Effects of the Mycoparasite *Sphaerellopsis filum* on Overwintering Survival of Stem Rust in Perennial Ryegrass

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Abstract

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Sphaerellopsis filum is a mycoparasite of *Puccinia graminis* subsp. *graminicola*, a rust fungus that causes widespread crop damage on perennial ryegrass grown for seed. In observations taken over the winter months, *S. filum* was found naturally colonizing 2% of *P. graminis* subsp. *graminicola* uredinia on first-year plantings and 25% of the uredinia on second-year plantings of perennial ryegrass. In controlled experiments conducted in glasshouses and growth chambers, *S. filum* applied to rust-inoculated plants reduced the lifetime spore production of *P. graminis* subsp. *graminicola* pustules by half, from 39,000 to 18,000 spores/pustule . Mist duration, temperature, and *P. graminis* subsp. *graminicola* pustule age at the time of *S. filum* inoculation had significant effects on the proportion of *P. graminis* subsp. *graminicola* pustules infected by *S. filum*. Fifty percent of all *P. graminis* subsp. *graminicola* pustules were infected when *S. filum* was inoculated onto erumpent pustules and incubated above 5°C for 48 h while exposed to mist. Plants inoculated with both fungi under controlled conditions and then planted into the field had a significantly greater proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum,* and a significantly reduced *P. graminis* subsp. *graminicola* overwintering population, compared with plants inoculated with *P. graminis* subsp. *graminicola* only. First-year stands of perennial ryegrass treated in the field with monthly applications of *S. filum* had more than 10 times the proportion of pustules infected with *S. filum* and 50% less *P. graminis* subsp. *graminicola* disease compared with the nontreated controls. In comparison, plants with one application of fungicide during the winter had 98% lower *P. graminis* subsp. *graminicola* severity than the *P. graminis* subsp. *graminicola*-only control There were no effects of *S. filum* or fungicide application on rust severity in 2- or 3-year-old perennial ryegrass stands.

Perennial ryegrass (*Lolium perenne* L.) is a cool-season seed crop intensively cultivated in the Pacific Northwest of the United States, New Zealand, and parts of Europe (3,37,38). The most damaging disease of perennial ryegrass in the Pacific Northwest is stem rust, caused by the fungus *Puccinia graminis* subsp. *graminicola* Z. Urb. If untreated with fungicide, stem rust can cause reductions in seed yields exceeding 90% (24).

Weather conditions and host growth during the cropping season (spring and summer) play a major role in determining the severity of the stem rust epidemic. These factors have been combined into a mathematical model that predicts disease severity based on daily weather inputs, host stage, and initial spring rust severity (25,26,28). The model predicts that disease severity during the course of the epidemic is proportional to the initial amount of rust in the spring, if all other variables are held constant. Other studies have found that autumn planting date, cultivar, and stand age all affect spring and summer epidemic severity of other rust diseases in the Pacific Northwest (6,19,33). Late-autumn plantings of perennial ryegrass have significantly less stem rust severity during the epidemic than those planted earlier in the fall, probably due to

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lower overwintering population of the pathogen (27). However, late planting results in a yield reduction, as demonstrated by a 23% lower seed yield compared with earlier planted stands when stem rust is kept equally low in both stand types through the use of fungicides (20). Reducing the overwintering *P. graminis* subsp. *graminicola* population with a biological control organism could reduce the severity of the spring epidemic without incurring the yield penalty of late planting.

Considerable research has been conducted in evaluating biological control strategies for reducing severity of foliar diseases (8,11,36). Some bacteria (*Pantoea agglomerans* and *Erwinia uredovora*; 10) and fungi (*Cladosporium tenuissimum*; 1) exude extracellualar lytic enzymes that inhibit the germination of urediniospores. Other fungi, including *Simplicillium lanosoniveum*, *Verticillium lecanii*, and *Aphanocladium album,* can colonize the rust sorus and decrease the number of urediniospores produced (11,14,36). Of the organisms studied, the mycoparasite *Sphaerellopsis filum* (Biv.) B. Sutton (teleomorph *Eudarluca caricis* (Biv.) O.E. Erikss.) has shown the greatest potential in controlling a wide range of rust diseases, in that it colonizes 369 rust species (13,15,21,23).

E. caricis (synonyms *Sphaeria filum*, *Darluca filum*, and *E. filum*) is an ascomycete in the order Pleosporales and family Phaeosphaeriaceae. The teleomorph is rarely found but has been well described living in the sori of the blackberry rust fungus (*Phragmidium violaceum*). It consists of black-brown stromata with pseudothecia 120 to 260 μm in size (39). Pycnidia of the anamorph, *Sphaerellopsis filum*, are black, 90 to 200 μm in diameter, and have distinct ostioles which excrete hyaline conidiospores [13 to 18 by 3 to 5 μm] in cirrhi (30). Spatial clustering of genotypes in the field, as well as a morphology characterized by gelatinous appendages on the ends of the conidia, indicate that conidia are splash dispersed (2). It is not certain whether the fungus regularly undergoes sexual recombination or what triggers it to enter the teleomorphic stage but there is considerable genetic variation in field populations (2,22).

S. filum grows and sporulates on artificial media containing malt extract or potato dextrose agar (PDA) (4,7,16,22,31,34). In controlled experiments, *S. filum* can infect 93% of *Cronartium fusiforme* and 76% of *Puccinia triticina* (syn. *P. recondita*) uredinia when conidia are applied to rust-infected plants and incubated under moist conditions (16,35). When infected with *S. filum*, *Melampsora epitea* urediniospore production is reduced by 64 to 98% (40). A simulation model which integrates wheat leaf area and *P. triticina*–*S. filum* biological interactions estimated that leaf rust severity would be reduced by 60 to 80% if *S. filum* infected 40 to 60% of the pustule surface (12).

Under scanning electron microscope analysis, *S. filum* hyphae are seen penetrating urediniospores and are interwoven throughout uredinia of leaf rust (*P. triticina*) and stem rust (*P. graminis* f. sp. *tritici*) (5,30). Shortened lifespan and reduced spore production of *S. filum*-infected uredinia are thought to be the underlying mechanisms responsible for observed suppression of oak rust (caused by *C. fusiforme*) and willow rust (caused by *M. epitea*) epidemics in the field (16,40).

Field studies of *S. filum* infecting rust uredinia have been limited in scope. In addition, infection parameters, including host age and the moisture and temperature conditions required for infection, have not been clearly established (16,35). The purpose of this study is to determine whether *S. filum* can infect *P. graminis* subsp. *graminicola* uredinia on perennial ryegrass during the winter and subsequently reduce the ryegrass stem rust severity of the spring epidemic. The experiments seek to quantify (i) the ideal infection conditions for *S. filum* on *P. graminis* subsp. *graminicola* uredinia on perennial ryegrass plants, (ii) the effects of *S. filum* on *P. graminis* subsp. *graminicola* urediniospore production and viability, and (iii) the effects of *S. filum* parasitism on the *P. graminis* subsp. *graminicola* population size in the field over the winter and into the spring epidemic.

Materials and Methods

Glasshouse plants. Seed of perennial ryegrass 'Jet', highly susceptible to stem rust, were planted in a soilless potting mix (Sunshine Growing Mix; Sun Gro Horticulture Inc., Bellevue, WA) in pots ("Cone-tainer", 3.8 by 21 cm; Stuewe & Sons Inc., Corvallis, OR), one seed per pot. Seed were kept constantly moist until three leaves emerged, at which point the pots were irrigated by placing them in water for 2 days every week, and fertilized every 2 weeks with aliquots from a solution of 6 mg of N, 5.4 mg of P_2O_5, and 6 mg of K_2O in 4 liters of water. Plants were grown in a glasshouse maintained at day and night temperatures of 20 and 15°C, respectively, with a 12-h day length, and they were periodically trimmed to a height of 8 cm until time of inoculation, 2 to 5 months after planting.

Controlled inoculation with *P. graminis* subsp. *graminicola*. Urediniospores were obtained each year from multiple-cultivar perennial ryegrass field plantings at the Oregon State University Hyslop Experimental Farm (44°38′ N, 123°12′ W). Spores were dried at 30% relative humidity overnight, stored at –60°C, and heat shocked at 43°C for 1.5 min before use (28). A spore suspension (approximately 2×10^5 spores/ml) was made for each inoculation by adding spores at 1 mg/ml of light mineral oil (Isopar M; ExxonMobile Chemical Co., Houston). To document spore viability, a 50-µl sample of the spore suspension was placed on water agar and incubated in the dark at 23 ± 2°C, and spore germination was determined after 24 h by counting the number of spores out of 100 that had a germ tube longer than the spore width. Each 500-µl aliquot of spore suspension was mixed immediately before being sprayed onto seven plants with a handheld Venturi atomizer operating at 21 kPa. After the oil dried (approximately 30 min), inoculated plants were placed in a mist chamber at 20 ± 4°C with a 15-h dark period immediately followed by a 4-h light period. Mist was run for 15 min at 2-h intervals during the dark period and continuously during the light period, to keep the leaves consistently moist.

