Pathogenicity and Formulation of *Typhula phacorrhiza*, a Biocontrol Agent of Gray Snow Mold

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ABSTRACT

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In previous testing, we found that gray snow mold, caused by *Typhula ishikariensis* or *T. incarnata*, could be controlled by applying *T. phacorrhiza* onto turf grass prior to snowfall. To test for phytopathogenicity of this biocontrol agent, the five most disease-suppressive isolates were cultured on mixed grains and applied to 12 turfgrass cultivars and 2 winter wheat cultivars in December 1995 and 1996. After snow melt in April 1996 and 1997, significantly greater winter injury was visible on plots treated with the pathogens compared to *T. phacorrhiza*-treated plots or untreated plots. Except for one cultivar in 1996, there were no significant differences between *T. phacorrhiza*-treated plots and untreated plots. Pelletized alginate formulations of *T. phacorrhiza* containing kaolin clay with various nutritional amendments were tested for viability and efficacy. After 64 weeks of storage, viability remained >85% at -15°C and >70% at 4°C, but <30% at 10°C and 25°C. Significant control of gray snow mold by *T. phacorrhiza* using small numbers of pellets (20 g/m²) was equivalent to using larger amounts of mixed grain inoculum (200 g/m²) or wheat bran inoculum (100 g/m²) in 2 years of field testing.

Typhula incarnata and *T. ishikariensis* are important fungal pathogens that cause gray snow mold of turf grasses and cereals. This disease is of particular concern on golf courses where intensively managed and high-value turf can be severely damaged. Although several synthetic fungicides containing active ingredients such as mercury or quinterozone can provide effective control of the disease (20), societal concerns regarding the environmental effects coupled with the cost of applying fungicides led us to investigate alternative management approaches.

T. phacorrhiza is considered to be a saprotroph overwintering on leaves, petioles, and herbaceous stems (16). In Ontario, the fungus has been observed most frequently on corn residues and other organic debris in winter wheat and corn fields after at least 80 days of snow cover (3,18). *T. phacorrhiza* was found to be non-pathogenic on turf (3) and winter wheat (2); however, Schneider and Seaman (18) reported that *T. phacorrhiza* was pathogenic on wheat based on controlled environment studies and a field study with

a single isolate. Pathogenicity of *T. phacorrhiza* has not been reported elsewhere, but it is essential to establish that a putative biocontrol agent is not pathogenic to targeted plants or to other economically important plants grown nearby.

T. phacorrhiza has been found to suppress the development of gray snow mold (3,10,11,14,24,25). From 1994 to 1996, we collected and tested over 300 isolates of *T. phacorrhiza*, and have selected more than 10 isolates that provided significant disease suppression and performed as well as a commercial fungicide (25). In earlier experiments, colonized mixed-grain inoculum of *T. phacorrhiza* was applied by hand in controlled environment and field studies. Lawton and Burpee (10) determined that at least 200 g/m² of mixed-grain inoculum was required to provide suppression of gray snow mold equivalent to that obtained with conventional applications of the fungicide quinterozone. However, the use of mixed-grain inoculum is impractical for large-scale commercial applications due to the volume of formulated inoculum required.

The objectives of this study were (i) to assess the pathogenicity of *T. phacorrhiza* to amenity turf grasses and winter wheat in the field, (ii) to develop suitable formulations of *T. phacorrhiza* as a biocontrol agent, and (iii) to evaluate these formulations for suppression of *T. ishikariensis*- or *T. incarnata*-induced gray snow mold of creeping bent grass in the field.

MATERIALS AND METHODS

Fungal isolates. Five isolates of *T. phacorrhiza* from southern Ontario, ranked as

the most suppressive to gray snow mold in previous field trials (25), were used in this study. Five isolates each of *T. incarnata* and *T. ishikariensis* were used as gray snow mold inocula. These pathogenic isolates were obtained from stock cultures, and have been used in other studies with gray snow mold (6).

