Efficacy of *Typhula phacorrhiza* as a biocontrol agent of grey snow mould of creeping bentgrass

C. Wu, T. Hsiang, L. Yang, and L.X. Liu

Abstract: Isolates of the fungus *Typhula phacorrhiza* Fries (TP) were evaluated in field tests over a 3-year period for suppression of grey snow mould caused by *Typhula ishikariensis* Imai (Tish) and *Typhula incarnata* Lasch ex Fr. (Tinc). Isolates of TP were collected across southern Ontario in the spring of 1994. In December 1994, 46 of these isolates, which had been cultured on mixed grains, were applied to creeping bent grass (*Agrostis stolonifera* L.) at a rate of 200 g/m^2 (4×10^5 colony forming units (cfu)/m^2) with inoculum of Tish or Tinc at 10 g/m^2 (2×10^4 cfu/m^2). In December 1995, 30 selected TP isolates were inoculated onto a new set of plots along with grey snow mould fungi. In November 1996, 22 of these isolates were re-inoculated onto the 1995 plots. All plots were rated for injury after snowmelt, 1995–1997. Isolates of TP varied significantly in their ability to suppress disease. No strong correlations were found between in vitro growth characteristics and field performance; however, significant positive correlations were found between the disease suppression trials for the 3 years, with several isolates showing statistically significant control of grey snow mould equal to a fungicide treatment.

Key words: biocontrol, turfgrass disease, fungi.

Résumé : Au cours d'expériences conduites aux champs et étalées sur 3 années, les auteurs ont évalué la capacité d'isolats du champignon *Typhula phacorrhiza* Fries (TP) à supprimer les champignons causant la moisissure nivéale grise *Typhula ishikariensis* Imai (Tish) et *Typhula incarnata* Lasch ex Fr. (Tinc). Les isolats du TP ont été cueillis dans le sud de l'Ontario au printemps 1994. En décembre 1994, 46 de ces isolats, préalablement cultivés sur mélange de grains, ont été appliqués sur l'agrostide courbé (*Agrostis stolonifera* L.) à un taux de 200 g/m^2 (4×10^5 cfu/m^2), avec des inoculums de Tish ou Tinc à 10 g/m^2 (2×10^4 cfu/m^2). En décembre 1995, les auteurs ont inoculé 30 isolats du TP sur un nouvel ensemble de parcelles, en même temps que les champignons de la moisissure nivéale grise. En novembre 1996, ils ont ré-inoculé 22 de ces isolats sur les parcelles traitées en 1995. En 1995–1997, ils ont évalué les dommages après la fonte des neiges. Les isolats du TP varient significativement dans leur capacité à supprimer la maladie. Il n'y a pas de forte corrélation entre les caractéristiques de croissance in vitro et le comportement aux champs; cependant, on observe des corrélations positives significatives entre les essais de suppression de la maladie des 3 années, plusieurs isolats montrant une suppression statistiquement significative de la moisissure nivéale grise, égale à celle obtenue avec traitement fongicide.

Mots clés : lutte biologique, maladie de la tourbe, champignons.

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Introduction

Grey snow mould, also known as Typhula blight, is a common disease of turfgrasses in areas where there are over 90 days of continuous snow cover during the winter (Smiley et al. 1992). This disease is caused by *Typhula incarnata* Lasch ex Fr. or *Typhula ishikariensis* Imai (Fushtey 1980) and has been commonly controlled in Canada with fungicides containing mercury or quintozene (Smith 1987). Although control of this disease can be achieved with such chemicals, the cost of applying fungicides coupled with health and environmental concerns requires investigation of alternative management approaches (Burpee et al. 1987).

