Effect of *Trichoderma harzianum* on Sporulation of *Cochliobolus sativus* on Excised Wheat Seedling Leaves

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ABSTRACT

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Wheat seedlings were spray-inoculated with a conidial suspension of *Cochliobolus sativus* (3×10^5 conidia per milliliter), placed in a mist chamber for 24 hr, and transferred to a greenhouse bench for observation of lesion development. Seven days later, a conidial suspension of *Trichoderma harzianum* (5×10^7 conidia per milliliter) was spray-applicated to the infected wheat seedlings. After drying, individual lesions of *C. sativus* were excised, and the fungus was induced to sporulate in petri dishes lined with moist filter paper. Six days later, conidia from the lesions were counted. The sporulation capacity (number of conidia produced per 15 lesions) of *C. sativus* in lesions treated with *T. harzianum* was significantly less than in lesions treated with distilled water at 26 and 32 C but not at 21 C. Sporulation capacity of the fungus was reduced when treated with *T. harzianum* as compared with water controls in lesions on winter wheat cultivars Baca, Newton, Scout 66, and Vona but not on durum cultivars Calvin and Vic. Subsequent tests revealed a significant inverse correlation in the sporulation capacity of lesions of *C. sativus* on cultivar Scout 66 with application of various conidial suspension concentrations of *T. harzianum*.

Additional key words: biocontrol, common dryland root rot.

Biological control has only recently been incorporated into and significantly increased the efficiency of several disease control programs (2,4). A living, multiplying, biological control agent potentially may provide continuous control of a pathogen, whereas chemical applications usually are effective for a limited time period. Several studies have demonstrated reduced disease after supplementing with nonphytopathogenic fungi, bacteria, and/or actinomycetes (2,6,13). Campbell (6) found that when certain nonphytopathogenic fungi were added to soil containing conidia of *Cochliobolus sativus* (Ito & Kurib.) Dreschsl. ex Dastur, wheat seedling root rot was reduced. Fokkema et al (13) demonstrated disease reduction when *Sporobolomyces roseus* and *Cryptococcus laruentii* var. *flavescens* were applied to rye plants before inoculating with *C. sativus*.

Several *Trichoderma* spp. have been shown to be effective mycoparasites (9), and subsequent studies have demonstrated they are active biocontrol agents in the soil (4). The biological control mechanism of *Trichoderma* may be hyperparasitism and/or competition (7,10,18,21,23). Conidia of *T. harzianum* Rifai have been applied as a seed coating (14), a spore suspension added to soil (12), and in a nutrient solution to specified plant parts (4,22). The efficacy of *Trichoderma* spp. as biological control agents on aerial plant parts has been reported for woody portions of plants, especially pruning wounds (4). Because *T. harzianum* is not normally abundant on leaves, it is seldom considered for biological control of foliar pathogens.

C. sativus causes plant and yield losses in most wheat and small grain-growing areas of the world as the causal agent or complex component of foot and root rot (11). The foliar disease phase of *C. sativus* usually is economically important only in areas with high humidity and warm temperatures.

Infection efficiency, lesion size, and sporulation capacity are relative parasitic fitness attributes that usually can be measured in host-parasite interactions (19). In a preliminary investigation, wheat seedlings spray-inoculated with a conidial suspension of *C. sativus* were sprayed with 3 ml of either a conidial suspension of *T. harzianum* or autoclaved, distilled water. After 7 days, no significant differences in infection efficiency (number of lesions per plant) or size of lesions were found between inoculated plants treated with the conidial suspension of *T. harzianum* and those treated with autoclaved, distilled water. Lesions excised from plants treated with the conidial suspension of *T. harzianum* produced less conidia when induced to sporulate in petri dishes lined with moist filter paper than lesions excised from plants treated with autoclaved, distilled water. Therefore, sporulation capacity, the average number of conidia produced per 15 lesions, was selected as the appropriate parasitic fitness attribute to determine the effect of *T. harzianum* on foliar lesions of *C. sativus* on wheat seedlings. The main objective of this study was to determine if treatment with *T. harzianum* would affect the sporulation capacity of lesions of *C. sativus* excised from wheat seedlings.

