Biological control of fusarium head blight of wheat with *Clonostachys rosea* strain ACM941

A.G. Xue, H.D. Voldeng, M.E. Savard, G. Fedak, X. Tian, and T. Hsiang

Abstract: Fusarium head blight (FHB), caused by *Gibberella zeae*, is a devastating disease of wheat. A strain of *Clonostachys rosea*, ACM941 (American Type Culture Collection ATCC 74447), was evaluated for antibiosis against *G. zeae* in vitro and for control of FHB under greenhouse and field conditions in comparison to the registered fungicide Folicur (tebuconazole). Strain ACM941 reduced mycelial growth of the pathogen by 52.6% in dual-culture after 6 days and completely suppressed spore germination for 6 h when cocultured with a macroconidial suspension of *G. zeae*. Strain ACM941 reduced *G. zeae* perithecial production by more than 99% in a leaf disk assay, 60%–77% on infected corn kernels, and 32%–57% on spikelet debris in the field. These effects were significant ($P < 0.05$) and not statistically different from those produced by tebuconazole. When strain ACM941 was sprayed onto wheat heads 2 days prior to inoculation with *G. zeae*, it significantly reduced infected spikelets (IS) by 64% and *Fusarium*-damaged kernels (FDK) by 65% in greenhouse experiments. Under simulated disease epidemic conditions during 2005–2007, strain ACM941 reduced the FHB index by 58%, IS by 46%, FDK by 49%, and deoxynivalenol (DON) in kernels by 21%. These effects were significant but lesser in magnitude than those achieved by tebuconazole, which reduced FHB index by 97%, IS by 82%, FDK by 73%, and DON by 62%. Results from this research suggest that strain ACM941 of *C. rosea* is a promising biocontrol agent against *G. zeae* and may be used as a control measure in an integrated FHB management program.

Key words: biological control, *Gibberella zeae*, fusarium head blight, *Clonostachys rosea*, wheat.

Résumé : La fusariose de l'épi (FHB), causée par *Gibberella zeae*, est une maladie dévastatrice qui s'attaque au blé. La souche ACM941 (ATCC 74447) de *Clonostachys rosea* a été évaluée, in vitro, sur le plan de l'antibiose pour lutter contre *G. zeae* et, en serre et au champ, pour lutter contre la FHB afin d'en comparer les effets à ceux du traitement fongicide homologué Folicur (tébuconazole). Au bout de six jours, lors de tests en culture duale, la souche ACM941 a réduit de 52,6 % la croissance mycélienne de l'agent pathogène et en a entièrement inhibé la germination des spores pendant six heures lorsqu'on l'a fait croître en présence d'une suspension macroconidiale de *G. zeae*. Lors d'un test biologique sur disque foliaire, la souche ACM941 a réduit la production périthéciale de plus de 99 %, de 60 % à 77 % lors d'un test sur des grains de maïs et de 32 % à 57 %, au champ, lors d'un test sur des débris d'épillets. Ces résultats étaient significatifs ($P < 0,05$) et semblables statistiquement à ceux obtenus avec le tébuconazole. Lorsque, au cours d'expériences menées en serres, la souche ACM941 a été pulvérisée sur des épis de blé deux jours avant de les inoculer avec *G. zeae*, les taux d'infection des épillets (IE) et les dommages subits par les grains affectés par *Fusarium* (DGF) ont été significativement réduits (64 % et 65 %, respectivement). Lors de la reproduction des conditions épidémiques prévalant de 2005 à 2007, la souche ACM941 a réduit l'indice de la FHB de 58 %, l'IF de 46 %, les DGF de 49 % et la teneur en désoxynivalénol (DON) dans les grains de 21 %. Ces résultats étaient significatifs, mais de moindre importance quant à ceux obtenus avec le tébuconazole qui a réduit l'indice de FHB de 97 %, l'IF de 82 %, les DGF de 73 % et la teneur en DON de 62 %. Les résultats de cette recherche suggèrent que la souche ACM941 de *C. rosea* est un agent de lutte biologique prometteur en ce qui a trait à la lutte contre *C. zeae* et qu'elle peut être utilisée comme mesure de contrôle dans un programme de lutte intégrée contre la FHB.

Mots-clés : lutte biologique, *Gibberella zeae*, fusariose de l'épi, *Clonostachys rosea*, blé.

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Introduction

Fusarium head blight (FHB), caused by *Gibberella zeae* (Schwein.) Petch (anamorph *Fusarium graminearum* Schwabe), is the most important disease of wheat (*Triticum aestivum* L.) in North America (Gilbert and Tekauz 2000; McMullen et al. 1997). The disease causes reduced grain yield and quality and kernel contamination with mycotoxins such as deoxynivalenol (DON), which are harmful to livestock and pose a safety concern in human food (Miller 1995; Placinta et al. 1999).