Many attempts have been made to examine biological control of snow mould diseases as an alternative to fungicides (Huber and McKay 1968; Harder and Troll 1973; Kaye 1985; Smith and Davison 1979; Smith 1981). Although laboratory studies on biological control of grey snow mould have been encouraging, field tests are limited (Burpee 1994). Burpee et al. (1987) were the first to examine biological control of grey snow mould with *Typhula phacorrhiza* Fries. Initially, they assumed *T. phacorrhiza* to be an unreported pathogen of turfgrass but later discovered that this *Typhula* species was not pathogenic to creeping bent grass (*Agrostis stolonifera* L.) in field tests with artificial inoculation. They also found that it had the ability to grow under snow and suppress the development of grey snow mould (Lawton et al. 1986; Burpee et al. 1987; Lawton and Burpee 1990). Their research has been frequently cited as an example of biological control on turfgrass (Nelson 1992; Burpee 1994), but there were limitations in the number of isolates or

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the number of field seasons of testing. More recently, isolates of a fungus like *T. phacorrhiza* was found to suppress snow mould of non-turf-type perennial ryegrass (*Lolium perenne* L.) in Japan (Matsumoto and Tajimi 1992).

Typhula phacorrhiza is a clavulate basidiomycete reported from northern temperate zones. It is considered to be a psychrophilic saprotroph (Remsberg 1940; Bruehl and Cunfer 1975; Berthier 1976; Burpee et al. 1987) and can be observed frequently on corn (*Zea mays* L.) residues and other organic debris after at least 80 days of snow cover (Burpee et al. 1987). Schneider and Seaman (1986, 1988) reported that some *T. phacorrhiza* isolates were pathogenic on wheat (*Triticum aestivum* L.) based on controlled environment studies and a field study with a single *T. phacorrhiza* isolate.

Since isolates of *T. phacorrhiza* have been found to exhibit potential as biocontrol agents (Burpee et al. 1987; Matsumoto and Tajimi 1992), further studies are desirable to achieve the goal of a commercially viable grey snow mould control product. Several areas were identified by Burpee (1994) or Matsumoto and Tajimi (1992) as requiring further research: (*i*) screening of new isolates of *T. phacorrhiza* that can exhibit greater potential for disease suppression; (*ii*) determining pathogenicity of *T. phacorrhiza* toward various amenity turfgrasses and wheat; (*iii*) evaluating long-term persistence of *T. phacorrhiza* in turfgrass; and (*iv*) testing formulations of *T. phacorrhiza* for application to turfgrass. The objective of this research was to collect and screen numerous isolates of *T. phacorrhiza* for grey snow mould suppression. Questions addressed in this study included the following: (*i*) Is there variation in field suppressiveness of *T. phacorrhiza* toward grey snow mould? (*ii*) Can in vitro growth or sclerotial production predict field efficacy? Preliminary reports have been presented elsewhere (Hsiang et al. 1995; Wu et al. 1996).

Materials and methods

Collection of *Typhula phacorrhiza* isolates

Corn fields in south-central Ontario were examined for the presence of sclerotia of *T. phacorrhiza* soon after snowmelt in April and May of 1994 and 1995. Samples of corn debris harbouring sclerotia of *T. phacorrhiza* were randomly collected and placed into plastic bags. In most cases, up to 10 samples were collected from each field, with the distance between any two samples greater than 100 m. Four sclerotia from each sample were surface sterilized for 30 s in 1% sodium hypochlorite followed by three 1-min rinses in sterile distilled water. Each sclerotium was then bisected and plated onto potato dextrose agar (PDA) amended with 0.1% yeast extract (PYDA). The plates were incubated in the dark at 10°C for 2 weeks. An agar plug (5 mm diam) from the edge of a colony was transferred to a fresh PYDA plate, and incubated in the dark at 10°C. Sclerotia began forming within 30 days after transfer. Isolates of *T. phacorrhiza* were identified by colony morphology and the presence of clamp connections, and confirmed as *T. phacorrhiza* by the rind patterns of mature sclerotia as described by Schneider and Seaman (1986). Confirmed isolates were subcultured once again to ensure purity, and stored on PDA slants at 10°C.