Controlled inoculation with *S. filum*. *S. filum* conidia were originally harvested from parasitized rust pustules infecting peren-

nial ryegrass leaves in the field in 2003. Three monoconidial isolates (SF1, SF2, and SF3) were obtained from inoculated rust pustules in the glasshouse, maintained separately on PDA, and used as a mixture for the experiments. Conidia were stored at –60°C in a 15% glycerol solution. Conidial suspensions were transferred to PDA amended with 300 mg of Bacto Tryptone and 150 mg of Bacto Yeast Extract, and incubated for 2 days under UV light (40-W blacklight; GE, Fairfield, CT) at 23°C and then under gro-lights (40-W, Gro-Lux; Sylvania, Danvers, MA) at 23°C for 2 to 3 weeks (20). Mucilaginous cirrhi of conidia were harvested from pycnidia by means of a sterile blade and placed into centrifuge tubes with 1 ml of sterilized water containing 0.05 M $MgSO_4$ to help prolong viability of the conidia (*unpublished data*). After mixing for 30 s, the suspension was transferred to a larger container and adjusted to 10^5 conidia/ml with 0.05 M $MgSO_4$. The resulting suspension was sprayed on each rust-infected plant (1.5 ml/plant) with an aerosol sprayer (Preval; Precision Valve Corporation, Yonkers, NY).

Effects of mist duration. Plants were inoculated with *P. graminis* subsp. *graminicola* 10, 5, or 0 days prior to the day on which all plants were inoculated with *S. filum.* These intervals constituted durations of 1.2, 0.6, and 0.0 *P. graminis* subsp. *graminicola* latent periods (25) as calculated by temperature measurements taken in the glasshouse. Immediately after the plants dried from the *S. filum* inoculation, they were placed on a bench in a glasshouse under a misting system that kept the leaves wet (misting leaves for 30 min every 2 h) with day and night temperatures of 15 and 20°C, respectively. At 0, 2, 4, 6, 12, 24, 48, 72, and 120 h after *S. filum* inoculation, 12 plants (4 replicate plants from each rust-inoculated age group) were transferred to a glasshouse bench where plants were not misted, thus preventing subsequent *P. graminis* subsp. *graminicola* infections from occurring. Additional control treatments, inoculated with *P. graminis* subsp. *graminicola* but not *S. filum,* were given 0 or 120 h of mist treatment. Trials were scored for *P. graminis* subsp. *graminicola* and *S. filum* severity approximately 1.6 ± 0.2 *P. graminis* subsp. *graminicola* latent periods after *S. filum* inoculation. The experiment was repeated once.

Effects of temperature. Plants were inoculated with *P. graminis* subsp. *graminicola* and *S. filum* as described in the mist experiment above. Immediately after the plant leaves dried following *S. filum* inoculation, the plants were placed in a growth chamber that misted plants for 2 min every 2 h, keeping the leaves wet for 48 h. Treatment temperatures were 5, 10, 15, 20, and 25°C. Because of limited growth chamber availability, only one of the listed treatments was conducted at a time, paired each time with a treatment at 15°C to allow normalization of results across trials. The choice of chamber to use for the 15°C was done by random-number selection independently for each trial. Each treatment consisted of 30 plants (5 inoculated with *S. filum* and 5 noninoculated plants from each *P. graminis* subsp. *graminicola*-inoculated age group). After the 48-h mist exposure, plants were kept without misting in the growth chambers at the specified treatment temperature to prevent subsequent infections. The experiment was scored as described in the mist-duration experiment and was repeated.

Effects of *S. filum* on *P. graminis* subsp. *graminicola* spore production and germination. To reduce variability in rust spore production that might arise from host genetic differences, plants raised in a glasshouse were vegetatively propagated so that 12 copies of each of 4 clones were obtained. All plants were inoculated with *P. graminis* subsp. *graminicola* and left on a glasshouse bench for 6 days (0.7 stem rust latent periods) while the infections incubated. Four replications of each of the four clones (16 plants) were then assigned to each of three treatments: (i) inoculated with *S. filum* conidia suspended in 0.05 M $MgSO_4$, (ii) sprayed with the $MgSO_4$ solution as a check, or (iii) left untreated. Immediately after the plants had dried, they were placed under a misting system for 48 h, as described in the mist-duration experiment, then transferred to a glasshouse bench without mist. Starting when pustules erupted, spores from four pustules per plant were collected every 2 to 3 days into gelatin capsules fitted on a small vacuum spore sampler (32). Pustules from four plants (16 pustules) made up one

measurement replication. Isopar M oil (100 μl) was added to each capsule and thoroughly mixed with the spores, and 20 μl of the resulting suspension was placed on a hemacytometer (Fisher Scientific, Pittsburg). The spores were counted and the total number of spores produced per pustule per day was calculated. An additional aliquot (20 μl) of the spore suspension was spread on a water agar plate, and the spore germination percentage was determined by microscopic observation after 24 h of incubation at 22°C in the dark. Two trials of the experiment were completed.

Field experiments. The effects of *S. filum* on *P. graminis* subsp. *graminicola* populations in the field over the winter were investigated at two sites with two types of experiments during the 2009–10 and 2010–11 field seasons. Site 1 was at the Oregon State University Hyslop Experiment Farm near Corvallis, OR (44°38′ N, 123°12′ W). Site 2 was a commercial perennial ryegrass field near Junction City, OR (44°23′ N, 123°20′ W). In experiment 1 ("controlled inoculation experiment") plants were inoculated with *P. graminis* subsp. *graminicola* and *S. filum* under controlled conditions, and then out-planted to the field. In experiment 2 ("field inoculation experiment") field plants were naturally infected with *P. graminis* subsp. *graminicola*, and *S. filum* conidia were applied in the field using a backpack sprayer. Weather conditions were collected at both sites with a data logger (CR10X; Campbell Scientific, Logan, UT) that had sensors for temperature and leaf wetness at the canopy level, rainfall, and relative humidity.

Field observations of naturally occurring infections. In 2000–03, first- and second-year plantings of perennial ryegrass were observed for the proportion of *P. graminis* subsp. *graminicola* uredinia infected with *S. filum* over the winter (December to March). Plants (six replications of 10 cm of row) from both first- and second-year perennial ryegrass plantings were randomly cut from field plantings. The leaves within each cut sample were mixed and observed under a dissecting microscope until 100 *P. graminis* subsp. *graminicola* pustules were counted. The numbers of *P. graminis* subsp. *graminicola* uredinia and uredinia containing *S. filum* pycnidia were tabulated and used to determine the proportion of uredinia infected with *S. filum*.

Field experiment 1: controlled inoculations transplanted to the field. Seed of perennial ryegrass Jet, 'Morningstar', and 'Linn' were planted in rows in an outdoor planting bed on 7 October 2009 and 15 September 2010. In the field experiments, the perennial ryegrass "plant" unit consisted of 10 cm of row (6 to 10 individual plants). The 10-cm plant units were dug from the rows and placed in pots (one unit per pot) on 11 December and 6 November for the 2009 and 2010 experiments, respectively. The potted plants were brought into a glasshouse and inoculated with *P. graminis* subsp. *graminicola* and *S. filum* as described in the spore production experiment; however, in this experiment, only *P. graminis* subsp. *graminicola* infections that were 7 days (0.8 stem rust latent periods) old were inoculated with *S. filum*. The three treatments were (i) noninoculated plants, (ii) *P. graminis* subsp. *graminicola*-inoculated plants, and (iii) *P. graminis* subsp. *graminicola*-inoculated plants inoculated with *S. filum*. After *S. filum* inoculation, all treatments were placed under the mist system for 48 h, then out-planted to field sites 1 and 2 as described below. Plants were scored weekly for *S. filum* and *P. graminis* subsp. *graminicola* severity while in the field.

At site 1, each experimental unit consisted of two identically treated plants (i.e., two 10-cm units) spaced 0.6 m (two rows) apart from one another. At site 1 in 2009, treatments were applied to plants of Jet and experimental units were set out 11 m apart from one another, randomized in a 2-by-45-m plot area in a field of Jet. In 2010, treatments were applied to Jet, Morningstar, and Linn, and experimental units were set out 1.2 m apart from one another into a plot of 'Manhattan 5' (moderately resistant to *P. graminis* subsp. *graminicola* infection). In each year, there were four replications per treatment in a completely randomized design.

At site 2, each experimental unit within a treatment consisted of a plant (10 cm of row). At site 2, experimental units were set out 2 m apart, randomized within a 35-by-35-m plot in a commercial field of 'Silver Dollar'. In 2009–10 at site 2, treatments were applied to plants of Jet and Morningstar. In 2010–11, treatments were applied to plants of Jet, Morningstar, and Linn. In each year, there were six replications in a completely randomized design. Plants were scored as previously described in the field during both years.

Field experiment 2: field inoculations. Field inoculation experiments were conducted at site 1 only. During the 2009–10 season, four blocks (2 by 45 m) of perennial ryegrass Jet were planted on 7 October 2009 and were designated as the first-year stand. Second-year stand plots of Jet had been planted on 29 September 2008. For the 2010 season, Jet and Linn were planted on 15 September and designated as first-year stand plots. Third-year stand plots of Jet and Linn had been planted on 29 September 2008. The first-year and established (second- or third-year) stand blocks were randomly placed in a larger field with a minimum distance of 4.5 m maintained between blocks to allow access for spraying and disease assessment without disturbing adjacent blocks. Planting and maintenance procedures following standard agronomic practices have been described previously (27).