The use of genetic resistance is considered one of the most practical and environmentally safe measures to control FHB (Bai and Shaner 1996; Gilbert and Tekauz 2000). Considerable efforts have been made by wheat breeders to develop FHB-resistant varieties (Comeau et al. 2003; Pandeya et al. 2003; Van Ginkel et al. 2003). However, these efforts have not been completely successful due to the fact that the expression of resistance to FHB is complex (Kolb et al. 2001) and complete resistance is not available. The improvements to date have been incremental and should eventually culminate in germplasm with elevated levels of FHB resistance, through pyramiding and introducing known resistance genes, and discovery of new genes from wheat or related wild species (Fedak et al. 2001; Oliver et al. 2005; Pumphrey et al. 2007). Other cultural practice options, such as crop rotation and tillage to reduce initial inoculum, have an effect but are often limited or unsatisfactory (Gilbert and Fernando 2004; Miller et al. 1998; Shaner 2003). Current management of this disease relies on fungicides to sustain productivity and improve grain quality in most wheat production regions (Jones 2000; Matthies and Buchenauer 2000; Tekauz et al. 2003). However, fungicide application presents additional costs to producers, and their long-term usage has become a public concern because their residues may remain in food and feed products, causing potential risk to human health and the environment. The availability of alternative means to control FHB would therefore be desirable.

Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatments (McSpadden Gardener and Fravel 2002). Several fungi, bacteria, and yeasts have been reported to have antagonistic effects against *G. zeae* (Luz et al. 2003). The antagonistic effects of these microorganisms have been demonstrated by in vitro antibiosis (Chan et al. 2003) and in situ disruption of spikelet infection leading to reduced disease severities (Fulgueira et al. 1996; Khan et al. 2001; Nourozian et al. 2006; Schisler et al. 2002; Stockwell et al. 2001), reduced systemic movement of *G. zeae* within infected spikes (Yuen et al. 2003), lower mycotoxin accumulation in grain (Dawson et al. 2004; Stockwell et al. 2000), and reduced pathogen survival and subsequent ascospore production in residues (Bujold et al. 2001; Fernandez 1992). The reported antagonists of *G. zeae* include several Brazilian isolates of *Bacillus* and *Paenibacillus* species that reduced FHB severity in the field by 50%–60% and increased yield by more than 700 kg/ha (Luz 2000). To date, only *Bacillus subtilis* (Ehrenberg) Cohn strain TrigoCor 1448 and *Cryptococcus laurentii* (Kufferath) Skinner isolate OH 182.9 (syn. *C. nodaensis)* have been evaluated under diverse growing conditions across the United States for FHB control (Milus et al. 2001; Stockwell et al. 2001). Efforts are underway to develop TrigoCor 1448 and OH 182.9 as commercially viable biocontrol agents (Dunlap and Schisler 2005; Pryor et al. 2007; Zhang et al. 2005). These studies demonstrate that biological control of FHB using antagonistic microorganisms is promising.

In previous studies, a fungal strain of *Clonostachys rosea* (Link:Fr.) Schroers, Samuels, Serfert and Gams (syn. *Gliocladium roseum* Bainier), strain ACM941 (American Type Culture Collection ATCC 74447), was identified as a mycoparasite of certain fungal pathogens in plants (Xue 2002, 2003a, 2003b). When applied as a seed treatment, strain ACM941 provided protection against a number of fungal pathogens under both growth-room and field conditions (Xue 2000, 2001, 2002). It was further revealed that strain ACM941 could survive for more than 35 days after seed application. The biocontrol agent propagates and quickly establishes in the rhizosphere and colonizes the surfaces of the seed, hypocotyl, and roots as the seed germinates and the seedling plant develops and grows (Xue 2003a). FHB is a monocyclic disease in which ascospores of *G. zeae* from residue-borne perithecia are the only source of initial inoculum in Eastern Canada (Bujold et al. 2001; Fernando et al. 1997; Paulitz 1996; Sutton 1982). A reduction in the number of perithecia of *G. zeae* on crop residues should have a significant impact on successful management of FHB by diminishing infection pressure during the growing season when weather conditions favour disease epidemics. The objectives of this research were to evaluate strain ACM941 for its in vitro antagonistic effects against *G. zeae*, its ability to inhibit perithecial production by *G. zeae*, and its efficiency in reducing FHB disease severity, *Fusarium*-damaged kernels (FDK), and DON levels under both greenhouse and field conditions in comparison with tebuconazole, a widely used fungicide for control of FHB.

Materials and methods

Strain ACM941 and pathogen inoculum production

Three isolates of *G. zeae* (DAOM 178148, DAOM 212678, and DAOM 232369) obtained from the Canadian Collection of Fungal Cultures at the Eastern Cereal and Oilseed Research Centre (ECORC), Ottawa, Canada, were used for this study. These isolates were chosen because they are known to be aggressive (Xue et al. 2004). Only isolate DAOM 232369 was used for the in vitro studies, but all three isolates were used for greenhouse and field components. The isolates were cultured on a modified potato dextrose agar (PDA, 10 g/L of dextrose amended with 34 μmol/L streptomycin sulfate) and incubated at 22–25 °C under mixed ultraviolet (UV) and fluorescent lighting on a 12 h light : 12 h dark cycle for 14 days. The modified PDA medium was used to reduce mycelium growth, possible mutation, and poor vigour and increase spore production by the pathogen (Xue et al. 2004). To prepare inoculum, 0.5 mL of a concentrated macroconidial suspension (approx. 10^7 spores/mL) obtained from the isolates was spread over the surface of the modified PDA in 9 cm Petri dishes and incubated as previously described for 48 h. Ten millilitres of sterile dis-

tilled water containing 0.01% Tween 20 (polyoxyethylene sorbitan monolaurate) was then added to each dish, and the surface was scraped gently with a sterile microscope slide to dislodge spores. The resulting conidial suspension was filtered through two layers of cheesecloth and adjusted to 5×10^4 spores/mL using a haemocytometer. Separate conidial suspensions were prepared for each isolate. The final suspension used for greenhouse and field inoculations consisted of a 1:1:1 mixture of each of the three *G. zeae* isolates.