Initial screening of *Typhula phacorrhiza* isolates

Isolates of *T. phacorrhiza* were evaluated for growth and sclerotial production on both BASM (1% malt in potato agar; Smith 1981) and mixed grains (equal weights of wheat, oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), and corn; Lawton and Burpee 1990). A 5 mm diameter agar plug taken from an actively growing colony was transferred to a 20-mL tube (1.5 cm diameter by 12 cm length) containing either autoclaved mixed grains or 6 mL of BASM (allowed to solidify horizontally). All tubes were incubated in the dark at 10°C with four replications per isolate on each medium. Beginning on day 6 after inoculation, hyphal growth was measured every 3 days until day 21 and then every 6 days up to day 57. After 10 weeks of incubation, the test tubes were assessed for sclerotial production.

On BASM, sclerotia tended to form on the agar surface and could be easily counted. For the mixed grain medium, sclerotia were much more abundant and some formed within the medium. To account for these hidden sclerotia and to reduce the labour involved, we devised a rating scale for sclerotial number. We visually rated 40 tubes with a range of visible sclerotia and then enumerated all sclerotia present in these tubes. The 0–9 rating scale with 0 being no sclerotia and 9 being 1300 sclerotia was transformed to sclerotial number for analysis. Because of logistical reasons, not all isolates could be tested in the field, so a sampling of isolates with various combinations of growth rate and sclerotial production were selected for field tests.

Isolates of grey snow mould fungi

Five isolates each of *T. ishikariensis* and *T. incarnata* were used to produce grey snow mould inoculum for the field studies described below. These isolates originated from southern Ontario and were obtained from stock cultures in this laboratory. They have been used previously as inoculum in fungicide tests involving grey snow mould (e.g., Hsiang and Cook 1995).

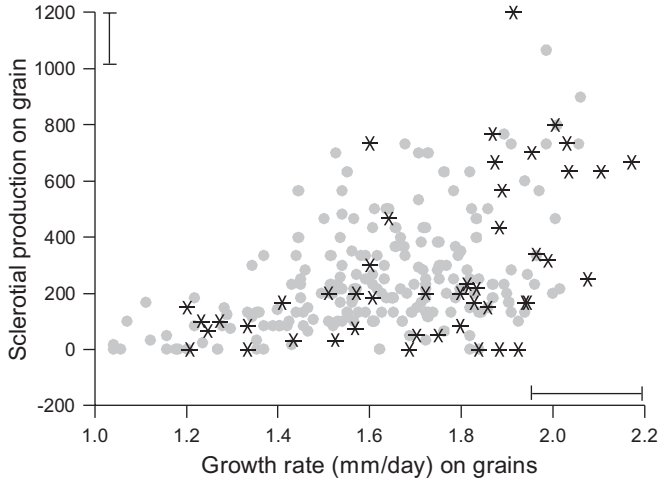
Preparation of inocula

Mixed grains were used to grow inoculum and serve as the carrier for biocontrol isolates and for grey snow mould fungi in field tests. Briefly, 200 g mixed grains plus 300 mL distilled water in a 1-L mason jar were autoclaved twice for 20 min 24 h apart. Five to 10 disks taken from the colony margin of an isolate growing on PDA were transferred to the jars and incubated for 7–8 weeks at 10°C. Immediately before use, the grain inoculum was air-dried for 36 h and chopped into small particles with a domestic blender. Inoculum consisted primarily of grains with fragments of fungal mycelium and sclerotia and was assessed to contain 2000 colony forming units (cfu) per gram for both biocontrol and pathogenic isolates at time of inoculation.

Winter 1994–1995 suppression trials

Tests on grey snow mould suppression by isolates of *T. phacorrhiza* were conducted at the Guelph Turfgrass Institute (GTI) in Ontario, Canada. Of 264 isolates collected in the spring of 1994, forty-six isolates of *T. phacorrhiza* were selected for field testing in late fall of 1994. Field plots were established on a creeping bent grass green, which had been built in spring 1994 and was seeded with Penncross creeping bent grass on 7 June 1994. Turfgrass maintenance was similar to that used for golf course putting greens in Ontario with irrigation as needed and a mowing height of 7 mm. No previous research on snow moulds had been conducted on these plots nor had any fungicides been applied. The experimental design consisted of a completely randomized design with four replications per treatment resulting in 200 plots. Each plot measured 0.5 × 0.5 m, and the turf was carefully inoculated by hand with 200 g/m^2 inoculum of *T. phacorrhiza* (4×10^5 cfu/m^2). Lawton and Burpee (1990) found that at least 200 g/m^2 of

Fig. 1. Scatterplot of growth rate by number of sclerotia produced on mixed grain media after 70 days at 10°C. *Typhula phacorrhiza* isolates chosen for field testing are represented by stars, and circles show the other isolates. LSD values (p = 0.05) are presented as bars for sclerotia number near the Y axis and for growth rate near the X axis.



mixed-grain inocula were needed to provide suppression equal to standard rates of the fungicide quintozene.