Each block was divided into four treatment plots, each measuring 2 by 11 m and arranged in a randomized complete block design. The four treatments were (i) a nontreated control, (ii) one application of *S. filum* in the winter, (iii) monthly applications of *S. filum* from late fall until late spring, or (iv) one application of the fungicide Quilt (7% azoxystrobin and 11% propiconazole; Syngenta Inc., Basel, Switzerland) in the winter. A 3-by-2.1-m buffer (sprayed with Quilt to minimize the spread of disease) was maintained between treatment plots in 2010–11.

S. filum was applied by first harvesting conidia from approximately 80 PDA plates into 12 liters of sterilized 0.05 M MgSO$_4$ in water to give a final concentration of 10^5 spores/ml. The conidial suspension was applied to plots at a rate of 10^9 spores/ha (12 liters per ha) by means of a CO$_2$-pressurized backpack sprayer operated at a pressure of 138 kPa (20 psi). The suspension was applied also to two PDA plates to test for contamination and to quantify the germination of the *S. filum* conidia.

One-time applications of *S. filum* were applied on 15 November 2009 or 30 November 2010. Monthly applications of *S. filum* were applied on 15 November, 15 December, 24 January, 21 March, 14 April, 3 May, and 26 May during the 2009–10 winter and on 24 October, 30 November, 27 December, 14 February, 7 March, and 6 April during the 2010–11 winter. Quilt was applied at a rate of 1,500 ml in 75 liters of water per hectare (0.3 kg a.i./ha) at a pressure of 207 kPa (30 psi) on 28 January in the 2010 experiment and 4 February in the 2011 experiment.

Treatment plots were observed for *P. graminis* subsp. *graminicola* and *S. filum* severity by counting the number of *P. graminis* subsp. *graminicola* pustules and the number of pustules infected with *S. filum*. Specifically designated plants in two replications per treatment were observed repeatedly during the experiment. These plants were marked by arbitrarily selecting 10 cm of row and repeatedly scoring two to four times a month, taking care not to disturb the plants.

Effects of fungicide on *S. filum* germination. The effects of three fungicides widely used to prevent *P. graminis* subsp. *graminicola* epidemic development were tested for their effect on the viability of *S. filum* spores. The fungicides used were Bravo (54% chlorothalonil; Syngenta Inc.), Quadris (22.9% azoxystrobin; Syngenta Inc.), and Tilt (41.8% propiconazole; Syngenta Inc.). For each fungicide, filter discs (5.5-mm-diameter Whatman 42 ashless filter paper; Kent, UK) were saturated with a dilution series of the formulated products (1, 1:10, 1:100, 1:500, 1:1000, and a water control). The fungicide-soaked discs were placed on water agar plates (three replications of each dilution) that had been sprayed with a conidial suspension of *S. filum* in 0.05 M MgSO$_4$ (as described in the controlled inoculation experiments above). The plates were closed and incubated for 2 days under UV light at 23°C and 3 days under gro-lights at 23°C, with 12 h of dark and light periods. The plates were scored for the zone of inhibition, where germination (germ tube longer than the spore width) oc-

curred in less than 10% of the spores. The distance between the edge of the fungicide filter disc and the delineated inhibition edge was measured with a ruler.

Data analysis. The mist-duration and temperature experiment counts of *P. graminis* subsp. *graminicola* pustules or pustules infected with *S. filum* were expressed as total number of *P. graminis* subsp. *graminicola* pustules or the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*. A multiple linear regression analysis on log-transformed data was performed to determine the relationship between mist duration and pustule age on the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*. The 0- and 120-h mist-duration observations were removed from the analysis to prevent skewness, because the effects of mist leveled off at 72 h and there were no *P. graminis* subsp. *graminicola* infected with *S. filum* in the 0-h treatment. In addition, the 0-day-old rust treatment, which was rarely infected by the mycoparasite and, therefore, had virtually invariant data, was omitted from analysis in the mist-duration experiment.

There were sufficient growth chambers available to conduct only two temperature treatments in each test; therefore, chambers were randomly reassigned to a different temperature for each trial and a 15°C treatment was included in each trial as a standard (25). Because inoculum conditions can vary among trials, we standardized observations by expressing the level of *S. filum* infection in each trial as a proportion of that produced in the standard 15°C treatment. Statistical analysis (analysis of variance [ANOVA]) of proportion pustules infected in the 15°C treatment in the different growth chambers among the multiple trials showed no effect of growth chamber and no interaction of chamber with plant age treatment ($P = 0.13$ and 0.14, respectively); therefore, any non-temperature effect of growth chamber was implicitly included as random experimental error. Temperature, *P. graminis* subsp. *graminicola* pustule age at time of *S. filum* inoculation, and trial were factors in the three-way ANOVA for the temperature experiment.

Weibull (four-parameter) curves were fit to the data for spore production and for spore production multiplied by spore viability

for each treatment, using SigmaPlot (SigmaPlot v. 11.0; Systat Software, San Jose, CA). Spore production and viability data were analyzed by three-way and four-way ANOVA, respectively. The factors in the analysis were treatment, host genotype, trial, and, for the germination experiment, days after inoculation.

Counts of rust pustules per 10 cm of row in the field experiment were plotted against time, and the area under the disease progress curve (AUDPC) was calculated from this graph (18) for the period between 1 March and the end of the observations (11 June, 2010 and 2 June 2011) by summing areas under the trapezoids produced from the data points joined by straight lines. This calculation of AUDPC was done to determine any treatment differences in rust severity that were manifested during the first part of the crop production season. The AUDPC values for proportion of *P. graminis* subsp. *graminicola* pustules with *S. filum* were obtained from data across the entire sample period (14 November to 11 June 2009–10 and 11 October to 2 June 2009–10). Field data were analyzed using three-way ANOVA. The factors were stand age, treatment, and block. Multiple comparisons were made using Tukey's HSD (honestly significant difference) method. Analysis was conducted using R (R Development Core Team [2011], R Foundation for Statistical Computing, Vienna).

Results

Effects of mist duration. Multiple linear regression analysis of log-transformed data showed that mist duration between 2 and 72 h and *P. graminis* subsp. *graminicola* pustule age at the time of *S. filum* inoculation had significant ($P < 0.006$ and $P = 0.02$, respectively) effects on the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* (Fig. 1). Mist duration between 2 and 72 h was positively correlated ($P < 0.006$) with the proportion of pustules infected with *S. filum*. Pustules that were 10 days old had a higher ($P = 0.02$) percentage of pustules infected with *S. filum* across all mist durations than the pustules that were 5 days old at the time of *S. filum* inoculation. There was not a significant interaction ($P = 0.06$) between mist duration and pustule age.
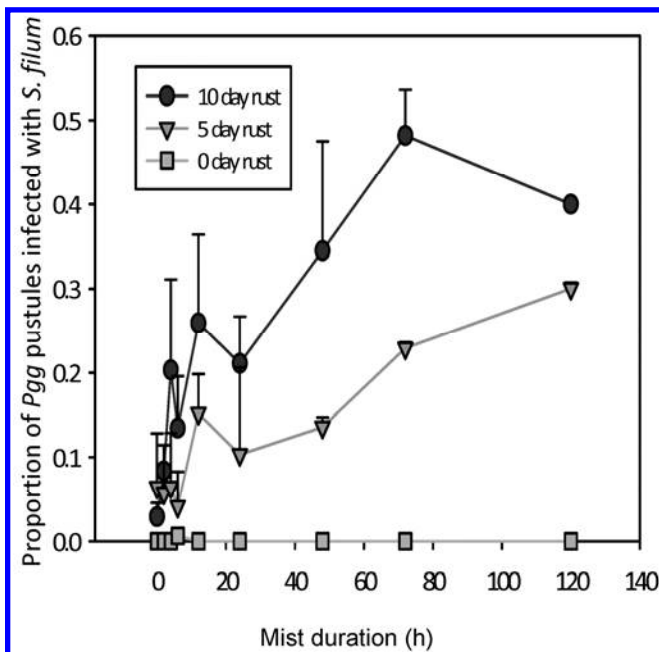
Fig. 1. Effects of pustule age and leaf wetness on the proportion of *Puccinia graminis* subsp. *graminicola* (*Pgg*) pustules infected with *Sphaerellopsis filum*. Perennial ryegrass plants were inoculated with *P. graminis* subsp. *graminicola* 10, 5, or 0 days before they were treated with a conidial suspension of *S. filum*. Plants were then incubated at day and night temperatures of 15 and 20°C, respectively. Treatments (four replications) were misted for 30 min every 2 h to maintain wet leaves for the durations shown, and scored after 1.5 *P. graminis* subsp. *graminicola* latent periods. Each data point is the mean and standard error of the mean between two experiments.
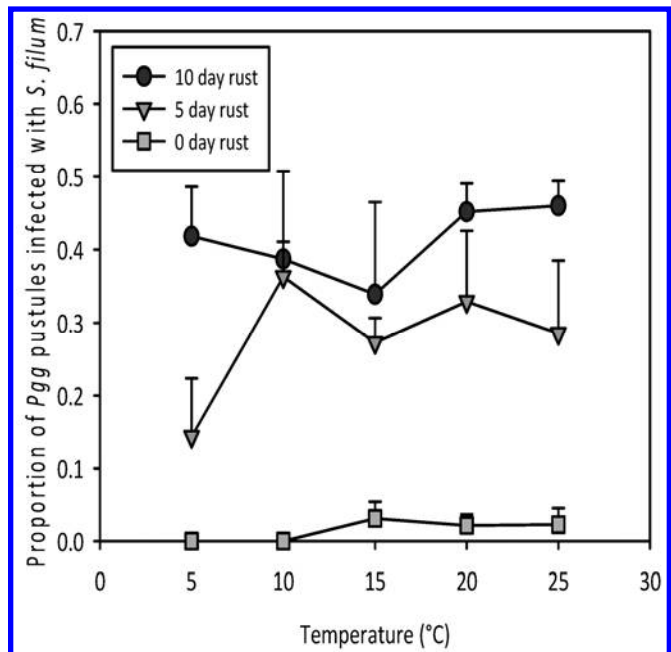
Fig. 2. Effect of temperature and pustule age on the proportion of *Puccinia graminis* subsp. *graminicola* (*Pgg*) pustules infected with *Sphaerellopsis filum*. Perennial ryegrass plants were inoculated with a conidial suspension of *S. filum* 10, 5, or 0 days after they were inoculated with *P. graminis* subsp. *graminicola*. Plants were placed in growth chambers at the constant temperatures shown with 48 h of mist. After 1.5 *P. graminis* subsp. *graminicola* latent periods, plants were scored for *P. graminis* subsp. *graminicola* and *S. filum* severity. Data points represent the mean (five replications) and standard error of the mean in one trial of the experiment. A second trial produced similar results and the same conclusions.