The *C. rosea* ACM941 strain used in this study was originally recovered from a field pea plant in 1994 (Xue 2000). The fungus was stored by freeze-drying and kept at –20 °C in ampoules. Cultures of the fungus were established by transferring freeze-dried fungal material at approximately 0.01 g per Petri dish containing PDA and incubating these dishes at 22–25 °C under the mixed UV and fluorescent lighting. Spore suspensions of ACM941 were prepared using the same method as that for the pathogen isolates. Cultures of the pathogen and ACM941 were stored at 4 °C and transferred at 3 month intervals during the course of this study.

In vitro antibiosis

The effect of strain ACM941 on mycelial growth of *G. zeae* isolate DAOM 232369 was tested using a dual-culture protocol by placing two 5 mm mycelial disks, each from margins of 7-day-old PDA cultures of ACM941 and *G. zeae*, on PDA in 9 cm Petri dishes with three replications. The two mycelial disks were spaced 4 cm apart and deposited within 30 min. As the control, a PDA disk was used instead of strain ACM941. The effect of strain ACM941 was compared to that of Folicur 432F (43.2% tebuconazole, Bayer CropScience Inc., Calgary, Alta.), the only fungicide registered for control of FHB in Canada at the time of these experiments; a 5 mm PDA disk amended with 65 μmol/L tebuconazole was used instead of strain ACM941. The test plates were incubated at 25 °C with a 12 h light : 12 h dark cycle, and on the sixth day the radius of the *G. zeae* colonies was measured on a straight line between the two disks. The experiments were repeated twice. Inhibition of growth was calculated using the following formula: % inhibition = $(a - b)/a \times 100$, where a is the *G. zeae* colony radius in the untreated control, and b is the colony radius in ACM941 or tebuconazole treatments.

The effect of strain ACM941 on the germination of macroconidia of *G. zeae* DAOM 232369 was examined by coculturing 50 μL of a *G. zeae* macroconidial suspension with 50 μL of a conidial suspension of ACM941 on a hanging-drop slide with three replications and incubated at room temperature (21–23 °C). The spore suspensions for both organisms were adjusted to 5×10^6 spores/mL using a haemocytometer. The percent germination after 6 h and 9 h of incubation was determined using a phase-contrast microscope (Nikon Canada Inc., Mississauga, Ont.) at 400× magnification. Controls included combinations of 50 μL of sterile distilled water or 50 μL of 65 μmol/L tebuconazole mixed with the *G. zeae* macroconidial suspension. At least 300 macroconidia were examined for each replication, and the experiment was conducted twice. Macroconidia were considered to have germinated when the germ tubes exceeded their lateral radius, as suggested by the American Phytopathological Society Committee on the Standardization of Fungicidal Tests (American Phytopathological Society 1943).

Effect of ACM941 on perithecial production

Leaf disk assay

Seedlings of wheat 'Roblin' grown in a controlled environment for 5–6 weeks were used to prepare leaf disks. The fully grown third and fourth seedling leaves were then cut in the middle of the leaf blade with a sterile cork borer to produce 6 mm diameter leaf disks. The leaf disks were sterilized in an autoclave at 121 °C for 15 min. Nine random leaf disks were placed on 1% water agar (Difco-Bacto) in a 9 cm Petri dish. Approximately 30 min before the placement of the leaf disks, each Petri dish received 1.0 mL of *G. zeae* isolate DAOM 232369 macroconidial suspension (5×10^6 spores/mL) evenly spread on the surface of the water agar using a sterilized glass rod. Petri dishes were incubated at 22–25 °C under mixed UV and fluorescent lighting and a 12 h light : 12 h dark cycle for 2 days to induce the production of perithecia on the disks. The leaf disks within a Petri dish were individually treated with three 20 μL droplets of strain ACM941 spore suspension (5×10^6 spores/mL), 65 μmol/L of tebuconazole, or sterile distilled water. The three droplets were adequate to deposit and spread the liquids on each disk but not to overflood the disk. Petri dishes were sealed and incubated as previously. The presence and number of perithecia on each leaf disk were examined using a dissecting microscope (Nikon SMZ 1500) at 4, 7, 12, 17, 22, 27, and 37 days after treatment. The experiment was conducted twice, and each was arranged in a completely randomized design with a total of 30 disks in 10 replicate Petri dishes per treatment.

