables (Table 1). Nevertheless, the inoculation method significantly influenced the amount of *Trichoderma* DNA detected in the roots (Table 1). All *Trichoderma* isolates were present in higher amount in the roots when the BCAs were applied to the soil as sorghum inoculum rather than when they were applied as seed treatment ($P = 0.0010$) (Table 1). Interestingly, *F. virguliforme* DNA was also detected in higher amounts when the BCAs were applied to the soil than what was detected when BCA-treated seed were used ($P = 0.0127$) (Table 1). Moreover, the amount of *F. virguliforme* detected in soybean roots from the plants treated with *Trichoderma* and *F. virguliforme* was not different from what was detected in plants only treated with *F. virguliforme* ($P = 0.1375$).

Fig. 2. Visualization of overgrowth and formation of inhibition zones during the antagonistic interaction between potential biocontrol isolates and *Fusarium virguliforme* Mont-1 using a dual-plate assay. *Trichoderma* spp. and *Mortierella elongata* isolates are represented. Higher growth can be observed in the control plates on the right, where *F. virguliforme* grows unchallenged by the biocontrol.

(Table 1). The results also indicated that both *T. harzianum* KMISO2-2-19A and WMICO1-2-26 and *T. asperellum* WKSSO2-4-18 were able to colonize soybean roots, because there were no significant differences in the amount of detected *Trichoderma* DNA among the different BCA treatments (Table 1).

Induced *Trichoderma* genes associated with mycoparasitism in the presence of *F. virguliforme*. An in vitro dual-plate assay was conducted to evaluate the expression of *T. harzianum* genes, usually associated with mycoparasitism, during interactions with *F. virguliforme*. The isolate *T. harzianum* KMISO2-2-19A was chosen due to its efficacy against *F. virguliforme* in the greenhouse and microplot experiments. The expression levels of the genes were assessed by RT-PCR before, during, and after contact with *F. virguliforme* during a dual-plate assay. The expression levels of the targeted genes were not affected before physical contact or upon early contact between *T. harzianum* KMISO2-2-19A and *F. virguliforme*. However, significantly higher expression levels of the targeted chitinase and glucanase genes were observed later during the interaction between the BCA and *F. virguliforme* (Fig. 4). This supports the possibility of the occurrence of mycoparasitism events during the interaction between *T. harzianum* KMISO2-2-19A and *F. virguliforme*. The expression levels of *agluc75* and *chit* were 26- and 23-fold higher, respectively, after interaction with *F. virguliforme*. The expression levels of *endo* and *chi42* were 15- and 2-fold higher, respectively, after the establishment of the interaction (Fig. 4).

F. virguliforme parasitism by KMISO2-2-19A was further confirmed by light microscopy and SEM (Fig. 5). Coiling of KMISO2-2-19A around *F. virguliforme* hyphae was observed as well as parallel growth closely associated with the pathogen. Hyphal penetration and degradation of the *F. virguliforme* cell wall in the coiling area could also be observed (Fig. 5), suggesting activity of CWDE as implied by gene expression.

***Trichoderma*-induced expression of SA and JA/ET-inducible defense-related genes in soybean roots.** A greenhouse experiment was conducted to evaluate the ability of *Trichoderma* isolates to induce defense-related genes in soybean seedlings. The *Trichoderma* isolates *T. harzianum* KMISO2-2-19A and *T. asperellum* WKSSO2-4-18 were selected for their efficacy in reducing *F. virguliforme* growth and improving soybean growth, as previously described. Gene expression assessment with RT-PCR 2 weeks after planting