Effects of temperature. The mean overall effect of the temperature (5 to 25°C) at which *S. filum* was incubated was significant (*P* = 0.03) on the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* (Fig. 2), although it was only among the 5-day-old pustules that infection was lower at 5°C than at the other temperatures. *P. graminis* subsp. *graminicola* pustule age at the time of *S. filum* inoculation had a significant effect (*P* < 0.001) across all temperatures on the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* (Fig. 2). The overall effect of pustule age averaged across the temperatures tested was that *S. filum* infected 13% more 10-day-old *P. graminis* subsp. *graminicola* pustules than 5-day-old pustules (*P* < 0.001), and 36% more 10-day-old pustules than 0-day-old pustules (*P* < 0.001). There was no significant interaction between temperature and age (*P* = 0.43), trial and temperature (*P* = 0.73), or trial and age (*P* = 0.74).

Rust spore production and germination. Nontreated *P. graminis* subsp. *graminicola* pustules produced 39,000 spores over their lifetime, more than double (*P* < 0.001) the 18,000 produced when infected with *S. filum* (Fig. 3A). There was no effect of host genotype on the number of spores produced (*P* = 0.064) and no interaction between host genotype and treatment (*P* = 0.99).

S. filum reduced the overall average viability of spores by 4.75% (*P* < 0.001). Spore viability multiplied by daily spore production was plotted against days since *P. graminis* subsp. *graminicola* inoculation (Fig. 3B). Weibull (four-parameter) curves were fit to each treatment and plotted with the averages of each trial. The standard form for the Weibull curve is $f(x) = a \times ((c-1)/c)^{((1-c)/c)} \times (abs((x-x0)/b + ((c-1)/c)^{(1/c)})^{(c-1)}) \times exp(-abs((x-x0)/b + ((c-1)/c)^{(1/c)})^c + (c-1)/c))$, for $(x \le x0 - b \times ((c-1)/c)^{(1/c)})$. The parameter estimates for the *P. graminis* subsp. *graminicola* spore curve as a function of days after infection are *a* = 5,500, *b* = 5.44, *c* = 1.01, and *x*0 = 14.30. The parameter estimates for the *P. graminis* subsp. *graminicola*-only treatment for the spore curve multiplied by spore viability are *a* = 5,000, *b* = 5.44, *c* = 1.11, and *x*0 = 14.30. The parameter estimates for the *S. filum* treatment for the rust spore curve multiplied by spore viability are *a* = 3,200, *b* = 4.2, *c* = 1.01, and *x*0 = 12.12. The coefficient and standard error of the first parameter of each curve (which set the maximum) for each treatment are (i) nontreated *P. graminis* subsp. *graminicola*, 5,500 ± 204; (ii) *P. graminis* subsp. *graminicola* with 0.05 M MgSO₄, 5,540 ± 263; and (iii) *P. graminis* subsp. *graminicola* with *S. filum*, 3,200 ± 370 (the adjusted r^2 values are 0.74, 0.63, and 0.83, respectively).

Field observations. In 2000–03, first- and second-year plantings of perennial ryegrass were observed for the proportion of *P. graminis* subsp. *graminicola* uredinia infected with *S. filum* over the winter (December to March). In all, 2% of the pustules were infected on the first-year plantings, whereas the second-year plantings had 25% of the pustules infected with *S. filum* (significantly different, *P* < 0.05). There was also a significant difference between the pustules infected with *S. filum* on living leaves and dead leaves. On the first-year plantings, there were 4% more pustules infected on the dead leaves than on live leaves (not significantly different at *P* > 0.05). On the second-year plantings, there were 43% more pustules infected with *S. filum* on the dead leaves than on the live leaves (different at *P* < 0.05).

Field experiment 1: controlled inoculations. Results of the controlled inoculations with *S. filum* on *P. graminis* subsp. *graminicola*-infected perennial ryegrass plants transplanted to the field are shown in Figure 4 (site 1) and Figure 5 (site 2). The area under the disease progress curve was calculated from 1 March through June for *P. graminis* subsp. *graminicola* pustule data, and across all observation dates in a given year for the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*. Means and the statistical conclusions from one- and two-way ANOVA are summarized for sites 1 and 2 (Table 1). Analysis is shown for data within a site but not between sites because of a significant interaction effect between sites. There were significant effects of *S. filum* infection on *P. graminis* subsp. *graminicola* pustule severity

and the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* at both sites in 2009–10. In 2010–11, the *P. graminis* subsp. *graminicola* population did not survive the winter at either site in any treatment; therefore, treatment effects on spring-time *P. graminis* subsp. *graminicola* severity could not be assessed.

In 2009–10 at site 1, the *S. filum* treatment had only a third as much disease (AUDPC, difference significant at *P* = 0.013) as the *P. graminis* subsp. *graminicola* treatment without *S. filum* (Fig. 4; Table 1). Similarly, at site 2 in that year, the *S. filum* treatment had only 5 to 10% as much disease (*P* < 0.001) as the *P. graminis* subsp. *graminicola*-only treatment on Jet and Morningstar, respectively (Fig. 5; Table 1). In 2009–10, the *S. filum* treatment had more *P. graminis* subsp. *graminicola* pustules infected with *S. filum* (*P* < 0.001) than the *P. graminis* subsp. *graminicola* treatment without *S. filum*. Jet had significantly more disease (*P* < 0.001) than Morningstar. Cultivar did not have a significant effect on the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*.

Field experiment 2: field inoculations. Four treatments were applied to first-year and older (second- or third-year) plantings of perennial ryegrass exposed to natural inoculum of *P. graminis* subsp. *graminicola* at site 1 in 2009–10 and 2010–11. Treatments
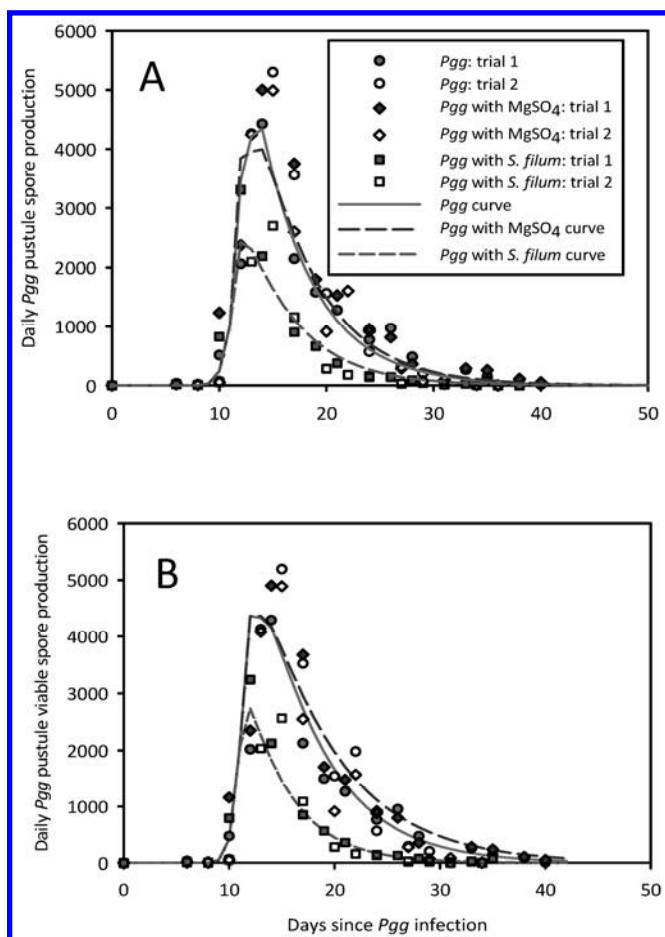
Fig. 3. A, *Puccinia graminis* subsp. *graminicola* (*Pgg*) daily spore production and **B,** spores produced multiplied by spore viability. Perennial ryegrass 'Jet' plants were inoculated with a *P. graminis* subsp. *graminicola* spore suspension. After 7 days (0.7 rust latent periods), three treatments were applied and plants were subjected to misting for the first 48 h at day and night temperatures of 20 and 15°C, respectively. The three treatments applied were (i) a nontreated control, (ii) 0.05 M MgSO₄, or (iii) *Sphaerellopsis filum* conidial suspension in 0.05 M MgSO₄. Spores were collected from individual rust pustules every 2 or 3 days and counted with a hemocytometer. Spore viability at each collection time was determined as percent germination on water agar. A Weibull (four-parameter) curve (see text) was fit to the data. Symbols represent means for data from each trial; lines represent Weibull curves calculated for each treatment.

were (i) nontreated control, (ii) one application of *S. filum*, (iii) monthly applications of *S. filum*, or (iv) one winter application of Quilt fungicide.