MATERIALS AND METHODS

The isolate of *C. sativus* used in this study was obtained from a naturally infected root of a winter wheat plant collected from a commercial field in Colorado. Several seedlings of winter wheat cultivar Vona were spray inoculated with a 3×10^5 conidial suspension of *C. sativus* in autoclaved, distilled water, placed in a mist chamber for 24 hr at approximately 23 C, and incubated on a greenhouse bench with no supplementary light. Seven days later, diseased leaves were collected, dried, and stored at room temperature. Inoculum for all experiments was obtained from this source by inducing lesion sporulation in petri dishes lined with moist filter paper, and transferring a single conidium to petri dishes containing potato-dextrose agar (PDA). Cultures were incubated in the laboratory for 7–10 days before being used for inoculation.

T. harzianum isolate (T95, ATCC 60850) was obtained from R. Baker, Colorado State University, Fort Collins, CO. The isolate was stored at 4 C on PDA slants and increased, when needed, by mass transfer to petri dishes containing PDA. Cultures were incubated under fluorescent and incandescent light ($38\ \mu E\ m^{-2}\ s^{-1}$) for 18 hr followed by 6 hr of dark at approximately 25 C. After 14–21 days, autoclaved distilled water was applied to the colony surface, and conidia were removed by gentle scraping with a glass microscope slide. The spore suspension was strained through

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cheesecloth and calibrated to 5×10^7 conidia per milliliter of autoclaved distilled water (unless otherwise indicated) with the aid of a hemacytometer.

Four greenhouse experiments were conducted to determine the effect of *T. harzianum* on the sporulation capacity of lesions of *C. sativus* on wheat seedling leaves. Five wheat seeds were sown per 6.5- × 6.5-cm plastic pot containing a sterilized mixture of soil, peat, perlite, and vermiculite (2:1:1:1, v/v). Seedlings were grown on greenhouse benches at approximately 22 C with no supplementary light for 30 days, and pots were irrigated to soil saturation every 2 days with a 20:20:20 (N:P:K) nutrient solution (0.6 g/L).

Wheat seedlings (four- to six-leaf stage) were sprayed until runoff with a 3×10^5 conidial suspension of *C. sativus* per milliliter of distilled water. Inoculated plants were transferred to a mist chamber for 24 hr and then transferred to greenhouse benches. After 7 days, 3 ml of either a conidial suspension of *T. harzianum* containing two drops of Tween 20/100 ml or autoclaved, distilled water was sprayed on each set of inoculated plants. Leaves were allowed to dry for 2 hr, and then 15 lesions were excised from each set of plants, which represented one replication. Each group of 15 lesions was placed in petri dishes containing moist filter paper and stored in the dark at approximately 26 C to induce sporulation. Six days later, each group of 15 lesions was placed in a 20-cm-diameter test tube containing 25 ml of 0.05% $CuSO_4$ solution to inhibit spore germination. After we shook the tubes to disperse conidia evenly, 0.1 ml of suspension was placed on a 1- × 2-cm strip of water agar, and conidia were counted under 20× magnification. Four 0.1-ml counts were made from the suspension in each test tube. The four counts were averaged and expressed as total number of conidia per 15 lesions. Variances were determined to be homogeneous before data were pooled. Significant differences of all analyses were determined at $P = 0.05$.

The first test determined the effect of treatment with conidia of *T. harzianum* on the sporulation capacity of *C. sativus* in lesions as compared with the sporulation capacity in lesions treated with distilled water. Winter wheat (*Triticum aestivum* L.) cultivar Scout 66 seedlings were used. There were five replicates of each treatment, and the test was repeated four times. A one-way analysis of variance was used to analyze the data.