Each 0.5 × 0.5 m plot was split, and one half was inoculated with *T. ishikariensis*, and the other, with *T. incarnata* at a rate of 10 g/m^2 (2 × 10^4 cfu/m^2). Three checks and a control were used as follows: (*i*) fungicide (Daconil 2787, 2 g a.i./m^2) plus *T. incarnata* or *T. ishikariensis* (10 g/m^2); (*ii*) *T. ishikariensis* or *T. incarnata* only (10 g/m^2); (*iii*) *T. phacorrhiza* only (isolate 23BDS; 200 g/m^2); and (*iv*) untreated control. Plots were inoculated in early December prior to snowfall. A snow fence was placed around the plots, and continuous snow cover was maintained for 3.5 months during winter by piling snow from surrounding areas onto the fenced plots.

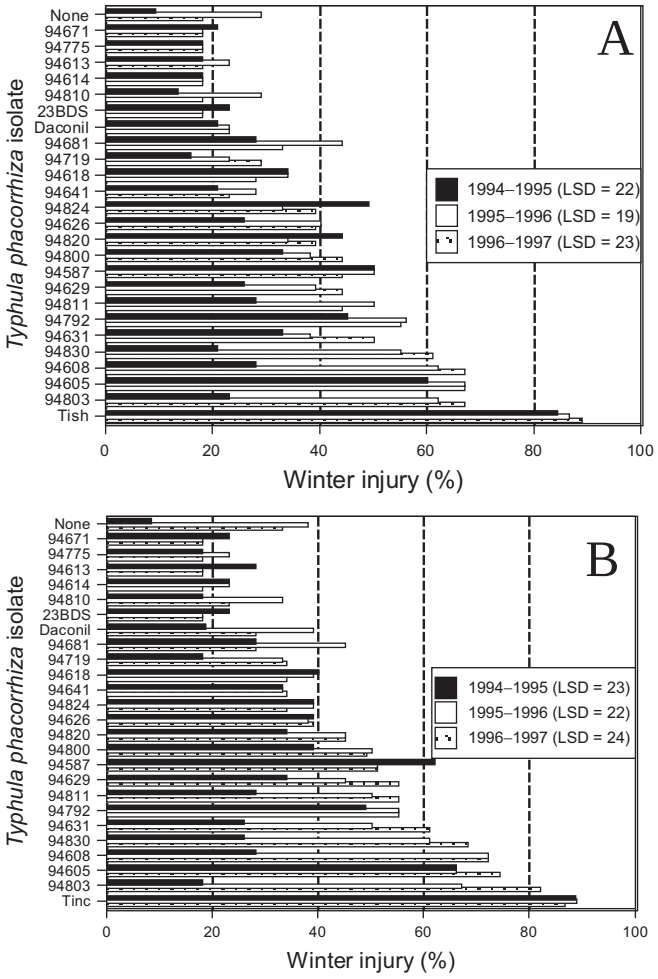
The Horsfall–Barratt rating scale (Horsfall and Cowling 1978) was used to estimate percent winter injury in the plots (1, 99%; 2, 95%; 3, 91%; 4, 82%; 5, 62%; 6, 38%; 7, 18%; 8, 9%; 9, 5%; 10, 1% winter injury). Although other factors, such as abiotic winter kill and pink snow mould (caused by *Microdochium nivale* (Fr.) Samuels & Hallett), also contributed to damage on the plots, the major component of winter injury was grey snow mould. This was most evident when sclerotia of the pathogenic *Typhula* species were visible. Ratings were taken immediately after snow melt and were transformed into percent injury prior to statistical analysis.