Preparation of grain inocula. A total of 200 g mixed grain (equal weights of wheat, oat, barley, and corn; 10) or 100 g wheat bran were mixed with 300 ml distilled water in 1-liter mason jars and autoclaved twice for 20 min on two successive days. Up to 10 5-mm-diameter mycelial plugs from the margin of a single isolate growing on potato dextrose agar (PDA, Difco Laboratories, Detroit) were placed in a jar and incubated for 7 to 8 weeks at 10°C in the dark. Immediately before use, inocula were air dried for 36 h and chopped into small particles (<1 mm) with a domestic blender (1 min at high speed in 100-g portions). Inocula consisted primarily of colonized grain or bran with fragments of mycelium and sclerotia, and was assessed to contain approximately 2,000 CFU/g by counting colonies after plating on PDA amended with 300 ppm streptomycin sulphate. Inocula of the five *T. phacorrhiza* isolates were applied separately, while inocula of five *T. incarnata* isolates were mixed together before application, as were inocula of five isolates of *T. ishikariensis*.

Preparation and viability of pellet inocula. Preparation of alginate pellets with mycelia or sclerotia of *T. phacorrhiza* followed the methods of Walker and Connick (22) and Lewis and Papavizas (12), with some modifications. To obtain mycelia, liquid cultures in 100 ml BASM broth (1% malt in potato broth; 19) were incubated on a rotary shaker (150 rpm) for 6 to 8 weeks at 4°C in the dark. Fungal biomass was separated from the liquid by filtration on Whatman No. 1 paper. To produce sclerotia, isolates were grown for 10 to 12 weeks in the dark at 10°C in 1-liter mason jars containing 200 g mixed grain medium. Sclerotia were harvested from cultures by hand. Fresh mycelia or sclerotia (4.0 g) were ground with 60 ml of 1.3% sodium alginate solution in a mini-sample blender (Fisher 14-509-18A, Unionville, Ontario, Canada) for 30 to 60 sec at high speed. The suspensions were then mixed with sterile distilled water plus clay and nutritional amendments to a final volume of 100 ml. The final concentration of the kaolin clay

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and the different nutritional amendments (cornmeal, oatmeal, wheat flour, wheat germ, or soya flour) was 10% each. The mixtures were blended for 30 s at high speed and then poured through a 1-ml pipette tip (3-mm-diameter opening) into a flask containing sterile 0.1 M CaCl_2 and 300 ppm streptomycin sulphate. Globules formed immediately upon contact with the CaCl_2 solution. These pellets were then collected by pouring the contents of the flask through a fine wire mesh screen. Pellets were air-dried in a laminar flow hood for 24 to 48 h at room temperature, and stored at 4°C for less than 2 months prior to use in field trials. Initial attempts to formulate pellets without the addition of clay were abandoned because pellets were not spherical after drying.

Pellets of five isolates of *T. phacorrhiza* formulated with 10% kaolin clay and 10% wheat flour were used immediately for a viability study. Pellets were placed into petri dishes (1,000 to 1,500 per plate) and sealed with parafilm for incubation at -15, 4, 10, and 25°C. Viability of pellets was tested by sampling 75 pellets per treatment per isolate at 1, 2, 4, 8, 12, 16, 32, and 64 weeks, and placing these pellets (25 per plate) onto PDA amended with 300 ppm streptomycin (17). Incidence of mycelial growth from the 25 pellets on each plate was assessed after incubation at 10°C for 2 to 3 weeks, and the data were subjected to analysis of variance. When significant treatment effects were found ($P \leq 0.05$), means were separated by the test of least significant difference ($P = 0.05$).

Effect of *T. phacorrhiza* formulation on gray snow mold. A field experiment at the Guelph Turfgrass Institute in Guelph, Ontario was conducted in winter 1995 to 1996 and repeated in winter 1996 to 1997.