Corn kernel assay

Mixed barley and corn kernels that were autoclaved, inoculated with isolate DAOM 232369 of *G. zeae*, incubated at 20–25 °C for 3 weeks, and air dried were used for this study. The production of mixed barley and corn kernel inocula was described by Xue et al. (2006). For easy assessments of perithecial production on tissue surface, we used corn kernels only. Ten randomly infected corn kernels were subsequently placed in a bag (6 cm × 10 cm) of black nylon mesh (0.2 cm × 0.2 cm) (Saint-Gobain Technical Fabrics, Albion, N.Y.), and the bags were individually sprayed with an ACM941 spore suspension at 5×10^6 spores/mL, 65 μmol/L tebuconazole, or sterile distilled water. The treatments were applied to incipient runoff using a DeVilbiss model 15 atomizer (The DeVilbiss Co., Somerset, Penn.). After the bagged kernels were air dried for 30 min, the bags were taken to the FHB disease nursery of the Central Experimental Farm, Ottawa, Ontario, and scattered evenly by hand on the soil surface between rows of growing wheat plants. Bags were each placed 10 cm apart in a random block design, and the placement of bags was done on 29 June in 2005 and 28 June in 2006 when plants were at the booting stage (Zadoks growth stage ZGS 43) (Zadoks et al. 1974). Bags were retrieved five times at 5 day intervals in 2005 and four times at 5 to 8 day intervals in 2006 starting 5 days after the placement. On each sampling date, four bags from each treatment block were selected at random,

rinsed under running water to wash off adhering soil, and dried using paper towels. The perithecial production on each kernel was examined under a dissecting microscope (Nikon SMZ 1500) and the number of perithecia, including both mature and immature perithecia, estimated using the method described by Bujold et al. (2001) within 12 h of the sample retrieval. Perithecia were counted on the upper surface of the corn kernel.

Debris assay

Naturally infected wheat spikes of FHB-susceptible wheat, 'AC Foremost', were collected from the FHB disease nursery of the Central Experimental Farm, Ottawa, in the 2004 and 2005 field seasons for use in the experiments in the 2005 and 2006 field seasons, respectively, as the "debris" for this assay. Individual spikelets at maturity from the diseased portion of spikes were cut and any awns removed. The spikelets were air dried and stored at 4 °C until use in June of the following year. Ten spikelets, taken at random, were placed in a black nylon mesh bag (6 cm × 10 cm) and treated with strain ACM941, tebuconazole, or sterile distilled water as described earlier for the corn kernel assay. The experimental design, field placement, sample collection, and method of assessing perithecial production were also the same.

Greenhouse disease management trials

A greenhouse experiment to manage FHB was conducted at ECORC, Ottawa, Ontario, during the winter of 2004 and repeated in spring 2005. The FHB-susceptible wheat 'Roblin' was used to test the efficacy of strain ACM941 as a biocontrol agent. Seed was planted in 15 cm diameter pots containing a mixture of loam soil, sand, and composted cow manure (1:1:1, *v*/*v*/*v*) and maintained at 23–25 °C during the day and 18–20 °C at night in a greenhouse. Supplemental light was provided by 300 W metal halide lamps to ensure a 16 h photoperiod and a minimum intensity of 360 $mol \cdot m^{-2} \cdot s^{-1}$. Plants were thinned to three individuals per pot shortly after emergence and fertilized with a 1% solution of 20–20–20 (N–P–K) after 5 weeks.

At anthesis (ZGS 65), wheat spikes were sprayed with a spore suspension of strain ACM941 at a concentration of 5×10^6 spores/mL, 65 µmol/L tebuconazole, or sterile distilled water. The treatments were applied to incipient runoff using a DeVilbiss model 15 atomizer. Following inoculation, plants were kept at room temperature for 3 h to allow the treated spikes to air dry and then were inoculated with a spore suspension of 5×10^4 conidia/mL from equal parts of the three isolates of *G. zeae*. The inoculum was sprayed on all spikes as described previously. Thirty minutes later, plants were transferred to a polyethylene humidity chamber in a growth room operated at 25 °C with a 12 h photoperiod at a light intensity of 250 $mol \cdot m^{-2} \cdot s^{-1}$ for 48 h. The humidity chamber was maintained at or near 100% relative humidity by the continuous operation of two ultrasonic humidifiers, and air temperature and humidity in the chamber were monitored with a portable datalogger (model 21XL Micrologger, Campbell Scientific Canada Corp., Edmonton, Alta.). Plants were subsequently returned to the greenhouse bench. For each treatment within an experiment, 24 replicate pots of three plants each were used. Pots were arranged in a randomized complete block design in both greenhouse and humidity-chamber settings.

Symptoms of FHB were rated 21 days after inoculation, when plants were at the soft dough stage, as the percentage of infected spikelets (IS) per spike for all spikes in a pot and then averaged per pot. Plants were hand harvested at maturity and hand threshed after air drying in the greenhouse. The percentage of *Fusarium*-damaged kernels (FDK; kernels with a chalky-white appearance) was determined with the assistance of a magnifying lamp for all kernels and averaged per pot.

Field disease management trials

Field experiments were conducted at ECORC, Ottawa, Ontario, in each of 2005, 2006, and 2007. The FHB intermediately resistant wheat 'AC Barrie' was used to test the efficacy of *C. rosea* strain ACM941 compared to tebuconazole. Experiments were arranged as a one-factor factorial design with four replicates per year. Plants were grown in two-row plots, 1.5 m long with 18 cm row spacing. Plots were seeded at a rate of 97 kg/ha in a clay loam soil in the second week of May each year and fertilized based on soil test recommendations. Appropriate herbicides for efficient weed control were applied according to standard management practices (OMAFRA 1999).

All plots were inoculated with two 50 g applications of infected barley and corn kernels that had been infected separately with the three isolates of *G. zeae* (Xue et al. 2006) about 3 and 2 weeks, respectively, before anthesis (ZGS 15–30). The infected kernels were scattered evenly by hand between the two rows of each plot. Sprinkler irrigation was applied daily for 0.5 h in the morning and afternoon (excluding rainy days), starting with the first inoculation and continuing until about 3 weeks after anthesis, when plants were at the soft dough stage.

