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(54) Title: FUSARIUM AND FUSARIUM MYCOTOXIN BIOCONTROL

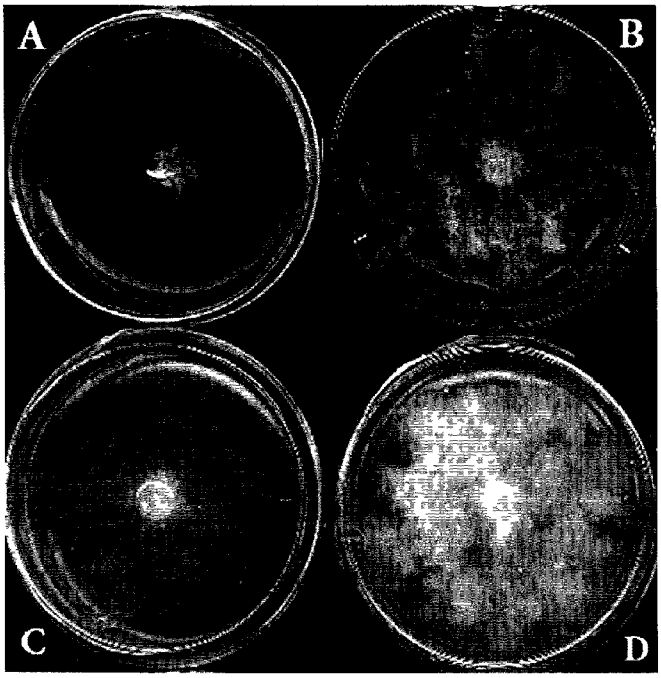

Figure 1

(57) Abstract: The present invention relates to a novel ascomyceteous fungus, *Sphaerodes mycoparasitia* strain IDAC 301008-01, for controlling *Fusarium* plant pathogens, disease symptoms, and mycotoxins *in planta* and *ex planta.* Uses, methods, compositions, sequences, and products are also disclosed herein.

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FUSARIUM AND FUSARIUM MYCOTOXIN BIOCONTROL

FIELD QF THE INVENTION

The present invention relates to novel biocontrol agents, related compositions with mycocidal effect and uses thereof. In particular, the present agent has a mycocidal effect on fungi of the genus *Fusarium.* The present invention further relates to the isolated fungal inoculant, genes, proteins and/or organisms as well as uses, methods, compositions, involving the same.

BACKGROUND TO THE INVENTION

Fusarium is a filamentous fungus widely distributed on plants and in the soil. Certain *Fusarium* species are plant pathogens. For example, *Fusarium oxysporum,* causes *Fusarium* wilt disease in more than a hundred species of plants. It does so by colonizing the plant xylem which can result in blockage and breakdown. When this occurs symptoms such as leaf wilting and yellowing appear in the plant eventually leading to the plant's death. This condition was the primary cause of the decline and disappearance of the Gros Michel banana cultivar from markets around the world. Recently a new strain has begun attacking plants of the dominant Cavendish cultivar leading to fears that, in the absence of a solution, this cultivar will too disappear from world markets.

Fusarium root rot is a major cause of seedling mortality in forest nurseries and also causes reduced survival after outplanting during the first growing season. The disease is caused by several *Fusarium* species and is common in many parts of the world. The disease is a particular problem in Western Canada and the United States and also in the North Central and Southern States. In addition, Pine pitch canker - caused by the fungus *Fusarium circinatum* - is a serious disease of pine trees and a threat to the forest industry in particularly in the US and New Zealand. Radiata or Monterey pine is highly susceptible to the disease with mortality rates in mature trees reaching 80% in some areas of California.

Fusarium Head Blight (FHB), also known as 'ear blight' or 'scab', is a disease of wheat, barley, oats and other small cereal grains caused by *Fusarium.* Corn and maize can be affected