Winter 1995–1996 and 1996–1997 suppression trials

Thirty of the 46 isolates used in the 1994–1995 suppression trial were chosen for the second round of testing in winter 1995–1996. New plots were established near the 1994–1995 suppression plots using the same experimental design and controls as the 1994–1995 trial. The area of these new plots had not been previously used for any research nor had any fungicides been applied previously. In early December 1995, plots were inoculated with *T. phacorrhiza* and the appropriate pathogen. Continuous snow cover on the plots was maintained for 3.5 months by piling snow, and the plots were evaluated for winter injury after snow melt in spring 1996 as described above.

In early December 1996, the 1995–1996 plots were re-inoculated with isolates of *T. phacorrhiza*, as well as the appropriate pathogen. Twenty-two isolates of the original 46 from 1994 were cultured and applied as described above. Continuous snow cover on the plots for 3.5 months was maintained by piling snow, and the plots were evaluated for winter injury after snow melt in spring 1997 as described above.

Fig. 2. Mean winter injury on creeping bent grass plots inoculated in fall of 1994–1996 with *T. phacorrhiza* and either (A) *T. ishikariensis* (Tish) or (B) *T. incarnata* (Tinc), except for control and check treatments: None, untreated control; 23BDS, *T. phacorrhiza* isolate alone; Daconil, fungicide check applied at 2 g active ingredient/m^2; Tish or Tinc, pathogen-inoculated plots without biocontrol agent. Each value is the mean of four replicate plots assessed in spring after snowmelt. Only the data involving the 22 TP isolates common to trials over the 3 years are presented.



Data analysis

All ratings were converted to estimates of sclerotial number on grains or percent winter injury, and data were subject to analysis of variance. When treatment effects were significant in the analysis of variance ($p \leq 0.05$), means were separated by the test of least significant difference (LSD, p = 0.05). These tests as well as correlation and multiple linear regression analyses were performed using SAS® version 6.04.

Results

Collection and selection of *T. phacorrhiza*

In 1994, we surveyed 35 corn fields throughout south-central Ontario and collected 264 isolates of

Table 1. Pearson correlation coefficients (r) of winter injury values from suppression trials of grey snow mould by *Typhula phacorrhiza*.

		1994–1995		1995–1996		1996–1997	
Trial[a]	Species[b]	Tish	Tinc	Tish	Tinc	Tish	Tinc
1994–1995	Tish	1.00	0.91	0.54	0.56	0.51	0.43
	Tinc		1.00	0.53	0.48	0.38[c]	0.32[c]
1995–1996	Tish			1.00	0.94	0.93	0.90
	Tinc				1.00	0.96	0.95
1996–1997	Tish					1.00	0.97
	Tinc						1.00

[a]Plots were inoculated with biocontrol isolates (*T. phacorrhiza*) and pathogens (*T. ishikariensis* or *T. incarnata*) in late fall of each year. In early spring of each following year, plots were rated for winter injury. Each value is a correlation between isolate means for winter injury data based on four replicate plots.
[b]Tish, *T. iskikariensis*; Tinc, *T. incarnata*.
[c]Except for these coefficients, all others were statistically significant at $p < 0.05$.

Table 2. Pearson correlation coefficients (r) of winter injury values from grey snow mould suppression trials against in vitro growth characteristics of *Typhula phacorrhiza*.

		Growth rate (mm/day)		Sclerotial no.	
Trial[a]	Species[b]	BASM	Grains	BASM	Grains
1994–1995	Tish	–0.21	0.24	0.38[c]	0.40[c]
	Tinc	–0.26	0.19	0.35[c]	0.40[c]
1995–1996	Tish	–0.27	–0.04	0.02	–0.09
	Tinc	–0.29	–0.04	–0.01	–0.11
1996–1997	Tish	0.25	–0.25	0.04	–0.29
	Tinc	0.38	–0.29	0.06	–0.23

[a]Plots were inoculated with biocontrol isolates (*T. phacorrhiza*) and pathogens (*T. ishikariensis* or *T. incarnata*) in late fall of each year. Four replicate plots per treatment were rated in the following spring for winter injury. Growth rate and sclerotial number were assessed during 70 days of incubation in the dark at 10°C with three replicates per isolate for each medium (BASM, 1% malt potato agar; grains, mixed grain medium). Means were used in correlation analysis.
[b]Tish, *T. ishkariensis*; Tinc, *T. incarnata*.
[c]Statistically significant at $p < 0.05$.