provided evidence that the evaluated *Trichoderma* isolates stimulated the induction of defense genes in soybean seedlings. Significant increases in the level of expression of the targeted defense-related genes associated with the SA and JA/ET pathways were observed when seed were coinoculated with both *Trichoderma* spp. and *F. virguliforme*. Inoculation with *T. asperellum* WKSSO2-4-18 strongly induced all six defense-related genes tested; *PR2* and *CHS*, which are involved in the SA defense pathway, were 2.7 and 10 times more expressed, respectively, when the plants were primed by WKSSO2-4-18 in comparison with the *F. virguliforme* control (Fig. 6). This isolate also induced the transcription of JA/ET-related genes, with increases amounting to 14.79- and 2.64-fold for the pathogenesis-related proteins *PR3* and *PR12*, respectively; 5.18-fold for *AOS*; and 16.77-fold for *IPER* (Fig. 6). *T. harzianum* KMISO2-2-19A also primed soybean defenses against *F. virguliforme* by increasing the level of *CHS*, *PR3*, *AOS*, and *IPER* expression by 3.85-, 7.42-, 2.06-, and 2.38-fold, respectively, compared with expression levels when the plants were only inoculated with the pathogen (Fig. 6).

Discussion

Trichoderma spp. have been increasingly used as BCAs to manage fungal pathogens, oomycetes, bacteria, and nematodes. They have also been commercialized as biofertilizers, with direct benefits to plant growth, nutrient acquisition, and plant response to abiotic stresses (Woo et al. 2014). However, the interaction between these organisms and plants is complex. Their beneficial effects are dependent on many factors, including the host plant, the timing of the interaction with the plant, the interaction with other organisms (including pathogens) in a specific biome, as well as other environmental factors. The extent of these beneficial effects may also depend not only on the associated *Trichoderma* spp. but also on the particular strain that is being used (Alonso-Ramírez et al. 2014; Medeiros et al. 2017; Mendoza-Mendoza et al. 2018; Morán-Díez et al. 2012; Pereira et al. 2014; Salas-Marina et al. 2014; Woo et al. 2006). For instance, Woo et al. (2006) has shown that *Trichoderma* expression is strongly and differentially altered while the fungus is colonizing a plant root or when a different host pathogen is detected. Therefore, the selection and identification of highly antagonistic isolates targeting a specific pathogen are crucial for the future development of biological control tools that could be included in disease management

Fig. 3. Boxplot from greenhouse assay testing potential biocontrol agents against *Fusarium virguliforme* Mont-1. Six biocontrol isolates (*Trichoderma harzianum* KMISO2-2-19A, WMNSO2-4-8, WNESO2-5-14, and KMISO2-2-15B; *T. asperellum* WKSSO2-4-18; and *Mortierella elongata* WMISO2-3-25) were used as seed treatments (soybean cultivar Spencer). **A**, Shoot length (mm); **B**, root length (mm); and **C**, *F. virguliforme* DNA (nanograms per 80 ng of total DNA) measured by quantitative PCR. Black dots are data outliers. Boxplots represented by the same letter are not statistically different by Tukey's honest significance test ($P < 0.05$). Asterisks (*) represent biocontrol treatments significantly different from the control (Fv MT-1) by Dunnett's *t* test ($P < 0.05$).