Strain ACM941 and tebuconazole treatments were applied twice in each growing season, at 50% anthesis (ZGS 65) and 3–4 days later. At each application, approximately 50 mL of a conidial suspension of strain ACM941 at 5×10^6 spores/mL, tebuconazole at the recommended rate for cereals, or sterile distilled water as the untreated control was sprayed evenly onto the spikes in each plot using a polyethylene tank compressed-air sprayer (Chapin Manufacturing Inc., Batavia, N.Y.). The treatments were applied in late afternoon approximately 2 h before sunset.

At heading, prior to the appearance of symptoms, 10 randomly selected spikes from each plot were labelled with tags. At the soft dough stage the percentage of IS was determined for the 10 selected plants. In addition, the plot disease severity using a population of approximately 200 spikes per plot was estimated by assessing both incidence (percentage of infected spikes) and severity (proportion of affected spikelets of the infected spikes). An FHB index (= incidence × severity/100) was derived to provide a measure of plot disease severity (Groth et al. 1999).

Plants were hand-harvested at maturity, air dried, and threshed during the second week of August each year using an LPT-Stationary Type Plot Thresher (ALMAGO, Nevada, Iowa) to ensure the capture of all small, shrivelled kernels. Percentage of FDK was determined on 300 randomly selected seeds per plot with the aid of a magnifying lamp.

Table 1. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) on mycelial growth of *Giberella zeae* in dual-culture experiments.

	Colony radius (mm)				
Treatment	Trial 1	Trial 2	Trial 3	Mean	Inhibition (%)
ACM941	16.8 b	32.5 b	20.2 b	23.2 c	52.6
Folicur	21.8 b	33.5 b	22.9 b	26.1 b	46.1
Untreated	42.0 a	56.1 a	45.8 a	48.0 a	0

Note: Colony radius was measured after 6 days of incubation in dual culture. Values within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected least significant difference (LSD) test.

Table 2. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) on conidial germination of *Giberella zeae* in coculture experiments.

	Conidial germination (%)					
	Trial 1		Trial 2		Mean	
Treatment	6 h	9 h	6 h	9 h	6 h	9 h
ACM941	0 b	44.7 b	0 b	52.9 b	0 b	48.8 b
Folicur	0 b	41.1 b	0 b	47.2 b	0 b	44.1 b
Untreated	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a

Note: To stabilize variances, the percent germination was arcsine square root transformed prior to analysis. Values are based on the mean of three counts and more than 300 microconidia per count. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected least significant difference (LSD) test.

Analyses to determine DON levels were done at the Mycotoxin Research Laboratory of ECORC using a 30 g seed sample from each plot. Samples were ground to a fine powder in a Retsch Ultra Centrifugal Mill Type ZM1 (Brinkman Instruments, Inc., Rexdale, Ont.) filtered with a 0.75 mm wire mesh. From each ground sample, a 1.0 g subsample was used for DON analysis. The concentration of DON was determined by the competitive direct enzyme-linked immunosorbent assay (CD-ELISA) procedure using monoclonal antibodies (MABs) as described by Sinha et al. (1995).

Statistical analyses

The mycelial radius and the number of perithecia were subjected to analysis of variance without transformation. Variance was stabilized using an arcsine square root transformation for percent spore germination, FHB index, IS, and FDK and logarithmic transformation of DON values (Snedecor and Cochran 1980). For greenhouse and field experiments, the residuals for each parameter and year were examined for normality and homogeneity of variances. A combined analysis over experimental repeats or years was conducted using the general linear models procedure in SAS (SAS version 8.1 1998, SAS Institute Inc., Cary, N.C.). Treatment means were separated by Fisher's protected least significant difference (LSD) test at a probability level of $P \leq 0.05$, where treatment effects were significant.

Results

In vitro antibiosis

Strain ACM941 significantly inhibited the mycelial growth of *G. zeae* in dual-culture in all three trials (Table 1). On average, strain ACM941 reduced colony mycelial growth by 52.6%. The effect was greater but not significantly different from that of tebuconazole. Both strain ACM941 and tebuconazole completely suppressed spore germination in coculture with a macroconidial suspension of *G. zeae* at 6 h, whereas the untreated control spore germination was 100% in both trials (Table 2). At 9 h, strain ACM941 inhibited spore germination by 51.2%, an effect that was lower but not significantly different from that of tebuconazole.

Leaf disk, corn kernel, and debris analysis

Abundant perithecia of *G. zeae* were produced on each of the leaf disks, corn kernels, and wheat spikelets of the untreated controls (Fig. 1A). When treated with strain ACM941 or tebuconazole, the number of perithecia was significantly lower on all the substrates used (Figs. 1B, 1C). In the leaf disk assay, strain ACM941 significantly inhibited perithecial production over the entire 37 day period after the plant tissue was treated in each of the two trials (Fig. 2A). Averaging both trials and all seven assessments in each trial, strain ACM941 reduced perithecial production by more than 99% based on the leaf disk assay. The effect was greater than, but not significantly different from, that of tebuconazole.