The sand-based plot area had been seeded with Penncross creeping bent grass (*Agrostis palustris*) in June 1994, and cultural treatments were similar to those used for the maintenance of golf course putting greens in Ontario, but without fungicides. Just prior to snow fall in 1995 and 1996, plots (0.25 by 0.5 m) were treated with inoculum of mixed grain (200 g/m²), wheat bran (100 g/m²), or alginate pellets (20 g/m²) of five isolates of *T. phacorrhiza* separately. To ensure disease pressure, plots were also treated with mixed grain colonized by either *T. ishikariensis* or *T. incarnata* at a rate of 10 g/m². This rate of 10 g/m² of mixed-grain inocula was previously determined to cause abundant gray snow mold disease under our trial conditions (6,25). The controls used were as follows: (i) mixed-grain inocula of either *T. incarnata* or *T. ishikariensis* only, 10 g/m²; (ii) fungicide (Daconil 2787, 2 g a.i./m²) plus *T. incarnata* or *T. ishikariensis*, 10 g/m²; and (iii) no treatment.

The experiment was organized in a completely randomized design with four replications per treatment including five isolates of *T. phacorrhiza*, and separate sets of plots for *T. ishikariensis* or *T. incarnata*. Continuous snow cover throughout winter was maintained by piling snow from surrounding areas onto the plots. After snow melt, residual pellets were sampled from the plots to test viability by plating between 50 and 100 pellets per treatment on PDA amended with 300 ppm streptomycin. Also after snow melt, the Horsfall-Barratt rating scale (5) was used to estimate injury of the plots, where 1 = 99%, 2 = 95%, 3 = 91%, 4 = 82%, 5 = 62%, 6 = 38%, 7 = 18%, 8 = 9%, 9 = 5%, and 10 = 1% injury. Injury was an overall measure of incidence of foliar necrosis (proportion of leaf blades

discolored) and the severity of damage (change in color). Although other factors, such as abiotic winter kill and pink snow mold (caused by *Microdochium nivale*), also contributed to damage on the plots, the major component of winter injury in pathogen-inoculated plots at this site was gray snow mold (25). This was most evident when sclerotia of the pathogenic *Typhula* sp. were visible. For data analysis, the scale ratings of 1 to 10 were converted to percent injury values. These were subjected to analysis of variance. When significant treatment effects were found ($P \leq 0.05$), means were separated by the test of least significant difference ($P = 0.05$).

Pathogenicity of *T. phacorrhiza*. Field tests on pathogenicity of *T. phacorrhiza* were conducted at Guelph Turfgrass Institute in winter 1995 to 1996 and repeated in winter 1996 to 1997 on the following turf grasses: redbow (*Agrostis alba*), Southshore creeping bent grass (*A. palustris*), Highland colonial bent grass (*A. tenuis*), Rebel Junior tall fescue (*Festuca arundinacea*), Nordic hard fescue (*F. longifolia*), MX-86 sheep fescue (*F. ovina* subsp. *glauca*), Treasure chewings fescue (*F. rubra* subsp. *commutata*), Vista creeping red fescue (*F. rubra* subsp. *rubra*), Competitor perennial rye grass (*Lolium perenne*), Rubens Canada blue grass (*Poa compressa*), Regent Kentucky blue grass (*P. pratensis*), and rough blue grass (*P. trivialis*). Turf grasses in the plot area had been established in 1994 on a native sand loam soil. During the growing season, they were maintained at a 5-cm mowing height, irrigated as needed, and fertilized annually in spring and fall with 2 kg/100 m² of sulfur-coated urea (N-P-K: 25-4-10). No fungicides were used. In addition, two cultivars of winter wheat (*Triticum aestivum*), Harus and Rebecca, were planted in adjacent plots in September of each year as part of these trials.