by a similar condition known as 'ear rot'. The aforementioned condition can reduce the yield and grade of the crop, and can potentially contaminate the grain with mycotoxins. It is estimated that FHB costs the cereal industry almost $5 billion annually. In recent years, FHB has proved a significant and growing problem for the commercially important wheat and barley crops in Western Canada. In the past *Fusarium oxysporum* and *Fusarium avenaceum* have been identified as the two species primarily associated with FHB in Canada wherein yields in some affected fields have been reduced by 30% or more. FHB and ear rot harvested grain is often contaminated with mycotoxins such as deoxinivalenol (DON) trichothecenes associated with feed refusal, general digestive disorders, diarrhea, hemorrhages. The emergence of the toxigenic 3-acetyldeoxynivalenol (3-ADON) *Fusarium graminearum* population in North America is a fairly recent phenomenon. It is replacing 15-ADON chemotype. Moreover, the mycotoxin profiles of *F. culmorum* - similar to *F. graminearum* under both laboratory and field conditions-represents an additional threat for corn, maize and wheat production worldwide. Currently, there are no effective control measures for FHB and associated mycotoxins. Additionally, there are also no resistant varieties of corn, wheat, barley, oats, or other small grain cereals. Fungicides can be effective but only temporarily suppress the disease.

In addition to being a common plant pathogen, *Fusarium* spp. can opportunistically infect animals and can be the causative agents of superficial and/or systemic infections in humans. *Fusaria* are one of the most drug-resistant fungi making *Fusaria* infections difficult to treat and invasive infections can prove fatal.

SUMMARY OF THE INVENTION

The present invention relates to a novel ascomyceteous fungus, *Sphaerodes mycoparasitia* identified as strain IDAC 301008-01 and alternatively as strain SMCD2220-01, that has a mycoparasitic and antimycotoxin effect. In addition, mycotoxic proteins have been isolated from *S. mycoparasitia* strain IDAC 301008-01. Uses, methods, compositions, sequences, and products are also disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a micrograph showing *Sphaerodes mycoparasitica* cultures after two weeks of incubation on (A) Modified Leonian's agar and (B) Potato dextrose agar-upper sides, (C) and (D) - down sides;

Figure 2 is a micrograph showing *Sphaerodes mycoparasitica* ascospore germination, showing single-polar and double-polar germination patterns as well as hyphal anastomosis (arrow) formation pattern;

Figure 3 is a micrograph showing *Sphaerodes mycoparasitica:* (A) Ascoma, (B) Hyaline seta arising from the neck (arrow), (C) Reticulate ascospores (arrows), (D) Smooth ascospore (arrow), (E) Triangular ascospore (arrow), (F) Phialides produced on ascoma surrounding hyphae, (G) Ampulliform phialide arising from the surface of ascoma peridial wall, (H) Formation of mature and starting ascomata, (I) Formation of hook-like structures by *S. mycoparasitica* parasitising on living hypha of *Fusarium oxysporum* (arrows) and (J) Large view of hook-like structure on living hypha of *F. avenaceum.* Bar scales for (A) and (H) are 50 pm; for (B), (C), (D), (E), (F), (G) and (J) are 10 pm; for (I) 25 pm;

Figure 4 (A) is a micrograph showing *Sphaerodes mycoparasitica* ascospores showing the conspicuous wall ornamentation and prominent irregular longitudinal ribs (arrows); (B) shows *Sphaerodes quadrangularis* ascospores. Bar scales 5 pm;

Figure 5(a) is a micrograph showing inhibition of mycelial growth of (A) *Fusarium oxysporum* and (B) *Fusarium graminearum* by extracellular proteins recovered from a *Sphaerodes mycoparasitica* culture; 5(b) is a chart showing the percent inhibition of mycelial growth of A) *Fusarium oxysporum* and (B) *Fusarium graminearum* by *S. mycoparasitica* extracellular proteins;

Figure 6 is a FPLC chromatogram showing the f1 and f2 extracellular proteins recovered from a *Sphaerodes mycoparasitica* culture;

Figure 7 is micrograph of SDS-PAGE gels of purified extracellular proteins recovered from a *Sphaerodes mycoparasitica* culture. Lane M contains marker proteins. Lane 1 contains the purified protein from peak f1. Lane 2 contains the purified protein from peak f2;

Figure 8 are micrographs showing the inhibition of *F. oxysporum* spore germination (A-a-b) and *F. graminearum* spore germination (B-1-b) by purified proteins f1 and £2 compared to controls (A-c and B-c);

Figure 9 is a chart showing the level of 3-ADON degradation by *Sphaerodes mycoparasitica* in potato-dextrose broth analysed by HPLC;