In both the corn kernel and wheat debris assays, perithecia were more abundant in 2005 than 2006 (Figs. 2B, 2C). There was a significant interaction between year and treatment for both the corn kernel and wheat debris bioassays. The inhibition of perithecial production on corn kernels by strain ACM941 was observed in both 2005 and 2006 (Fig. 2B). The biocontrol agent significantly reduced the number of perithecia by 60.3% based on the average of

Fig. 1. Perithecial production by *Gibberella zeae* on leaf disks, corn kernels, and wheat debris (spikelets) 25 days after inoculation: (A) untreated controls (left); (B) treated with strain ACM941 of *Clonostachys rosea* (middle); (C) treated with Folicur (tebuconazole) fungicide (right).

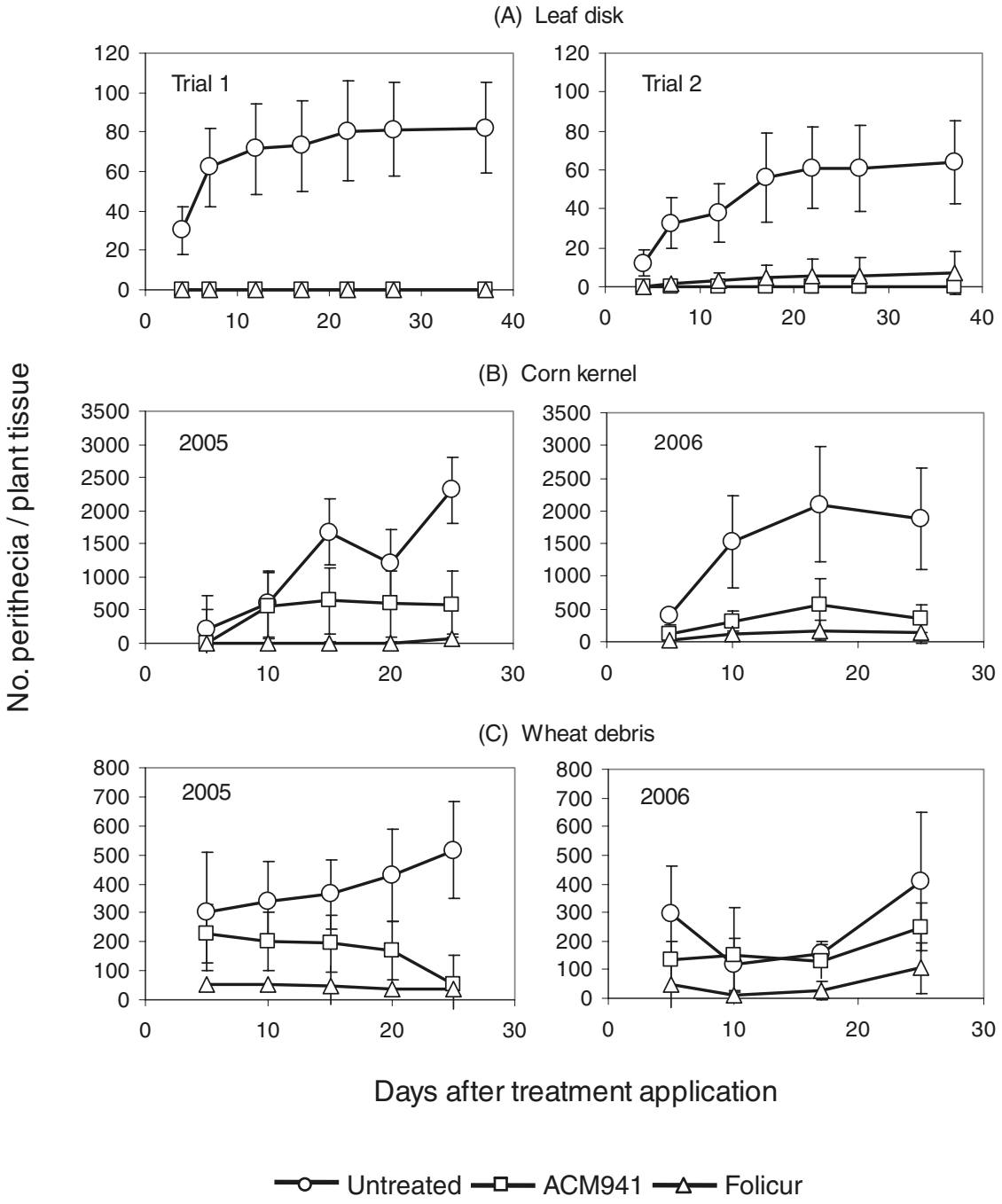
A B C

five assessments over the period of 25 days in 2005 and by 77.1% based on four assessments in 2006 (Table 3). In wheat debris assay, the significant effect of strain ACM941 in inhibiting the production of perithecia was observed over all five assessments in the 25 day period in 2005, and in two of the four assessments over the 25 day period in 2006 (Fig. 2C). Averaged over all assessments, strain ACM941 reduced the number of perithecia produced on infested wheat spikelets by 56.7% in 2005 and 32.3% in 2006 (Table 3). Except for corn kernels in 2005, the inhibition of perithecial production by strain ACM941 was less than, but not significantly different from, that of tebuconazole in both the corn kernel and debris assays.