Prior to snow fall, plots (0.5 by 0.5 m) were treated separately with 200 g/m² of mixed-grain inoculum of five isolates of *T. phacorrhiza*, along with the following checks and controls: (i) *T. incarnata*, 10 g/m² mixed-grain inocula; (ii) *T. ishikariensis*, 10 g/m² mixed-grain inocula; and (iii) no treatment. The experiment was organized in a completely randomized design with four replications. Winter injury was rated immediately after snow melt, and data analyzed as above.

RESULTS

Characteristics of *T. phacorrhiza* formulations. Pellets were initially 4 to 5 mm in diameter when freshly prepared, but dried down to 2 to 3 mm, remaining roughly spherical. Each liter of a mycelial or sclerotial alginate-clay mixture produced approximately 120 g of air-dried pellets. Greater viscosity of the initial suspension was observed with wheat germ, oatmeal, or cornmeal compared with wheat

Fig. 1. Effect of storage time at different temperatures on viability of isolates of *Typhula phacorrhiza* formulated as alginate pellets amended with 10% kaolin clay and 10% wheat flour. At each time interval (1, 2, 4, 8, 12, 16, 32, and 64 weeks), viability is the mean of five isolates with three replicate observations per isolate based on 25 pellets per observation. Bars above the X-axis represent pooled least significant difference values ($P = 0.05$).

flour or soya flour. Wheat or soya flour formulations passed through the funnel faster, and the pellets gelled more firmly and were more regularly spherical than with the other amendments.

Viability of pellet inocula. Pellets of *T. phacorrhiza* retained 90 to 100% viability after 16 weeks of storage at all temperatures (Fig. 1). Only pellets stored at -15°C maintained viability $>85\%$ up to 64 weeks. Pellets stored for 64 weeks at 4°C exhibited $>70\%$ viability. After 64 weeks, only 20 to 35% viability was detected for pellets stored at 10°C , and viability was even lower at 25°C . There were statistically significant variations among the five isolates, particularly at higher temperatures and longer storage times, but these differences were minor compared to the differences between temperatures (Fig. 1).

Effect of *T. phacorrhiza* formulations on gray snow mold. In plots treated only with mixed-grain inocula of *T. ishikariensis*

or *T. incarnata*, severe gray snow mold symptoms were visible after snow melt in early 1996 and 1997 (Table 1). Although up to five isolates of *T. phacorrhiza* were used for the different formulations, none differed significantly from isolate Tp94810 in either 1996 or 1997, so only the data of this isolate is presented (Table 1). In both years, snow cover was maintained on the plots for the requisite 3 months. There were no significant differences in suppression of gray snow mold among mixed-grain, wheat bran, and alginate formulations, between mycelial and sclerotial pellet formulations, or even among formulations with different nutritional amendments. In each year, intact pellets could be observed on the plots after snow melt, and the viability of 50 to 100 residual pellets per treatment recovered after snow melt was above 90%.

Susceptibility of plant species. There were significant differences between host

species for the amount of winter injury due to snow mold pathogens (Table 2). In both years, *A. tenuis* had the highest injury from either pathogen; however, this was not significantly different from the injury of eight other host species in either year. In 1996, *P. pratensis* showed the least snow mold injury, but not in 1997. Overall, *F. rubra* subsp. *commutata* showed the least winter injury induced by either pathogen, but this did not differ significantly from that of four other host species. For winter injury on untreated control plots, *A. tenuis* again showed the highest injury, followed by *L. perenne*, *A. alba*, and *F. arundinacea*, but again injury was not always significantly different from that of other cultivars.