The second test determined the effect of treatment with conidia of *T. harzianum* on the sporulation capacity of *C. sativus* in lesions under constant temperatures of 1, 21, 26, 32, or 40 C. Seven replications were used under each temperature regime in two separate runs, with cultivar Scout 66. Student's *t* test was used to analyze the data of each temperature regime.

The third test was performed to determine the concentration effect of conidia of *T. harzianum* on the sporulation capacity of *C. sativus*. Three concentrations of conidia of *T. harzianum* were applied to five sets of diseased Scout 66 wheat seedlings. Treatments consisted of 5×10^3, 5×10^5, and 5×10^8 conidia of *T. harzianum* per milliliter of autoclaved, distilled water, and an autoclaved, distilled water control. The initial run included three replications of each treatment, whereas a second run included five replications.

Winter and durum (*T. turgidum* L. var. *durum*) wheat seedlings infected with *C. sativus* were tested also. Winter wheat cultivars were Baca, Newton, Scout 66, and Vona and durum cultivars were Calvin and Vic. Five pots of seedlings of each cultivar were sprayed with 3 ml of a 5×10^7 conidia per milliliter of suspension of *T. harzianum* or autoclaved, distilled water. Lesions of *C. sativus* were induced to sporulate and the conidia counted as previously described. The test was repeated once. Student's *t* test was used to analyze the data of each cultivar.

In an effort to determine the control mechanism of *T. harzianum*, lesions of *C. sativus* from cultivar Scout 66 were treated with supernatant from a conidia and mycelium wash. Sporulation capacity was compared with lesions treated with autoclaved, distilled water. Sporulation, conidial counts, and inoculum preparation were made as previously described. The wash treatment was prepared from a 5×10^7 conidial suspension by straining through filter paper until no conidia or mycelial fragments were observable under a light microscope. Light and scanning electron microscopy also were used to determine if evidence of mycoparasitism existed.

RESULTS

The average number of conidia of *C. sativus* produced in 15 lesions was significantly reduced from 39,562 in the water control to 6,625 conidia after treatment with *T. harzianum* (Table 1). In all runs, treatment with *T. harzianum* resulted in significantly less sporulation than treatment with water. Similar results were obtained with several other isolates of *C. sativus*.

The effect of *T. harzianum* on sporulation of lesions of *C. sativus* under several temperature regimes was variable. No sporulation was observed at 1 or 40 C (Table 2). Maximum sporulation occurred at 26 C, whereas sporulation was significantly decreased by *T. harzianum* under the 26 and 32 C regimes by 47 and 83%, respectively, as compared with the controls. No significant decrease in sporulation of lesions of *C. sativus* by *T. harzianum* was observed at 21 C.

Mean sporulation of *C. sativus* was 21,500, 14,500, 11,000, and 2,250 conidia per 15 lesions after treatment with 0, 5×10^3, 5×10^5, and 5×10^8 conidial suspensions of *T. harzianum*, respectively (Fig. 1). All treatments with *T. harzianum* resulted in significantly less sporulation than treatment with water. The regression slope for $\log_{(10)}$ transformed value was -0.13 with a correlation of -0.84, which indicated that the concentration of conidia of *T. harzianum* was significantly and inversely related to the subsequent average number of spores produced by lesions of *C. sativus* (Fig. 1).

Conidial suspensions of *T. harzianum* reduced sporulation capacity in Newton, Vona, Baca, Scout 66, Vic, and Calvin by 76, 53, 64, 59, 18, and 20%, respectively (Table 3). Significant reduction in sporulation of *C. sativus* occurred on all winter wheat cultivars tested. Significant sporulation reductions were not observed on the durum cultivars Vic and Calvin.