First-year stands of perennial ryegrass treated with one application of *S. filum* did not have significantly less *P. graminis* subsp. *graminicola* disease than the nontreated control in either year (Fig. 6). First-year stands treated with monthly applications of *S. filum* had only about 50% (*P* = 0.02) of the disease severity of the control treatment in 2009–10 (Table 2) but, in 2010–11, the lower disease in this treatment (56% of the check) was not statistically significant (*P* = 0.24). In both years, first-year stands receiving monthly applications of *S. filum* had a significantly greater proportion (*P* < 0.03) of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* than the proportion infected in the control treatment.

Quilt fungicide treatments on first-year stands had a *P. graminis* subsp. *graminicola* severity that was only 20% and 1% that of the control treatment in 2009–10 and 2010–11, respectively (significant at *P* < 0.001 in both years) (Table 2). The fungicide treatment did not have a significantly different proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* than the nontreated control in either year.

Established stands of perennial ryegrass had substantially less *P. graminis* subsp. *graminicola* disease than the first-year stands in both years (significant at *P* < 0.001; Table 2). There were no significant treatment effects (*S. filum* or fungicide) within these older stands in 2009–10 or 2010–11.

Favorable field conditions for *S. filum* infection. Conditions favorable for *S. filum* infection were determined from daily weather measurements (Fig. 7) by finding days between the planting date and 15 April that had a minimum temperature above 5°C and six or more hours of leaf wetness. In this period in 2009–10, 24% of the days were favorable for infection and, in 2010–11, 25% of these days were favorable for infection.

Effects of fungicide. *S. filum* germination inhibition was measured on water agar against three fungicides along a dilution series (Fig. 8). At concentrations below 0.01% of the active ingredient (field concentrations are between 0.005 and 0.0005%), chlorothalonil and azoxystrobin inhibited *S. filum* germination with a zone of inhibition between 6 and 8 mm. There was no zone of inhibition around these same concentrations of propiconazole. At high concentrations (>0.01% of the active ingredient), all three of the fungicides inhibited *S. filum* germination but inhibition distance was 8 mm greater for axoxystrobin (*P* = 0.003), and 7 mm greater for chlorothalonil (*P* = 0.012) than for propiconazole.

Discussion

Our experiments showed that leaf wetness duration played a significant role in the proportion of *P. graminis* subsp. *graminicola*
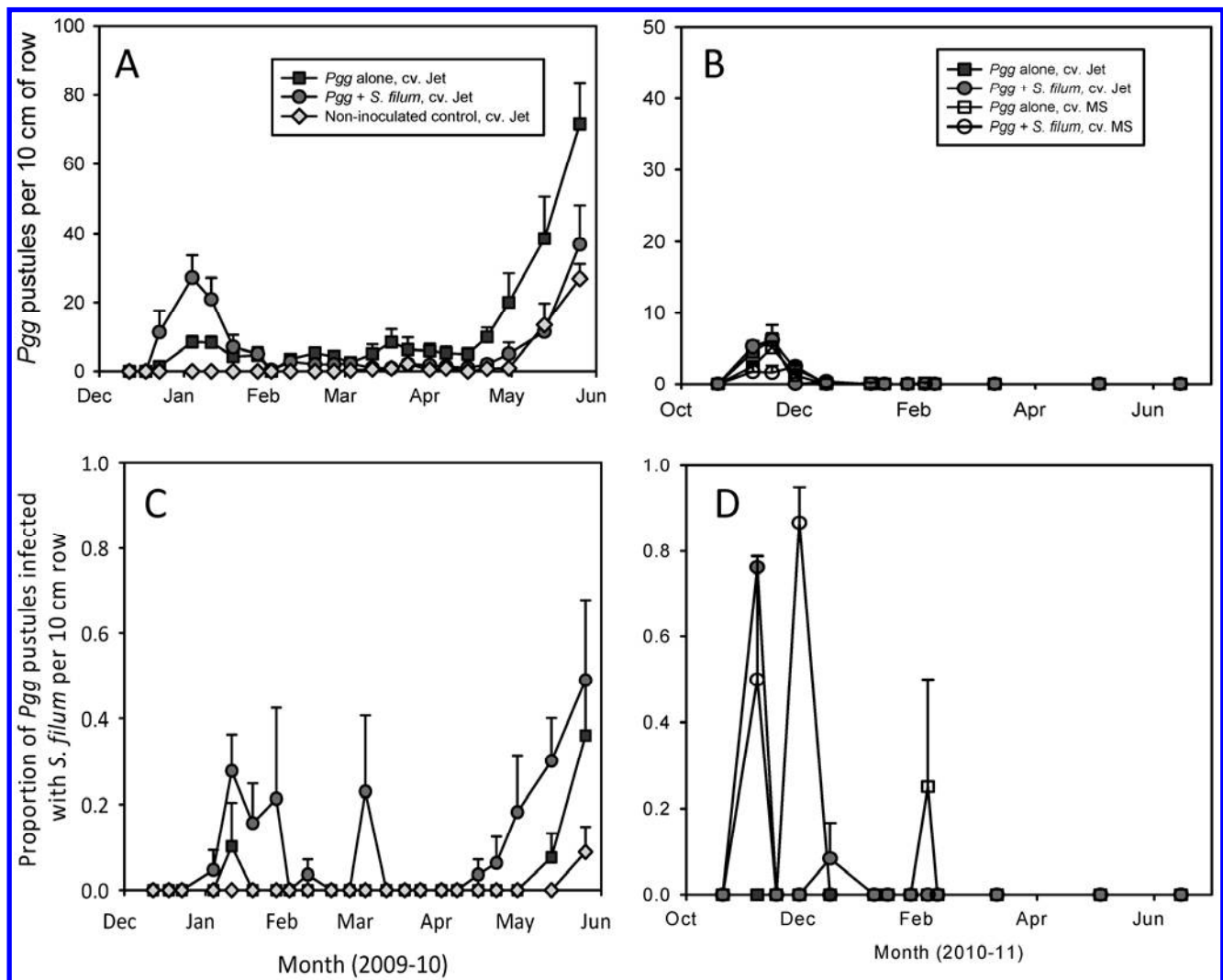
Fig. 4. Site 1 field experiment of *Puccinia graminis* subsp. *graminicola* (*Pgg*) and *Sphaerellopsis filum* applied to ryegrass plants before outplanting. **A** and **B,** Number of *P. graminis* subsp. *graminicola* pustules and **C** and **D,** the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* on plants observed repeatedly over the winter. The experiment was conducted in **A** and **C,** 2009–10 and **B** and **D,** again in 2010–11. Data points are the means and standard error of the mean on six replications of 10 cm of row. In 2009–10, 'Jet' was used. In 2010–11, Jet, 'Morningstar' (MS), and 'Linn' (*not shown*; results similar to Jet) were used. Noninoculated plants did not develop *P. graminis* subsp. *graminicola* pustules over the winter in 2010–11.

pustules infected with *S. filum,* as others had shown previously for *S. filum* infection on *C. fusiforme*, *P. triticina (*syn. *P. recondita*), and *M. larici-epitea*. *S. filum* infected 42% fewer *C. fusiforme* pustules with 4 h of moisture than with 16 h of moist incubation (16). We found a significant ($P = 0.006$) positive linear relationship, using multiple linear regression, of the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* as a function of mist duration, and some infection occurred with as little as 2 h of mist.

Previous research by others revealed that age of the rust infection can influence success of colonization by *S. filum*. *S. filum* does not infect *C. fusiforme* pustules when both fungi are inoculated simultaneously (16) but readily infects *M. epitea* on willow (21) or *P. triticina* on wheat (35) when conidia and urediniospores are inoculated onto leaf tissue simultaneously. Our results were closer to those reported for *S. filum* on *C. fusiforme* than for *S. filum* on *P. triticina* or *M. epitea*. *S. filum* infected 13% more 10-day-old *P. graminis* subsp. *graminicola* pustules than 5-day-old pustules and 36% more 10-day-old pustules than 0-day-old pustules. *P. graminis* subsp. *graminicola* infections that were 0 days old at the time of *S. filum* inoculation were generally not infected by the mycoparasite.