T. phacorrhiza. Significant differences ($p < 0.05$) in linear growth and sclerotial production on two media were found among isolates (Fig. 1). Sclerotial number on grain was significantly correlated with growth rate on grain ($r = 0.48$, $p < 0.01$) and sclerotial number on BASM ($r = 0.626$, $p < 0.01$). Forty-six isolates with various combinations of growth rate and sclerotial production (Fig. 1) were chosen for field suppression trials in winter 1994–1995.

Winter 1994–1995 suppression trials

In control plots receiving no treatment, the mean winter injury severity on 20 March 1995 was 9% (*T. ishikariensis*) or 8% (*T. incarnata*), which were the lowest among their respective treatments; however, plots inoculated with pathogens alone had winter injury of 84% (*T. ishikariensis*; Fig. 2A) or 88% (*T. incarnata*; Fig. 2B).

Isolates of *T. phacorrhiza* varied significantly in their ability to suppress grey snow mould (Fig. 2). For *T. phacorrhiza* isolates on *T. ishikariensis* plots, winter injury differed between the highest and lowest values by over 70% with an LSD ($p = 0.05$) of 22 (Fig. 2A). For *T. incarnata* plots, there was a difference of over 60% with an LSD ($p = 0.05$) of 23 (Fig. 2B). The biocontrol isolates had a correlation coefficient (r) of 0.91 ($p < 0.01$; Table 1) between the winter injury values of the *T. ishikariensis* versus *T. incarnata* trials in 1994–1995. For five isolates of *T. phacorrhiza*, the winter injury values did not differ significantly ($p = 0.05$) from the pathogen-inoculated checks, whereas 27 (against *T. ishikariensis*) and 29 (against *T. incarnata*) isolates of *T. phacorrhiza* had winter injury values that did not differ significantly from that of the fungicide treatment. Of these, seven (against *T. ishikariensis*) and four (against *T. incarnata*) had mean winter-injury values lower than the mean of the fungicide-treated plots.

Winter 1995–1996 suppression trials

On 20 March 1996, mean winter injury values of *T. phacorrhiza* treatments ranged from 18% to 67% for plots treated with *T. ishikariensis* (Fig. 2A) and from 18 to 72% for plots treated with *T. incarnata* (Fig. 2B). Correlation was high between the two trials (*T. ishikariensis* vs. *T. incarnata*) with $r = 0.94$ ($p < 0.01$; Table 1). The results of 1995–1996 were significantly correlated with those of 1994–1995 with a mean correlation coefficient of 0.53 (Table 1).

Winter 1996–1997 suppression trials

On 1 April 1997, mean winter injury values of *T. phacorrhiza* treatments ranged from 18 to 67% for plots treated with *T. ishikariensis* (Fig. 2A) and from 18 to 82% for plots treated with *T. incarnata* (Fig. 2B). Correlations were high between the results of the two trials (*T. ishikariensis* vs. *T. incarnata*) with $r = 0.97$ ($p < 0.01$; Table 1). Results of the 1996–1997 suppression trials showed very high correlations with those of the 1995–1996 trials with an average $r = 0.95$. Correlations to the results of the 1994–1995 trials were much lower (average $r = 0.41$), and two of the four coefficients were not statistically significant (Table 1).

Growth characteristics and field performance

In correlations between winter injury and in vitro growth characteristics, the only statistically significant relationships were found between the results of the 1994–1995 trials and sclerotial number (Table 2). Even though they were significant, the coefficients were moderately low ranging from 0.35 to 0.40 (Table 2).