Treatment of lesions of *C. sativus* with the supernatant from the conidial and mycelium wash of *T. harzianum* did not significantly

TABLE 1. Average number of conidia of *Cochliobolus sativus* produced per 15 lesions in vitro after a spray treatment with a conidial suspension of *Trichoderma harzianum* or an autoclaved, distilled water control in four runs of the experiment

	Conidia in lesions treated with	
Run[a]	Water	*T. harzianum*[b]
1	21,000	2,500
2	38,250	10,750
3	64,000	3,500
4	35,000	9,750
$\overline{X}$	39,562	6,625[c]

[a]Average of five replicates per run.
[b]Calibrated to 5×10^7 conidia per milliliter of autoclaved, distilled water.
[c]Significant difference between mean of water and *T. harzianum* treatments over experiment at $P = 0.05$.

TABLE 2. Average number of conidia of *Cochliobolus sativus* produced per 15 lesions in vitro under five temperature regimes after a spray treatment with autoclaved, distilled water or a conidial suspension of *Trichoderma harzianum*

Temperature	Conidia in lesions treated with[a]	
(C)	Water	*T. harzianum*[b]
1	0	0
21	10,000	9,500
26	31,500	16,750*
32	5,750	1,000*
40	0	0

[a]Average of seven replicates in each of two runs; * = Significant difference between the treatment means of water and *T. harzianum* at $P = 0.05$.
[b]Calibrated to 5×10^7 conidia per milliliter of autoclaved, distilled water.

affect subsequent sporulation. In two separate runs with eight and 10 replications, average conidial production was 19,125 for the water treatment and 17,500 for the supernatant treatment.

Light and scanning electron microscopy revealed a curling of hyphae of *T. harzianum* around hyphae of *C. sativus*. Small holes were observed in the hyphae walls of *C. sativus*, but evidence that they were caused by *T. harzianum* could not be found.

DISCUSSION

T. harzianum significantly decreased the sporulation capacity of *C. sativus* on wheat seedlings in vitro. Various environmental factors affected both the sporulation of *C. sativum* and the inhibitory effects of *T. harzianum*. No clear evidence was obtained to determine if the inhibitory activity was due to competition or hyperparasitism.

The optimum in vitro sporulation temperature of *C. sativus* in wheat seedlings was 26 C, which corresponds with the optimum temperature range for conidial germination, infection, and disease development (8,16,20,24). Temperature parameters have also been reported for the optimum antagonistic activity of *T. harzianum* in the soil (12). In this investigation, *T. harzianum* was not antagonistic to sporulation of *C. sativus* at 21 C, but appeared to be antagonistic at 26 and 32 C on the leaf surface. These results differ with those of Elad et al (12) in that they found decreased antagonist activity of *T. harzianum* at 27 C in the soil. The apparent loss of antagonism may have been caused by the increased virulence of the pathogens, *Rhizoctonia solani* and *Sclerotium rolfsii* at 29 C in Elad's experiment. The percentage of sporulation capacity reduction of lesions of *C. sativus* when treated with *T. harzianum* was 47% at 26 C and 83% at 32 C. The sporulation capacity of *C. sativus* decreased fivefold at 32 C relative to 26 C. Antagonistic activity appeared to increase when the temperature regime was not optimum for the pathogen.