Greenhouse and field trials

Strain ACM941 significantly reduced IS and FDK levels in both 2004 and 2005 greenhouse trials (Table 4). Over the two trials, strain ACM941 reduced IS by 64.1% and FDK

Fig. 2. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) fungicide on number of perithecia of *Gibberella zeae* produced in vitro using a leaf disk assay (A) and in the field using a corn kernel assay (B) and a wheat debris (spikelets) assay (C).



by 65.0% compared with the untreated controls. These effects were less pronounced than those of tebuconazole, which reduced IS by 94.7% and FDK by 98.9%.

A moderate to severe epidemic of FHB developed in the FHB disease nursery at ECORC in each of 2005, 2006, and 2007. The treatment effects were highly significant ($P < 0.01$) for all parameters measured in each year (Table 5). A significant effect of year ($P < 0.05$) was also observed for FHB index, FDK, and DON and a year × treatment interaction for FHB index and IS. The effect of year × treatment interactions, although significant, was relatively small, with less than 1% of the treatment effect. Strain ACM941 treatment significantly reduced IS, FHB index, and FDK in all 3 years and DON in 2006 and 2007 (Table 6). Over 3 years, strain ACM941 reduced FHB index by 57.9%, IS by 45.9%, FDK by 49.1%, and DON by 21.1% compared with the untreated controls. These effects were less than those of the tebuconazole, which reduced FHB index by 96.9%, IS by 81.8%, FDK by 72.8%, and DON by 62.0%.

Table 3. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) on production of perithecia of *Giberella zeae* on leaf disks, infested corn kernel, and wheat debris (spikelets) in assays done in vitro or under simulated field epidemic environments in 2005 and 2006.

	No. of perithecia					
	Leaf disk (diameter d = 5 mm)		Corn kernel		Spikelet	
Treatment	Trial 1	Trial 2	2005	2006	2005	2006
ACM941	0.0 b	0.0 b	476.9 b	337.3 b	168.9 b	166.1 b
Folicur	0.2 b	3.9 b	18.7 c	111.8 b	45.5 b	46.7 b
Untreated	68.7 a	46.4 a	1202.1 a	1472.2 a	390.4 a	245.5 a

Note: Values are the average number of perithecia per leaf disk, corn kernel, or spikelet from seven assessments for each trial of leaf disk assay and five and four assessments, respectively, for 2005 and 2006 for both corn kernel and wheat debris assays. The assessments started at 5 days after inoculation, and there were 5 to 8 day intervals between assessments. Values within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected least significant difference (LSD) test.

Table 4. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) on percentage of infected spikelets (IS) and fusarium damaged kernels (FDK) of 'Roblin' wheat in greenhouse trials.

	Trial 1		Trial 2		Mean	
Treatment	IS (%)	FDK (%)	IS (%)	FDK (%)	IS (%)	FDK (%)
ACM941	19.3 b	26.3 b	11.7 b	12.6 b	15.5 b	19.5 b
Folicur	3.8 c	0.9 c	0.8 c	0.3 c	2.3 c	0.6 c
Untreated	45.7 a	64.8 a	40.7 a	46.5 a	43.2 a	55.7 a

Note: Values within a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected least significant difference (LSD) test.

Table 5. Mean squares from individual and combined analysis of variance for effects of treatment and year on the efficacy of strain ACM941 of *Clonostachys rosea* in reducing FHB index, percentage of infected spikelets (IS), percentage of fusarium-damaged kernels (FDK), and deoxynivalenol (DON) content in kernels of 'AC Barrie' wheat under a simulated disease epidemic environment in field trials from 2005 to 2007.

Source of variance	df	FHB index	IS	FDK	DON
Individual analysis					
2005					
Replicate	3	4.3	3.7	20.4**	0.3*
Treatment	2	241.2**	324.5**	84.2**	0.5**
Error	6	11.4	17.1	0.6	0.0
2006					
Replicate	3	14.2	22.8	36.3	0.0
Treatment	2	1406.6**	1041.0**	105.6**	0.9**
Error	6	18.2	25.8	14.0	0.0
2007					
Replicate	3	93.0	101.5*	13.0	0.2
Treatment	2	404.1**	280.4**	124.4**	1.6**
Error	6	30.8	13.7	12.3	0.1
Combined analysis					
Replicate	3	45.9	27.8	32.6	0.1
Year	2	297.0*	220.9	138.0*	11.4**
Error A	6	32.8	50.1	18.6	0.2
Treatment	2	1780.4**	1465.7**	292.8**	2.7**
Year × treatment	4	135.7*	90.0*	10.7	0.1
Error B	18	20.1	18.8	9.0	0.1

Note: To stabilize variances, the FHB index and IS and FDK data were arcsine square root transformed and DON content was logarithm transformed prior to analysis. df, degrees of freedom. *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

Table 6. Effect of strain ACM941 of *Clonostachys rosea* and Folicur (tebuconazole) on FHB index, percentage of infected spikelets (IS), percentage of fusarium damaged kernels (FDK), and deoxynivalenol (DON) content in kernels of 'AC Barrie' wheat under a simulated disease epidemic environment in field trials from 2005 to 2007.