Pathogenicity of *T. phacorrhiza*. The *T. phacorrhiza* treatments were composed of five isolates with four replicate plots per isolate per year. Except for two instances, there were no significant differences among *T. phacorrhiza* isolates, and so the

Table 1. Formulation of *Typhula* spp. and suppression of gray snow mold of creeping bent grass by *T. phacorrhiza*

Treatment ^w	Formulation	Inoculum ^x	Amendments ^y	Rate	Winter injury ^v	
					1996	1997
Tp94810	Mixed grains	M + S	... ^z	200 g/m ²	28 bc	23 bc
Tp94810	Wheat bran	M + S	...	100 g/m ²	23 bc	28 bc
Tp94810	Alginate	S	Kaolin Clay (K)	20 g/m ²	23 bc	28 bc
Tp94810	Alginate	S	K + cornmeal	20 g/m ²	28 bc	28 bc
Tp94810	Alginate	S	K + oatmeal	20 g/m ²	23 bc	18 c
Tp94810	Alginate	S	K + wheat flour	20 g/m ²	18 c	33 b
Tp94810	Alginate	S	K + wheat germ	20 g/m ²	23 bc	23 bc
Tp94810	Alginate	S	K + soya flour	20 g/m ²	28 bc	28 bc
Tp94810	Alginate	M	K + wheat flour	20 g/m ²	28 bc	23 bc
No treatment	...	...	...	...	21 bc	18 c
Tish	Mixed grains	M + S	...	10 g/m ²	92 a	84 a
Tinc	Mixed grains	M + S	...	10 g/m ²	91 a	87 a
Fungicide	Daconil 2787	...	...	2 g a.i./m ²	28 bc	28 bc

^v Winter injury values followed by the same letter are not significantly different at $P = 0.05$ (least significant difference) and are the mean of eight plots (pooled results for *T. ishikariensis* and *T. incarnata*).

^w Tp = *Typhula phacorrhiza*, Tish = *T. ishikariensis* and Tinc = *T. incarnata*. Inocula of Tish and Tinc were applied in December 1995 and 1996 to all except the untreated control plots.

^x M = mycelium and S = sclerotia.

^y Amendments were added to fungal suspensions for a final 10% concentration (wt/vol) of each prior to forming pellets.

^z ... = not applicable.

Table 2. Pathogenicity of *Typhula* spp. to various turf grasses and winter wheat assessed as winter injury after snow melt in 1996 and 1997

Species	Cultivar	Winter injury 1996 (%) ^z					Winter injury 1997 (%)				
		None	Tp	Tinc	Tish	LSD _{row}	None	Tp	Tinc	Tish	LSD _{row}
<i>Agrostis alba</i>		61	58	93	95	19	39	38	92	90	18
<i>A. palustris</i>	Southshore	16	24	87	90	12	26	25	91	90	13
<i>A. tenuis</i>	Highland	92	92	99	99	3	50	53	96	95	16
<i>Festuca arundinacea</i>	Rebel Jr.	33	41	93	93	14	44	36	96	96	18
<i>F. longifolia</i>	Nordic	8	15	73	74	14	9	12	84	91	5
<i>F. ovina</i>	MX-86	23	37	87	87	13	33	15	79	79	20
<i>F. rubra commutata</i>	Treasure	8	17	67	62	10	14	11	66	68	14
<i>F. rubra rubra</i>	Vista	14	21	67	71	14	14	15	82	88	10
<i>Lolium perenne</i>	Competitor	92	91	96	96	4	38	45	94	73	24
<i>Poa compressa</i>	Rubens	9	12	67	72	8	18	21	71	90	17
<i>P. pratensis</i>	Regent	7	10	50	38	7	16	21	90	79	10
<i>P. trivialis</i>		23	37	87	94	17	23	25	92	84	12
<i>Triticum aestivum</i>	Harus	23	30	90	92	12	23	28	90	93	12
<i>T. aestivum</i>	Rebecca	14	21	91	90	11	33	33	89	94	13
LSD _{column} ($P = 0.05$)		11	6	13	13		21	7	18	19	

^z Winter injury values are the mean of four replicates per treatment. None = untreated control, Tp = *T. phacorrhiza*, Tinc = *T. incarnata*, Tish = *T. ishikariensis*, and LSD = least significant difference. For *T. phacorrhiza*, the results were from five isolates were combined. Inocula were applied as infested mixed grains prior to snow fall.