programs. In this study, we demonstrated the ability of selected *Trichoderma* isolates to reduce SDS severity as well as root rot caused by *F. virguliforme* in soybean seedlings. Our results suggest that these isolates use combined mechanisms during their interaction with *F. virguliforme* and with soybean, affecting the ability of the pathogen to cause disease. The antagonistic effect exhibited by the tested BCAs against *F. virguliforme* seems to be associated not only with direct competition and mycoparasitism but also with priming plant defenses through the activation of JA- and SA-dependent pathways. Little is known about the effect of *Trichoderma* treatments on soybean plants and its priming of defenses against *F. virguliforme*. *Trichoderma* is known to produce a variety of microbe-associated molecular patterns during root colonization such as hydrophobins, glycoside hydrolases, and cerato-platanins that act as potent elicitors of plant defense responses (Hermosa et al. 2012). In the *Trichoderma*–plant–pathogen interaction, *Trichoderma* plays a major role by triggering the activation of ET- and JA-dependent ISR and SA-associated SAR (Brotman et al. 2013; Mendoza-Mendoza et al. 2018). We observed a potential primed soybean response against *F. virguliforme* when seeds were treated with the BCAs, demonstrated by the higher expression levels of all six defense genes upon coinoculation, in contrast to lower expression levels of these genes in plants inoculated with *F. virguliforme* alone. Similarly, Pereira et al. (2014) reported that *T. harzianum* ALL-42 seems to potentiate the response of common bean to *Rhizoctonia solani* by increasing the expression levels of the *glu1* and *peroxidase* (*pod3*) genes, in comparison with the response of the host when exposed to the pathogen alone. In this study, we documented that *T. harzianum* KMISO2-2-19A and *T. asperellum* WKSSO2-4-18 were able to trigger systemic resistance in soybean roots. Both isolates increased expression levels of the JA/ET-responsive genes *PR3*, *PR12*, and *AOS* and the SA-responsive genes *PR2*, *CHS*, and *IPER*. These findings agree with previous studies that have reported simultaneous activation of SAR and ISR defenses, as well as the enhancement of plant resistance against phytopathogens by *Trichoderma* spp. Cucumber roots colonized by *T. harzianum* T-203 exhibited increased activity of chitinase, β -1,3-glucanase, cellulase, and peroxidase 72 h postinoculation (Yedidia et al. 2000). *Trichoderma* spp. induced genes involved in sesquiterpene phytoalexins biosynthesis and hypersensitive response in 32-day-old pepper seedlings (Bae et al. 2011). *Trichoderma harzianum* T-79 has also been reported to differentially prime JA and SA in tomato seedlings during the interaction with the root knot nematode *Meloidogyne incognita*. During the initial interaction, T-79 limited nematode root invasion by primed SA-regulated defenses. Later, T-79 enhanced JA-regulated defenses, reducing the nematode fecundity and gall formation (Martínez-Medina et al. 2017).

Trichoderma spp. are well known mycoparasites with documented activity against a range of fungi (Harman et al. 2004a). However, little is known about the interaction between *Trichoderma* and *F. virguliforme*. Chitin and β -1,3-glucan are main constituents of the cell walls of phytopathogenic fungi. Consequently, CWDEs such as chitinases and glucanases have been reported to be directly involved in the mycoparasitic activity of *Trichoderma* spp. by facilitating the lysis of the fungal host cell wall during parasitism, enabling the penetration of the fungal host (Harman et al. 2004b; Howell 2003; Vinale et al. 2008). Furthermore, genome comparison between the mycoparasitic *T. virens* and *T. atroviridae* and the saprophytic *T. reesei* revealed a higher number of chitinases and glucanases in the mycoparasitic species (Kubicek et al. 2011). In the current study, *T. harzianum* KMISO2-2-19A exhibited an increase in the expression level of several CWDEs upon interaction with *F. virguliforme* (Fig. 4). The overexpression of *agluc75*, *chit*, *chit42*, and *endo* was visible after the contact between the two organisms, at a point when *T. harzianum* overgrew *F. virguliforme*. The significant increase in the expression of chitinases and α -1,3-glucanase during the interaction of *T. harzianum* KMISO2-2-19A with *F. virguliforme* suggests that these enzymes are involved in the mycoparasitic activity of the BCA against *F. virguliforme*. In other studies, the production and secretion of β -1,3-glucanase and chitinase were

shown to play a role in the mycoparasitism of *F. oxysporum* by *Trichoderma* spp. (Ojha and Chatterjee 2011) as well as in the mycoparasitism of *Sclerotinia sclerotiorum* by *T. harzianum* T-aloe (Zhang et al. 2016). Overall, the increase in the expression of CWDEs and the impact of these enzymes on mycoparasitic interactions of *Trichoderma* spp. against plant pathogens are well documented in the literature (Lorito et al. 2010).