Treatment	FHB index (%)	IS (%)	FDK (%)	DON (ppm)
2005				
ACM941	7.1 b	13.8 b	4.5 b	1.3 a
Folicur	1.2 c	4.2 c	1.1 c	0.8 b
Untreated	13.7 a	24.6 a	6.7 a	1.7 a
2006				
ACM941	16.7 b	27.8 b	9.1 b	8.6 b
Folicur	0.6 c	4.7 c	5.3 b	4.8 c
Untreated	44.8 a	49.3 a	15.8 a	12.0 a
2007				
ACM941	8.2 a	16.2 b	3.7 b	6.9 b
Folicur	0.7 b	10.6 b	2.8 b	2.5 c
Untreated	17.8 a	33.3 a	11.6 a	7.7 a
Mean				
ACM941	10.7 b	19.3 b	5.8 b	5.6 b
Folicur	0.8 c	6.5 c	3.1 c	2.7 c
Untreated	25.4 a	35.7 a	11.4 a	7.1 a

Note: To stabilize variances, the FHB index, IS and FDK data were arcsine square root transformed and DON content was logarithm transformed prior to analysis. Values within a column in each year followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected least significant difference (LSD) test.

Discussion

Strain ACM941 of *C. rosea* was previously reported to be a mycoparasite of a number of fungal plant pathogens (Xue 2002, 2003a, 2003b), but it has not been shown to produce any antifungal compounds. Inhibition of growth (clear zones) was observed between the colonies of *G. zeae* and strain ACM941 in the dual-culture experiments, as was the suppression of spore germination in the coculture experiments in the present study. These antifungal effects might have been due to one or more diffusible inhibitory substances produced by the biocontrol agent. Further research is needed to evaluate the antagonistic mechanisms involved and the identification and purification of any antifungal compounds produced by strain ACM941.

Strain ACM941 of *C. rosea* significantly suppressed perithecial production on leaf disks, corn kernels, and wheat debris over a period of 25 or 37 days after the previously *G. zeae* infected plant tissues were treated with the biocontrol agent. Our results are similar to those reported for the antagonist *Micropshaeropsis* sp. (isolate P130A) against *G. zeae* (Bujold et al. 2001), except that in the present study strain ACM941 was applied only after the plant substrates had become infested by the pathogen. Previous research demonstrated that strain ACM941 is a good plant surface colonizer (Xue 2002, 2003a, 2003b). An early application of strain ACM941 would allow the biocontrol agent to occupy the plant tissues before the establishment of the pathogen. Consequently, the efficacy of strain ACM941 in suppressing perithecium production might be greater when it was applied to the plant tissues before the pathogen.

The reduction in *G. zeae* perithecial production on infested corn kernels and wheat spikelets following the treatment with strain ACM941 under field conditions may have a significant practical implication for biological control of FHB through the management of crop residues. Ascospores from residue-borne perithecia are the only significant inoculum source in Eastern Canada (Bujold et al. 2001; Fernando et al. 1997; Paulitz 1996; Sutton 1982). Results from this study suggest that it may be possible to reduce the levels of *G. zeae* initial inoculum by applying a biocontrol agent to crop residues. This approach has been previously used successfully in wheat against the tan spot pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (Pfender 1988; Pfender et al. 1993) and in rose against the botrytis blight pathogen *Botrytis cinerea* Pers. (Morandi et al. 2003). Further experimentation will be needed to confirm the efficacy of strain ACM941 in reducing the *G. zeae* initial inoculum under natural field conditions and its impact in an integrated FHB management program.

As part of this study, we developed an in vitro method for the production of perithecia of *G. zeae* on wheat leaf disks. The leaf disk assay was an efficient method to quantify suppression of perithecial production by strain ACM941 with little variation among the replicates within a treatment for all the sampling dates. In addition, the leaf disk assay offers several advantages in comparison to the corn kernel and wheat debris assays. The pathogen produced a smaller number but a more uniform and even distribution of perithecia on leaf disks than on other substrates, making the quantification of perithecia more accurate and time efficient. The leaf disk procedure requires less space and labour for substrate preparation and its inoculation compared with those for the corn kernel or wheat debris assay. Since the assay was conducted in a controlled environment, this avoided the possible confounding effects of other inoculum sources or abiotic stresses present in the field. The leaf disk assay should be suitable for screening large numbers of candidate biocontrol agents against *G. zeae*. This technique had been used effectively in a previous study using *Microsphaeropsis* sp. (isolate P130A) to inhibit ascospore production by *Venturia inaequalis* (Cooke) Wint., the causal agent of apple scab (Philion et al. 1997).

Strain ACM941 significantly reduced IS and FDK in greenhouse studies and the FHB index, IS, FDK, and DON in field studies. However, strain ACM941 was less effective than tebuconazole. Similarly, Milus et al. (2001) reported that the biocontrol products TrigoCor1448 and OH 182.9, selected as the most effective antagonists against *G. zeae*, were less effective than chemical fungicides in reducing FHB incidence and DON levels under field conditions in the United States. The efficacy of biocontrol agents relative to commercial fungicides has not been evaluated for other reported biocontrol agents on wheat (Dawson et al. 2004; Khan et al. 2001, 2004; Nourozian et al. 2006; Schisler et al. 2002). In separate studies, we have demonstrated that the efficacy of strain ACM941 to control FHB can be enhanced when applied to moderately resistant wheat cultivars by fine-tuning the timing of application, practicing effective

crop residue management, and combining the biocontrol agent with a reduced rate of fungicide (Xue et al. unpublished). Work on optimizing the fermentation process and developing commercial formulations of strain ACM941 as a registered biological control product in Canada is underway.

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