Incubation temperature had a statistically significant overall effect on the number of *P. graminis* subsp. *graminicola* pustules infected by *S. filum* but the effect was not marked and all temperatures between 5 and 25°C were permissive for infection. Weather data from field site 1 showed that 25% of the days between the planting date and 15 April in two different years were favorable for *S. filum* infection (temperatures above 5°C and >6 h of leaf wetness). This research was conducted in Oregon's Willamette Valley, which has a maritime climate where many winter days are sufficiently warm and wet to be conducive for *S. filum* infection. However, summer conditions are dry and, therefore, less conducive for *S. filum* infection. In our climate, *S. filum* is most likely to be effective as a biocontrol agent during the overwinter period, when moisture is common and the rust pathogen is relatively disadvantaged by the cooler conditions. *S. filum* might be applied effectively in other climates in seasons that have relatively warm and moist weather patterns. In continental climates where winter conditions are colder and drier, there may be less opportunity to use *S. filum* as a biocontrol during overwinter survival of the rust fungus.

S. filum colonizes 20.5% of *M. larici-epitea* pustules on detached willow leaves and reduces spore production by 38.4% per pustule (23). In our experiments, *S. filum* infected between 20 and 60% of *P. graminis* subsp. *graminicola* pustules and reduced spore production in colonized pustules by 54%. The reduction in *P. graminis* subsp. *graminicola* spore production is presumed to be the mechanism by which *S. filum* reduces rust epidemics (20). A simulation model that integrated wheat leaf area and *P. triticina* and *S. filum* biology concluded that *S. filum* would reduce the severity of a leaf rust epidemic by 60 to 80% when 40 to 60% of
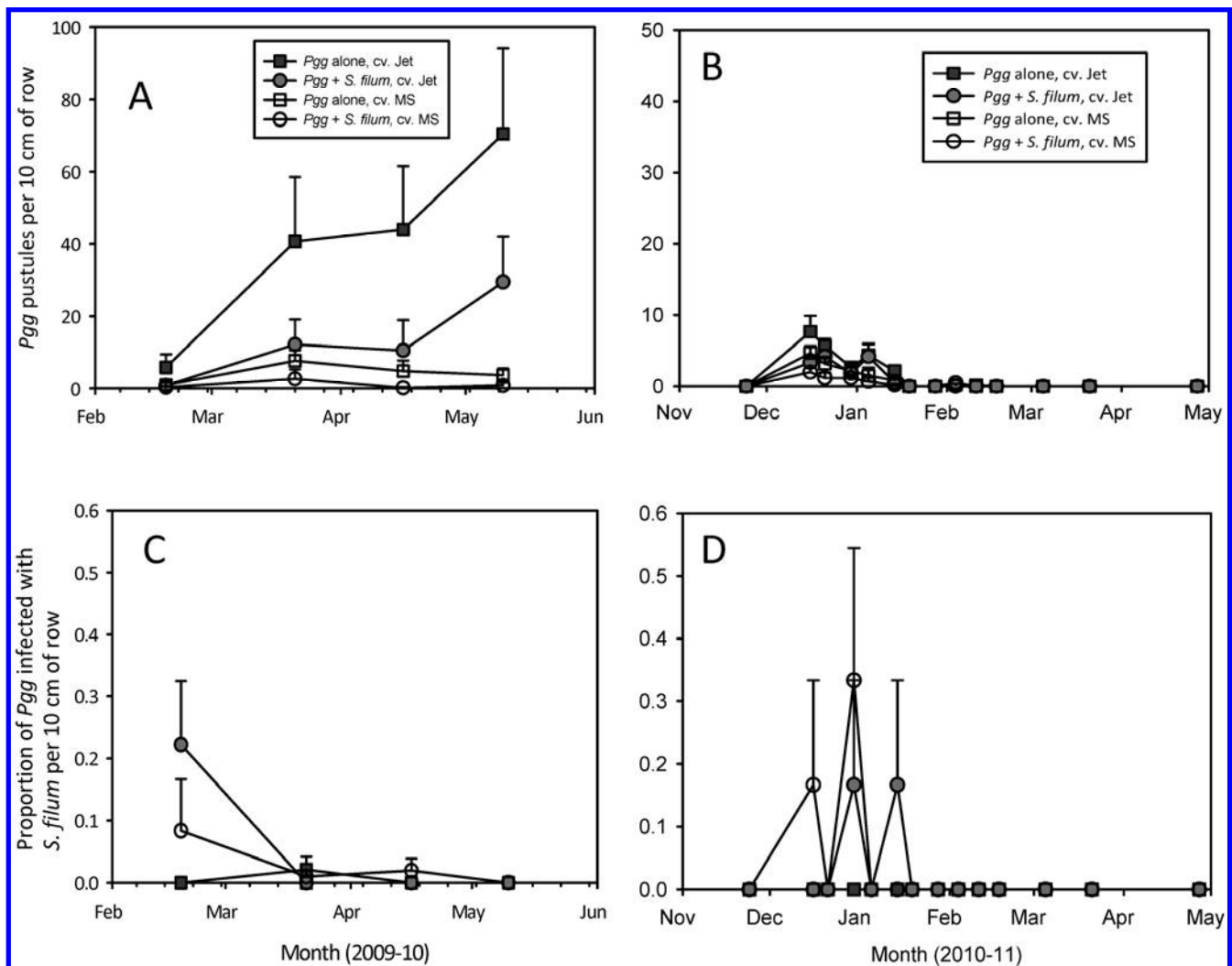
Fig. 5. Site 2 field experiment of *Puccinia graminis* subsp. *graminicola* (*Pgg*) and *Sphaerellopsis filum* applied to ryegrass plants before outplanting. **A** and **B,** Number of *P. graminis* subsp. *graminicola* pustules and **C** and **D,** the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* on plants repeatedly observed over the winter. The experiment was conducted in **A** and **C,** 2009–10 and **B** and **D,** again in 2010–11. Data points are the means and standard error of the mean on six replications of 10 cm of row. In 2009–10, 'Jet' and 'Morningstar' (MS) were used. In 2010–11, Jet, MS, and 'Linn' (*not shown*; results similar to Jet) were used. Noninoculated plants did not develop *P. graminis* subsp. *graminicola* pustules over the winter in 2010–11.

the pustules were colonized (12). An overwintering model that we developed for *P. graminis* subsp. *graminicola* on perennial ryegrass (9) predicted that *S. filum* would reduce the early spring epidemic by 20 to 70% when 20 to 60% of the *P. graminis* subsp. *graminicola* pustules were infected. Virulent strains of *S. filum* can infect 98% of *M. epitea* pustules and reduce spore production by 98% (40). Our model (9) predicts that, if an *S. filum* isolate infected 98% of *P. graminis* subsp. *graminicola* pustules and reduced spore production by 98%, the rust population would approach zero by spring.

S. filum has been reported as a component of several rust pathosystems even without human intervention. Naturally occurring levels of *S. filum* infection in *C. strobilinum* uredinia in North Florida oak stands was between 7 and 93% in 1975 (16). Levels of *S. filum*-infected *Melampsora* spp. uredinia on willow plantations in Germany was between 15 and 26% in 2000 to 2002 (17). A *Melampsora* spp. epidemic was suppressed by *S. filum* without application of the mycoparasite in the United Kingdom in 1990 (20). Over the winters of 2000 to 2003, we observed naturally occurring *S. filum* infection levels of 2 and 25% of stem rust pustules in first- and second-year plantings, respectively, of noninoculated perennial ryegrass. *S. filum* was found in 43% more pustules on dead leaves than on live leaves on the second-year plantings. Although the presence of *S. filum* in these pustules on dead leaves is of little consequence with respect to rust spore production (active *P. graminis* subsp. *graminicola* requires living host tissue), the infected pustules could represent a reservoir of overwintering inoculum for the biocontrol fungus.

The results of these experiments provide evidence that lower *P. graminis* subsp. *graminicola* overwintering populations give rise to less severe epidemics in the spring, supporting earlier indications of this effect (27,33). Therefore, the disease management effectiveness of treatments we tested can be assessed by their ability to produce a reduction in overwinter pathogen population. Our controlled inoculations, which established *S. filum* in emerging *P. graminis* subsp. *graminicola* uredinia before plants were outplanted to the field, showed that the *S. filum* population survived

the winter on *P. graminis* subsp. *graminicola* uredinia. *S. filum* reduced the *P. graminis* subsp. *graminicola* pustule count through late spring at two sites during one year (2009–10). In another year, *S. filum* infected *P. graminis* subsp. *graminicola* uredinia in the fall but the uredinial population (whether in *S. filum*-inoculated treatments or not) crashed in February and, therefore, it was impossible to observe a late spring effect of *S. filum* on rust severity. We were unable to locate *S. filum* growing outside *P. graminis* subsp. *graminicola* uredinia and presume that the *S. filum* population also crashed in the absence of its host.

In the experiments where *S. filum* was applied monthly to plants in the field, there was a significant increase in the proportion of pustules infected in both years on the first-year stand of perennial ryegrass over the noninoculated checks. Results from the repeated observations on designated plants, as reported here, demonstrated that monthly applications of *S. filum* decreased the severity of the *P. graminis* subsp. *graminicola* epidemic in both years. These experiments had additional assessment methods described elsewhere (9) consisting of destructively sampled plants, as well as plants removed intact from the field for periodic observation during subsequent growth and pustule development in a glasshouse. In all cases except one of the destructive-sampling methods in one year, these observations supported the conclusion drawn in this report, that multiple applications of *S. filum* reduce overwintering and early summer stem rust populations on perennial ryegrass. In none of the experiments, as measured by any methods, did a single application of *S. filum* in the fall produce a statistically significant reduction in overwintering stem rust population.