During mycoparasitism, *Trichoderma* has been reported to coil around the fungal host hyphae and to produce appressoria-like and hook-like structures that allow its firm attachment to the fungal host (Howell 2003; Zeilinger and Omann 2007). Our microscopy images showed evident coiling of KMISO2-2-19A around *F. virguliforme* hyphae as well as parallel growth with close hyphal association, which is also an indication of mycoparasitism, as reported by Monteiro et al. (2010). In their study, Monteiro et al. (2010) documented a distinct behavior of *T. harzianum* ALL42 when parasitizing different plant pathogens. When confronted with *R. solani* and *Macrophomina phaseolina*, ALL42 coiled around their hyphae, forming appressoria-like and hook-like structures. However, when confronted with *Fusarium* spp., ALL42 just touched the hyphae without coiling. In this study, both coiling and touching were observed during the interaction between KMISO2-2-19A and *F. virguliforme*. These results indicate that the mechanisms of host recognition by *Trichoderma* spp. can differ with different *Trichoderma*–host interactions, and that they can be specific to that interaction. In the current study, a possible CWDE activity was detected in the SEM images, where it was possible to note degradation of *F. virguliforme* cell walls and hyphal depression in the area encircled by KMISO2-2-19A.

In the microplot study, the amount of *F. virguliforme* DNA in soybean roots of plants coinoculated with *Trichoderma* and *F.*

Table 1. Root rot, disease severity, and fungal DNA quantitative PCR quantification to evaluate the performance of three selected *Trichoderma* isolates using two different inoculation methods against *Fusarium virguliforme* in a microplot trial^w

Factors	Root rot (1-to-5 scale)	Disease severity (0 to 100%)	<i>F. virguliforme</i> DNA (ng) ^x	<i>Trichoderma</i> DNA (ng)
Treatments ^y				
<i>F. virguliforme</i> Mont-1	4.0 a	22.8 a	0.150	0.000 b
<i>T. asperellum</i> WKSSO2-4-18	2.4 b	12.0 ab	0.137	0.010 a
<i>T. harzianum</i> KMISO2-2-19A	2.6 b	5.0 b	0.139	0.007 ab
<i>T. harzianum</i> WMICO1-2-26	3.1 b	10.0 b	0.210	0.013 a
Inoculation method ^z				
Seed treatment	2.9	13.5	0.127 b	0.002 b
Soil inoculation	3.1	10.5	0.192 a	0.013 a
Main factor (<i>P</i> values)				
Treatment	0.0003	0.0394	0.1375	0.0302
Inoculation method	0.4023	0.6078	0.0127	0.0010
Treatment × inoculation	0.4098	0.5610	0.2900	0.1039

^w Least significant means followed by the same letter within each column are not statistically different ($P < 0.05$).

^x Fungal DNA quantified in seedling roots by quantitative PCR in nanograms per 80 ng of total DNA. Soybean cultivar AG4632 was used.

^y Each *Trichoderma* isolate was coinoculated with *F. virguliforme*; the control received only *F. virguliforme*.

^z *Trichoderma* was applied as seed treatment or as soil inoculation (via sorghum inoculum).

Fig. 4. Normalized expression levels of five mycoparasitism-related genes measured by reverse-transcription PCR during the interaction of *Trichoderma harzianum* KMISO2-2-19A and *Fusarium virguliforme* Mont-1 in a dual-plate assay. Gene expression was evaluated at three different interaction times: T1 = prior to mycelia contact, T2 = at contact, and T3 = after contact (overgrowth). The $2^{-\Delta\Delta Ct}$ method was used for normalization of each target gene using the control (*T. harzianum* KMISO2-2-19A growing against itself) and α -tubulin as the reference gene.