Figure 10 is a schematic illustration of a system for growing wheat plants in a container (4 x 4 x 16 cm) with different layers of soil-less growing mixes;

Figure 11 is a micrograph of a gel showing SmyITSF/R primers amplified PCR products for *S. mycoparasitica* (SM), five *Fusarium* strains (Fa = F. *avenaceum,* Fo = F. *oxysporum,* Fs = F. *sporotrichioides,* Fg3 = *F. graminearum* chemotype 3, and FgI 5 = *F. graminearum* chemotype 15), two *Trichoderma* species (T22 = *T. harzianum* T22 and Tv = T viride), two *Cladosporium* species ACC = *C. cladosporioides* and CM = C. *minourae),* and *Penicillium aurantiogriseurn* (PA) were electrophoresed on 1% agarose gel at 100 V for 20 minutes. The size of the band is around 300 to 400 bp;

Figure 12 are charts showing standard linear curves for (A) *Sphaerodes mycoparasitica* (in the range of 3.8 x 10^2 to 3.8 x 10^{-2} ng in ten-hold decreasing manner); (B) *Fusarium graminearum* 3-ADCIN (in the range of 2.7 x 10^3 to 2.7 x $10^{-'}$ ng in ten-hold decreasing manner); (C) *Trichoderma harzianum* T-22 (in the range of 7.0 x 10^2 to 7.0 x 10^{-2} ng in ten-hold increasing manner);

Figure 13 is a chart showing RT-PCR sigmoidal coloured curves for *Sphaerodes mycoparasitica* (SMCD 2220-01), with 0.025 fluorescence line, in the ranges of 3.8 x 102 to 3.8 x 10-2 ng in a ten-hold decreasing manner;

Figure 14 are charts showing quantities of genomic DNAs for (A) *F. graminearum* (Fgra); (b) *S. mycoparasitica* (SM); and (C) Trichoderma harzanium (T-22) monitored in spring

wheat roots using genus-specific quantification real-time PCR. All values were means of 6 replicates. Error bars indicate SD;

Figure 15 is a chart showing real-time fluorescence curves of tri5 gene sequences amplified by using Tox5- 1/2 primer set from total DNA extracted from dual-culture assays of *Fusarium graminearum* strains and pre-inoculated *Sphaerodes mycoparasitica* SMCD2220-01 (SNI) or singly grown *F. graminearum* 3-ADON and 15-ADON chemotypes;

Figure 16 are micrographs of *Sphaerodes mycoparasitica* ascomata and ascospores: (A) formation of *S. mycoparasitica* ascomata on a colony of *F. avenaceum* (Arrows indicate that ascomata were produced near or surrounding the *Fusarium* culture); (B) production of numerous *S. mycoparasitica* ostiolated perithecia being produced on a *F. oxysporum* colony (Arrows indicate pericthecia were formed on the *Fusarium* isolate); (C) ungerminated dark-brownish reticulated *S. mycoparasitica* ascospore; (D) germinating ascospore of *S. mycoparasitica* in F. *oxysporum-f úrate* suspension showing one polar germ pore: (E) single polar germinating spore of *S. mycoparasitica* after 3d suspension in a *F. oxysporum-f úrate;* (F) ascospore of *S. mycoparasitica* illustrating two polar germination in *F. oxysporum-f úrate* suspension after 3d with an additional Id on PDA; (G) pattern of *S. mycoparasitica* two polar germination in *F. avenaceum-f úrate* suspension with additional Id on PDA: (H) single polar germination in *S. mycoparasitica* spore suspended 3d in *F. avenaceum-f úrate* with Id incubation on PDA; (I) single and double polar germinations demonstrated by *S. mycoparasitica* ascospores after 3d incubation in a *F. avenaceum-f úrate* suspension plus 1 additional day incubation on PDA. Scale bars for (C) to (H) are 1 Oμm and for (I) is 20μm. SM, Fa, and Fo represent *S. mycoparasitica, F. avenaceum,* and *F. oxysporum,* respectively;

Figure 17 is a chart showing spore germination patterns of *Sphaerodes mycoparasitica* biotrophic mycoparasitic fungus with spores isolated from a *F. oxysporum* colony. Single (■) and two (□) polar germination in a *F. avenaceum-f úrate* suspension; and Single (ø) and two (E) polar germination in a *F. oxysporum-f úrate* suspension. I = Id suspension, 2 = Id suspension plus Id on PDA incubation, 3 = 3d suspension, and 4 = 3d suspension plus Id on PDA incubation. Each incubation day for *S. mycoparasitica* was analyzed separately;