data of the *T. phacorrhiza* treatments were pooled (Table 2). *T. phacorrhiza* treatments did not incite greater winter injury than untreated plots except for one instance, with *F. ovina* in 1996 (Table 2). Although significant at $P = 0.05$, the difference was not significant at $P = 0.01$. When data of all cultivars were pooled, there was a significant difference in winter injury between untreated versus *T. phacorrhiza*-treated plots in 1996 (30 versus 36%), but not in 1997 (both 27%). In nearly all cases, pathogen-treated plots showed significantly greater injury than untreated or *T. phacorrhiza*-treated plots, with an average difference of 53% between pathogen and biocontrol plots.

DISCUSSION

Formulations of fungal propagules in sodium alginate have been shown to be an effective means for application of biocontrol agents (4,7,12,15). Preliminary work showed that isolates of *T. phacorrhiza* could be formulated as alginate pellets (9). Our results confirm that alginate pellets can be used as an effective formulation for the application of *T. phacorrhiza*. The disease-suppressive potential of *T. phacorrhiza* isolates in pellets at low rates (20 g/m²) was similar to higher rates of air-dried preparations of mixed grain (200 g/m²) or wheat bran (100 g/m²). One of the major advantages of pellet formulation over mixed grain and wheat bran is that it can provide inoculum with a minimum of bulk and can be distributed and incorporated using standard turf grass maintenance equipment such as fertilizer spreaders.

Some food bases, such as ground wheat bran and cornmeal, have been reported to enable the propagules of biocontrol fungi such as *Trichoderma* and *Gliocladium* spp. to germinate and colonize the soil (13,23). In this study, the amendments did not affect the ability of *T. phacorrhiza* isolates to suppress gray snow mold. These amendments might play some role as a nutrient base for the antagonist during the early stages of proliferation after application to the field. Perhaps if application rates had been reduced, some differences between formulations may have been detected.

The recovery of intact pellets after snow melt with >90% viability indicated that the fungus in alginate pellets could survive through a winter under field conditions. Under lab conditions, there was considerable loss of viability after 64 weeks at 10 and 25°C; however, the pellets stored at 4 or -15°C maintained high viability. More research on formulation stability, shelf life, and particle size to increase pellet effectiveness is required.

There were significant differences between host species for winter injury in the different treatments. Different species are

known to vary in susceptibility to gray snow mold injury. Vargas (21) ranks major turf species as follows (most to least susceptible): perennial rye grass, creeping bent grass, Kentucky blue grass, and red fescues. However, within each species, there can be very large differences in susceptibility between cultivars (8,21). Because there was only one cultivar per grass species, we cannot make strong conclusions about the relative susceptibility of different species to gray snow mold from our data.

From field trials between 1994 and 1997, we observed that creeping bent grass treated with *T. phacorrhiza* alone did not exhibit significantly greater damage than untreated controls (25), which is in agreement with past observations that *T. phacorrhiza* is saprophytic (1,16) and not pathogenic on grasses (2,3). The results from the current study showed that isolates of *T. phacorrhiza* collected from corn fields were not pathogenic to various turf grasses or winter wheat, even when applied at high rates. Schneider and Seaman (18) reported that isolates of *T. phacorrhiza* could cause damage to winter wheat in Canada. Possibly the isolates of *T. phacorrhiza* collected from wheat fields differ in pathogenicity from our isolates from corn fields. The *T. phacorrhiza* isolates used in this study were selected after a season of field testing for suppression of gray snow mold. Some of the isolates which showed no suppression in those tests may have characteristics quite different than the ones studied here. Further investigations on geographically diverse collections of *T. phacorrhiza* from different hosts may be required before final conclusions on the plant pathogenicity of *T. phacorrhiza* as a species can be drawn.

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