One winter application of the fungicide Quilt significantly reduced the spring *P. graminis* subsp. *graminicola* population on the first-year stands. Integration of biocontrol and fungicide for disease management could be possible if the biocontrol agent is relatively insensitive to the fungicide controlling the rust pathogen. We determined in laboratory tests that *S. filum* germination and growth is inhibited by azoxystrobin (Quadris) and chlorothalanil (Bravo) fungicides on water agar plates but shows little inhibition in the presence of the propiconazole fungicide (Tilt), especially at field-

Table 1. Controlled inoculation experiment analysis[x]

| Year, cultivar | Treatment | Area under the disease progress curve | | | |
| | | Site 1 | | Site 2 | |
		Pustules[y]	Proportion (%)[z]	Pustules[y]	Proportion (%)[z]
2009–10					
Jet	Control	399 b	0	13 b	1 b
	P. graminis subsp. *graminicola*	1,510 a	0	1,868 a	3 b
	Infected with *S. filum*	521 b	2 a	99 b	11 a
Morningstar	Control	…	…	2 b	0
	P. graminis subsp. *graminicola*	…	…	790 ab	0
	Infected with *S. filum*	…	…	78 b	1 b
2010–11					
Jet	Control	0	0	0	0
	P. graminis subsp. *graminicola*	0	0	0	0
	Infected with *S. filum*	0	2 a	0	12 b
Morningstar	Control	0	0	0	0
	P. graminis subsp. *graminicola*	0	0	0	2 c
	Infected with *S. filum*	0	5 a	0	19 ab
Linn	Control	0	0	0	0
	P. graminis subsp. *graminicola*	0	0	0	5 c
	Infected with *S. filum*	0	1 a	0	33 a

[x] Area under the disease progress curve (AUDPC) means and statistical analysis for the number of pustules of *Puccinia graminis* subsp. *graminicola* and the proportion of *P. graminis* subsp. *graminicola* pustules infected with *Sphaerellopsis filum*. The two fungi were inoculated under controlled conditions and plants were transplanted to sites 1 and 2 in 2 years of experiments (2009–10 and 2010–11). Data show the mean area under the disease progress curve of *P. graminis* subsp. *graminicola* pustules from 1 March to the end of the sampling period, and the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* over the entire sampling period, on 10 cm of perennial ryegrass row. Significant differences between cultivar and inoculation treatments (determined by one- and two-way analysis of variance, $\alpha = 0.05$) are denoted by different letters within a column for each year. Analysis was conducted within each site but not between sites.

[y] *P. graminis* subsp. *graminicola* pustules. Area under the disease progress curve of number of *P. graminis* subsp. *graminicola* pustules per 10 cm row versus time, from 1 March to 11 June in 2009–10 and 2 June in 2010–11.
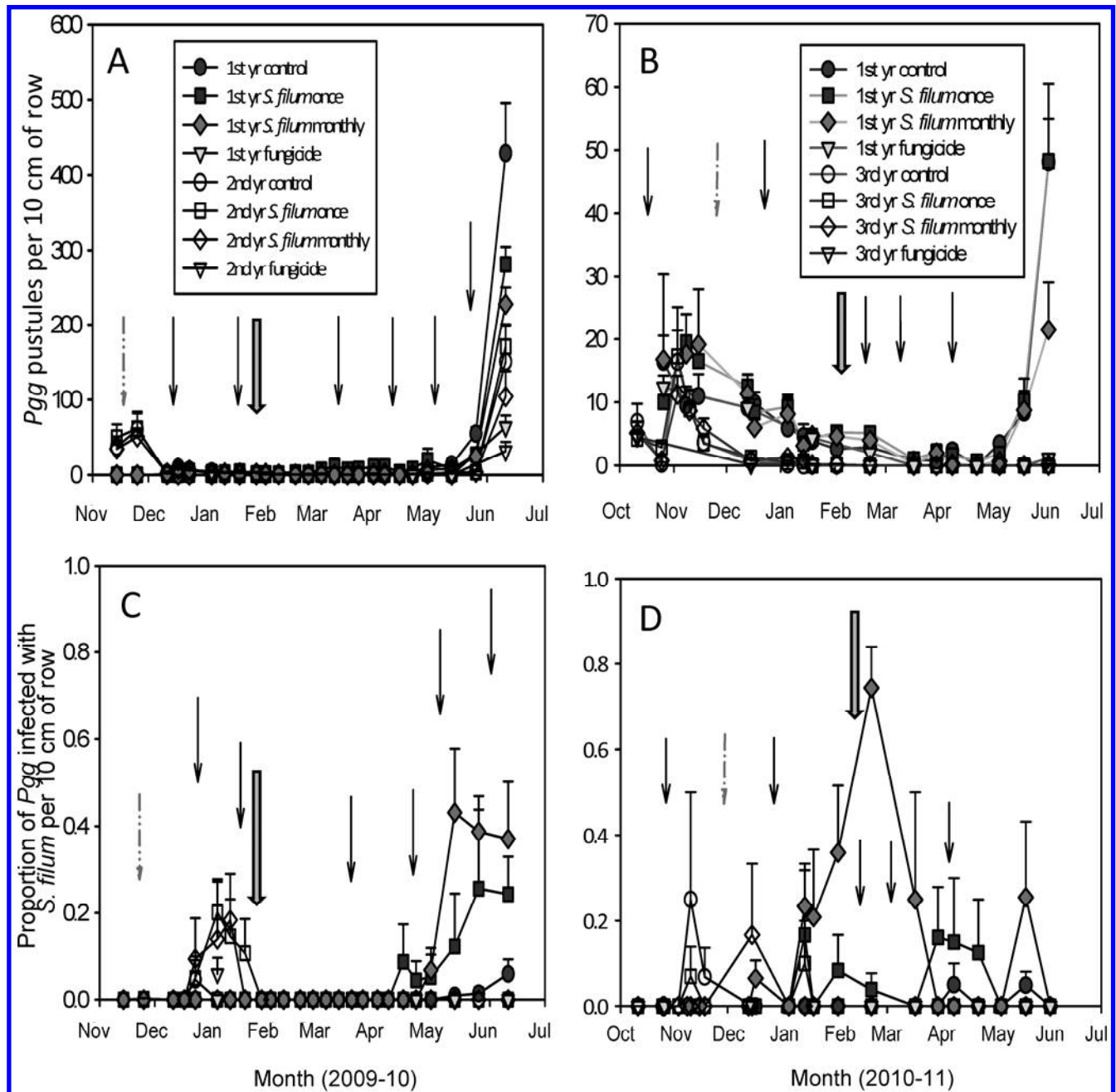
[z] Proportion of *P. graminis* subsp. *graminicola* pustules with *S. filum*. Area was calculated from the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* over the full duration of the experiment.

rate concentrations. Propiconazole has been shown to reduce latent *P. graminis* subsp. *graminicola* infections and prevent future *P. graminis* subsp. *graminicola* infections by inhibiting rust spore germination (29). *S. filum* and a propiconazole fungicide may be more effective at controlling rust disease when introduced together than when introduced independently. Considerations mitigating against the practical utility of winter fungicide application are the unpredictability of the need for fungicide (the *P. graminis* subsp. *graminicola* population sometimes crashes during the winter without any intervention), and the fact that a majority of winter days in the study region are too wet to permit fungicide application.

This experiment provided further evidence that second- and third-year stands of perennial ryegrass have lower rust populations in spring and early summer than do first-year stands (27). In 2009–

10, first-year stands had 2.8 times greater *P. graminis* subsp. *graminicola* severity as calculated from the area under the disease progress curve from 1 March until 11 June than second-year stands across all treatments and observation methods. In 2010–11, the first-year stand had a spring *P. graminis* subsp. *graminicola* severity (calculated from the area under the disease progress curve from 1 March to 2 June) that was more than 300 times greater than the third-year stand. We observed that *S. filum* can infect *P. graminis* subsp. *graminicola* pustules in first-year field stands of perennial ryegrass during Oregon winter weather conditions, but that a greater proportion of *P. graminis* subsp. *graminicola* pustules (in all treatments) on the second-year plantings than the first-year plantings were infected initially. This suggests that *S. filum* might be one of the factors that reduced the *P. graminis* subsp.

Fig. 6. Field experiment of *Sphaerellopsis filum* application to ryegrass naturally infected with *Puccinia graminis* subsp. *graminicola* (*Pgg*). **A** and **B,** Number of *P. graminis* subsp. *graminicola* pustules and **C** and **D,** the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*. Data were taken on 10 cm of row that was repeatedly observed at site 1 in **A** and **C,** 2009–10 and **B** and **D,** 2010–11. Treatments include (i) a nontreated control, (ii) one application of *S. filum*, (iii) monthly applications of *S. filum*, or (iv) Quilt fungicide applied one time on newly planted (first-year) or established (second- or third-year) stands of perennial ryegrass. Application dates for the different treatments are indicated by arrows, and are listed by treatment type: (i) one *S. filum* application: thin dashed arrow, (ii) monthly *S. filum* applications: thin solid arrows, or (iii) Quilt fungicide application: thick solid arrow. Note different y-axis scales in A and B.