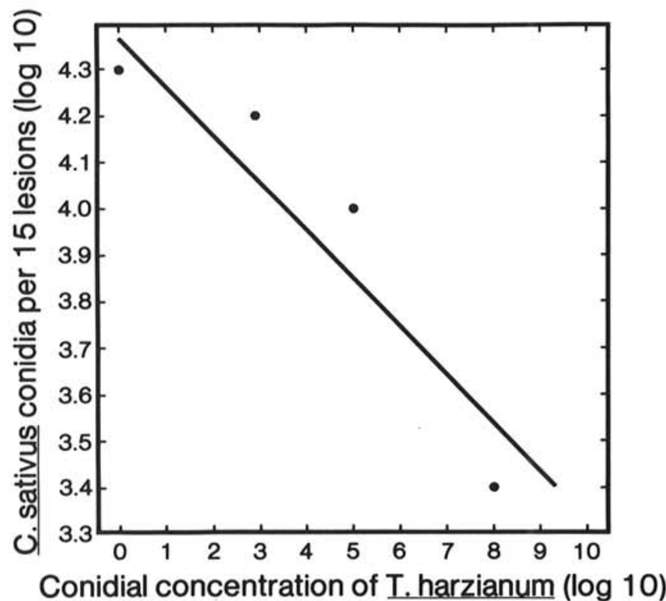

Fig. 1. The average number of conidia of *Cochliobolus sativus* produced per 15 lesions in vitro after a spray treatment with one of three conidial suspensions of *Trichoderma harzianum* or an autoclaved, distilled water control. The regression equation is $y = 2.04 - 0.131x$ and correlation coefficient is -0.84.

TABLE 3. Average number of conidia of *Cochliobolus sativus* produced per 15 lesions in vitro after a spray treatment with autoclaved, distilled water or a conidial suspension of *Trichoderma harzianum*[a]

	Conidia in lesions treated with	
Cultivar	Water	*T. harzianum*[b]
Winter wheat		
Newton	14,750	3,500*
Vona	10,500	5,000*
Baca	8,250	3,000*
Scout 66	9,000	3,750*
Durum wheat		
Vic	11,000	8,000
Calvin	11,250	9,000

[a]Calibrated to 5×10^7 conidia per milliliter of autoclaved, distilled water.
[b]Average of five replications in each of two runs; * = Significant difference between the treatment means of water and *T. harzianum* at $P = 0.05$.

T. harzianum did not significantly reduce the sporulation capacity of lesions of *C. sativus* on cultivars Vic or Calvin. Different physiological or morphological characteristics of the spring durums may have had an effect on activity of *T. harzianum*. The physical arrangement of leaves is more upright in the durum seedlings, and, possibly, the conidia of *T. harzianum* may be subject to a greater chance of runoff than the larger, faster germinating conidia of *C. sativus*. Metabolic by-products of other phyllosphere microorganisms, chemical components of the host wax and cuticle, and metabolites from cells within the leaf have also been shown to affect the colonization of microorganisms on a leaf surface (3). Infection efficiency and sporulation capacity of *C. sativus* were similar on durum and winter wheat cultivars, but the antagonistic properties of *T. harzianum* were deterred on the durums.

Mechanism of suppression of sporulation of *C. sativus* by *T. harzianum* was not determined. On PDA, *T. harzianum* inhibited and then grew over *C. sativus*, which indicates that *T. harzianum* is the more aggressive saprophyte in culture. When we observed hyphal interaction on WA and the leaf surface, coiling and possible penetration were also observed (5). Coiling, however, does not necessarily imply hyperparasitism as discussed by Dennis and Webster (10). Competition for the available substrate or antibiosis (17) could be other possible biological control mechanisms. *T. harzianum* isolate T-95 is highly cellulytic (1), and when the excised lesions of *C. sativus* were placed on filter paper, which has a high cellulose content, *T. harzianum* could have been stimulated. To clarify the mechanism of control, experiments investigating the necessary nutrients for sporulation of *C. sativus* on the leaf surface and the use of nonantagonistic saprophytic fungi in lesion treatments should be conducted.

The epidemiological application of this type of biological control in the field may result in decreased disease spread. Small reductions in inoculum increase would create a large accumulative effect over several generations. Applying this type of control measure when the disease first appears could reduce secondary spread and result in reduced primary inoculum available for the next season. If *T. harzianum* does not hyperparasitize *C. sativus*, it may compete for nutrients with *C. sativus* in the lesion. Such competition may extend through overwintering in the debris and perhaps reduce the amount of primary inoculum in the next season. Another possibility is the use of a systemic fungicide along with a fungicide-tolerant isolate of *T. harzianum*. For example, imazalil has little effect on sporulation of *C. sativus*, but significantly reduces infection efficiency (15). In this case, both infection efficiency and sporulation capacity may be reduced, which would inhibit disease development over time.

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