Fig. 5. Parasitism of *Trichoderma harzianum* KMISO2-2-19A against *Fusarium virguliforme* observed under **A** and **B**, light microscopy and **C**, **D**, **E**, and **F**, scanning electron microscopy. **A**, KMISO2-2-19A hyphae (Th) coiled around *F. virguliforme* hyphae (Fv) (solid black arrows) at $\times 20$ magnification; **B**, KMISO2-2-19A hyphae growing in parallel and closely associated with *F. virguliforme* (empty black arrows) and *F. virguliforme* macroconidia (Fvm), $\times 40$ magnification; **C** and **D**, *F. virguliforme* hyphae encircled by the antagonist with hyphae penetration (hp); **E**, encircling with greater magnification showing *F. virguliforme* hyphal depression (hd) caused by KMISO2-2-19A in the coiling area; and **F**, beginning of coiling with apparent degradation of *F. virguliforme* cell wall (dcw) in the interaction area.

virguliforme was not different from that detected in plants treated with *F. virguliforme* in the absence of BCAs (Table 1). Therefore, in the microplot, the observed reduction in the severity of foliar symptoms and root rot in the presence of the BCAs may be related to indirect mechanisms such as the induction of plant defenses, promotion of plant health, or suppression of disease symptoms. Interestingly, different results were observed under greenhouse conditions, where treatment with BCAs affected the amount of *F. virguliforme* DNA detected in the roots. Less *F. virguliforme* DNA was detected in the BCA-treated plants (Fig. 3C). Root colonization of *F. virguliforme* and *Trichoderma* spp. in the greenhouse might have been affected by the faster germination and development of the root system. In the greenhouse, close to 100% germination was observed within 3 days after planting whereas, in the microplot, seed took longer to germinate (about 5 to 8 days) (observations during the experiments, data not shown). Gongora-Canul and Leandro (2011) suggested that accelerated root growth reduces seedling susceptibility to *F. virguliforme* infections. Therefore, the slower germination or root development in the microplot might have favored root infection by *F. virguliforme* despite coinoculation with *Trichoderma* spp. Although not measured, the observed differences may also be related to the different soil used (potting mix in the greenhouse versus soil mix in the microplot) and the different soybean cultivar used (Spencer in the greenhouse versus AG4632 in the microplot).

For successful introduction of BCAs in production systems, the method of application of the BCA is crucial. It can affect the activity of the BCA by altering the way it interacts with the plant and the pathogen. In this study, the two methods of application tested proved to have an effect on the amount of BCA and pathogen able to colonize plant roots. Even though the delivery methods tested here were very simple and crude, a clear effect of the BCAs was still observed in terms of the development of disease symptoms. More significant results could probably be attained by optimizing the formulation of the BCAs and the method used to dispense them. Research is needed for the development of optimized BCA delivery systems that, for example, could provide a competitive advantage to the BCAs or offer control on the timing of the BCA release based on the pathogen's activity.

The identification of BCAs highly effective against plant pathogens provides a tool that can be explored to help in the management of plant diseases, especially in the case of important phytopathogens that are difficult to control such as *F. virguliforme*. Our results provide a basis for the future inclusion of BCAs in management strategies targeting SDS in soybean. More research still needs to be conducted to elucidate some of the aspects of the plant–*Trichoderma*–pathogen interactions and to develop improved formulations and application techniques that would optimize the effects of these beneficial organisms.

Fig. 6. Normalized expression levels of defense-related genes measured by reverse-transcription PCR in soybean roots (cultivar Spencer). Biocontrol isolates *Trichoderma harzianum* KMISO2-2-19A and *T. asperellum* WKSSO2-4-18 were applied as seed treatment and seedlings were grown in the presence or absence of *Fusarium virguliforme* Mont-1. Gene expression was measured 2 weeks after sowing. Control plants received only *F. virguliforme* Mont-1 inoculation (F.v). The $2^{-\Delta\Delta Ct}$ method was used for normalization of each target gene using noninoculated plants and soybean actin as the reference gene.

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