Table 2. Field inoculation experiments with applications of *Sphaerellopsis filum* or Quilt fungicide at site 1 in 2009–10 and 2010–11[v]

Year, age, effect[w]	Treatment[x]	Area under the disease progress curve	
		Pustules[y]	Proportion (%)[z]
2009–10			
1	Control	4,630 a	2 b
1	Once	3,389 a	9 ab
1	Monthly	2,402 b	15 a
1	Fungicide	924 bc	0.00
2	Control	1,285 bc	1 b
2	Once	1,463 bc	4 ab
2	Monthly	951 bc	4 ab
2	Fungicide	459 c	3 ab
Block effect	…	No	No
Interaction	…	Yes	No
2010–11			
1	Control	543 a	1 b
1	Once	564 a	9 b
1	Monthly	308 ab	35 a
1	Fungicide	8 bc	0.0 b
3	Control	1 c	2 b
3	Once	0.0 c	2 b
3	Monthly	1 c	5 b
3	Fungicide	0.00	0.0 b
Block effect	…	No	No
Interaction	…	Yes	Yes

[v] Area under the disease progress curve means and statistical analysis for *Puccinia graminis* subsp. *graminicola* pustules from 1 March to the end of the sampling period, and for the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* over the entire sampling period are shown. Data are the mean area under the disease progress curve of *P. graminis* subsp. *graminicola* pustules, or the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum*, along 10 cm of perennial ryegrass row repeatedly observed across time (four replications). Significant differences (two-way analysis of variance, α = 0.05) are denoted by different letters within a column and a year.

[w] Stand age in years. Block effect = statistically significant effect of block and Interaction = statistically significant interaction between treatment and stand age.

[x] Treatments are (i) a noninoculated control, (ii) one application of *S. filum*, (iii) monthly applications of *S. filum*, or (iv) Quilt fungicide applied one time in the winter.

[y] Number of *P. graminis* subsp. *graminicola* pustules. Area was calculated from *P. graminis* subsp. *graminicola* pustule severity from 1 March to 11 June in 2009–10 and to 2 June in 2010–11.

[z] Proportion of *P. graminis* subsp. *graminicola* pustules with *S. filum*. Area was calculated from the proportion of *P. graminis* subsp. *graminicola* pustules infected with *S. filum* over the full duration of the experiment.

graminicola severity in the spring on the second-year stands. Spring levels of rust were not significantly different in check and *S. filum*-treated plots in second- and third-year stands. A possible reason that treatment effects were not observed in these established stands is that the *P. graminis* subsp. *graminicola* population crashed in late winter (February) in established plants of all treatments, including the check. Our previous observations over several years (*unpublished data*) show that the *P. graminis* subsp. *graminicola* population typically reaches its lowest level, in some years being visually undetectable, in February to March. The mechanism is unknown but perhaps leaves (and, therefore, their rust occupants) in the second-year stands have a shorter average lifetime than do leaves in first-year stands during the winter. Additionally, leaf tissue in older stands may be more resistant to *P. graminis* subsp. *graminicola* infection, or *S. filum* infections in the fall could drastically reduce the *P. graminis* subsp. *graminicola* population over the winter.

At our current level of knowledge, it would be difficult to implement use of *S. filum* as a conventional biofungicide. Growth in liquid culture is slow, and our current method of growing the fungus on PDA, harvesting the spores by hand, and immediately applying them under moist conditions is time intensive and impractical for large acreages. More effective control of *P.*
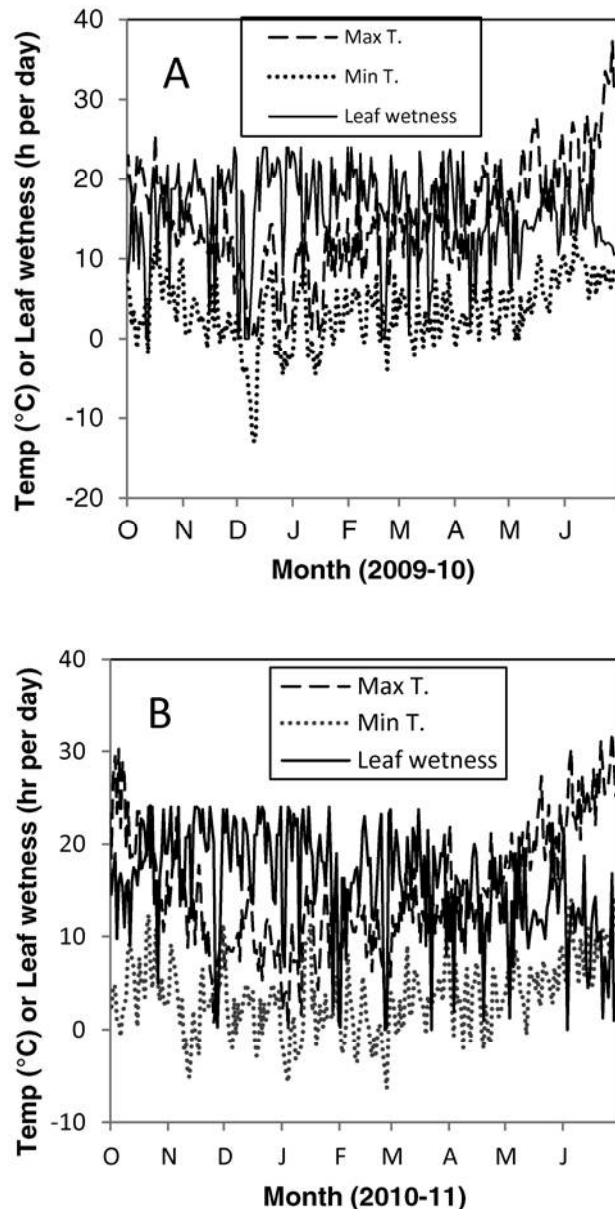
Fig. 7. Site 1 leaf wetness hours, plotted with daily high and low temperature values, for **A,** 2009–10 and **B,** 2010–11. Weather data were taken every 15 min on a data logger and analyzed for daily minimum (dotted line) and maximum (dashed line) air temperatures and hours of leaf wetness (solid line).

graminis subsp. *graminicola* with *S. filum* could be obtained by overcoming these current constraints. Finding more virulent strains of the fungus that can infect a greater proportion of *P. graminis* subsp. *graminicola* pustules and reduce the number of spores that each pustule produces could be one area to further investigate. Additionally, finding a way to apply the mycoparasite in a manner that is simple and inexpensive and insures high *S. filum* inoculum at optimal infection conditions would be necessary if this control tactic were to be used on a larger scale. A mycoparasite of powdery mildew (*Ampelomyces quisqualis*) was successfully introduced onto infected grapevines when the fungus was grown on cotton wicks saturated in malt agar (8). We observed (9) that *S. filum* can be grown on whole-grain media, and propose that such colonized grain could be applied to a field along with fertilizer applications. Increasing background levels of S. filum on rust pustules growing outside the field might also prove to be an effective means of introducing the mycoparasite. *S. filum* has a wide host range, and could be applied to rusts that do not infect perennial ryegrass growing near a commercial field.
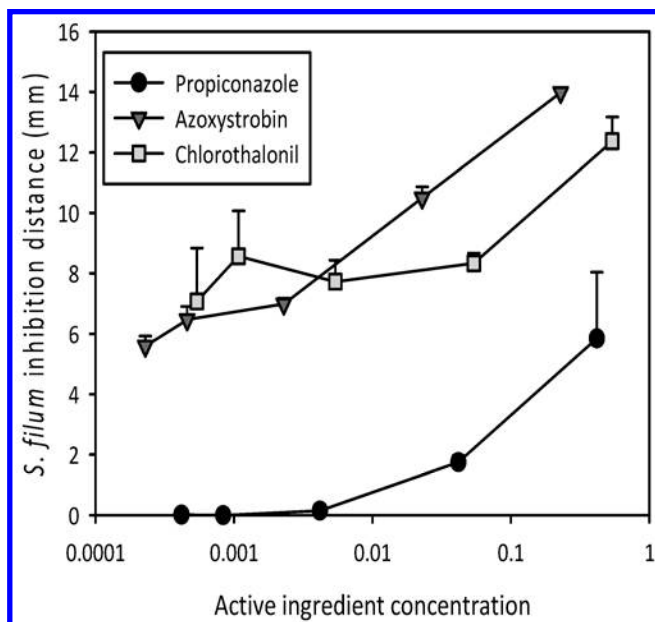
Fig. 8. *Sphaerellopsis filum* conidiospore germination inhibition distance by three fungicides. The three fungicides and their active ingredients were Tilt (propiconazole, 41.8%), Quadris (azoxystrobin, 22.9%), and Bravo (chlorothalonil, 54.0%). Filter discs (5.5 mm in diameter) saturated in serial dilutions of each fungicide (three replications at each concentration) were placed onto water agar plates that had been sprayed with a suspension of *S. filum* conidia. Plates were incubated with 12-h dark and light periods at 23°C for 5 days. The distance between the edge of the filter disc and the point where there was ≥10% germination was measured. Data shown are the mean and standard error of the mean of two trials.

Acknowledgments

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