Discussion

This study confirmed the findings of Burpee et al. (1987) and Matsumoto and Tajimi (1992) that grey snow mould could be effectively controlled by some isolates of *T. phacorrhiza*. Lawton and Burpee (1988) found differences among 37 isolates of *T. phacorrhiza* from one season of testing on plots that had not been inoculated with the pathogen. Matsumoto and Tajimi (1992) reported that some isolates, considered to be *T. phacorrhiza*, suppressed disease caused

by *T. ishikariensis* on perennial ryegrass, but suppression of *T. incarnata* by *T. phacorrhiza* was not tested.

In our 3-year field study, isolates of *T. phacorrhiza* were found to vary significantly in their ability to suppress the development of grey snow mould. Some *T. phacorrhiza* isolates did not provide suppression of grey snow mould, and their ratings did not differ significantly from plots inoculated with the pathogen alone. There were also some isolates that provided an intermediate level of suppression, but even though significantly different from the pathogen-inoculated controls, the level of suppression was likely not aesthetically acceptable. More than five isolates of *T. phacorrhiza* showed statistically significant disease suppression over three winters, with low winter injury ratings averaging 17% for *T. ishikariensis* plots and 19% for *T. incarnata* plots. These isolates provided suppression to the same level as the fungicide-treated controls in all suppression trials.

Because field testing of *T. phacorrhiza* is limited to periods with several months of snow cover, we could not screen all the isolates in our collection. We attempted to pinpoint some laboratory characteristics as indicators of field performance. We hypothesized that growth rate and sclerotial production of *T. phacorrhiza* were related to ability to suppress grey snow mould. The isolates used in the field tests were selected to represent a range of growth rates and sclerotial production. However, the only significant correlations were between sclerotial number and the winter injury values of 1994–1995, although at a low level ($r < 0.4$). We also attempted multiple linear regression of field performance with stepwise selection of growth variables. Some statistically significant equations were generated, but the significant autocorrelation between the dependent variables poses problems for this type of analysis (Snedecor and Cochran 1989). In any case, the coefficient of determination (r^2) values were all low, and variables were not consistently represented. We conclude that these particular in vitro growth characteristics are not good predictors of field performance.

Because of the limited opportunities to conduct field tests throughout the year, additional research is needed to develop an in vitro screening system or to discover attributes that may be good predictors of field performance. Burpee et al. (1987) reported that the mechanism by which *T. phacorrhiza* suppresses grey snow mould disease is not a direct effect such as hyperparasitism or cellular lysis. They suggested that nutrient competition may be the mode of action. Our preliminary results (Wu and Hsiang 1997) support this hypothesis. Elucidation of the mode of action and the substrate or site for which these organisms compete may allow discovery of attributes that are good predictors of field performance.

The Horsfall–Barratt rating system was previously employed to estimate grey snow mould intensity by Burpee et al. (1987). They measured disease severity in the field by rating percentage necrotic foliage per plot after close inspection. We used the same rating system in this study to estimate winter injury rather than disease severity. In some cases, obvious signs of disease such as mycelia or sclerotia were not present, and disease ratings could have been confounded by other damaging factors such as winter stress or pink snow mould. Abiotic winter injury is commonly observed locally, and according to our data, it may account for a baseline of around 15–20% of the overall winter injury ratings, since very few ratings were below 15%. Although we did not directly test it, the data implied that the selected *T. phacorrhiza* isolates also provided at least some suppression of pink snow mould. Burpee et al. (1987) have speculated that *Typhula* species may colonize leaf blades through natural openings or wounds, and thus, *T. phacorrhiza* may inhibit other snow mould fungi that infect similarly.

In conclusion, we found that there is a wide variation in the ability of *T. phacorrhiza* isolates to suppress grey snow mould and that some *T. phacorrhiza* isolates can provide disease control equivalent to a standard commercial fungicide as tested over three field seasons. Among the remaining 200+ isolates collected in 1994 and the hundreds collected since then, there may be even better performing isolates, but the determination would require labourious field testing or development of predictors or in vitro assays. Other research remains to be conducted on appropriate formulation and delivery systems, nontarget pathogenicity, and residual efficacy of *T. phacorrhiza*.

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