Figure 18 is a chart showing germination of *Sphaerodes mycoparasitica* ascospores in filtrates of six *Fusarium* strains and water suspension treatments at four different incubation days. Suspension in different treatments were: Fave-filtrate = *F. avenaceum-filtrate;* Foxy-filtrate = *F. oxysporum-filtrate;* Fgra3-filtrate = *F. graminearum* chemotype 3 filtrate; Fgra15-filtrate = *F. graminearum* chemotype 15 filtrate; Fpro-filtrate = *F. proliferatum* filtrate; Fspo-filtrate = F. *sporotrichioides* filtrate; and water = control. Day of incubations were: 1d suspension = spores suspended for 1d in different suspension treatments; 1d sus+PDA = spores suspended for 1d in suspension treatment and then inoculated onto PDA medium for an additional day; 3d suspension = spores suspended for 3d in different suspension treatments; and 3d sus+PDA = spores suspended for 3d in suspension treatment and then inoculated onto PDA medium for an additional day;

Figure 19 are charts showing linear mycelial growths of (A) *Fusarium graminearum* chemotype 3, and (B) *Fusarium graminearum* chemotype 15 in dual-culture assays challenged with *Sphaerodes mycoparasitica* in co-inoculation (same day) (--*--) and pre-inoculation (1 day prior to *Fusarium* inoculation) (···■···) treatments as well as control (without mycoparasite) (—♦—) for 5 days;

Figure 20 are micrographs of interactions between *F. graminearum* 3-ADON (Fgra3) and 15-ADON (Fgral5) chemotypes on slide culture assays with *S. mycoparasitica* (S) biotrophic mycoparasitic fungus: (A) Single mycelium of *S. mycoparasitica,* (B) *F. graminearum* mycelium with red complex, (C) Absorption of red complex from *F. graminearum* by *S. mycoparasitica* (arrow), (D) Excretion of red complex in crystal-forms (arrows) by *S. mycoparasitica* from mycelium interacting with *F. graminearum* chemotype 3 only, (E) Formation of series of hook-like structures by *S. mycoparasitica,* (F) Parasitism of *F. graminearum* mycelium by *S. mycoparasitica* with formation of hook-shaped structures, excretion of crystal-like compounds (arrows), and internal haustorium, (G) Initiation of penetration-peg formation by *S. mycoparasitica* on *F. graminearum,* (H) Infected or penetrated and non-infected myclial cells, (I) Branching of haustorium inside *Fusarium* host, (J) Formation of extensive short branching structures by *F. graminearum* chemotype 15 only at the contact zone with *S. mycoparasitica.* Bar scales: (A) to (I) in 5μm, and (J) in 20μm;

Figure 21 is a chart showing the differences in diameters between infected and non-infected *F. graminearum* chemotype 3 (Fgra3) and 15 (Fgral5) host cells in presence of *Sphaerodes mycoparasitica* on slide culture assays. Basr demarked by different lowercase letters represent significant difference in size of infected vs. non-infected hyphae for the two different *F. graminearum* chemotypes at $P = 0.05$ using a T-test;

Figure 22 are charts of the standard curves of *Fusarium graminearum* chemotype 3 and 15 genomic DNA concentration standards versus cycle threshold (Ct) with PCR reactions performed in triplicate using primer sets; (A) *Tox5-l/2,* with genomic DNA ranging from 270 ng (Logio = 2.90) to 0.27 ng (Logio = -0.60), readings at 0.005 fluorescence line; (B) *Tox5-l/2,* with genomic DNA ranging from 30 ng (Logio = 1.48) to 0.03 ng (Logio = -1.52), readings at 0.005 fluorescence line; and (C) *Fgl6NF/R,* with DNA template ranging from 270 ng (Logio = 2.43) to 0.027 ng (Logio = -1-57); in 10-fold dilution series, readings at 0.025 fluorescence line. Error bars indicate standard deviation for the mean of *F. graminearum* chemotype 3 and 15 standard curves derived from *tri5* gene and *F. graminearum* specific primer set;

Figure 23 is a schematic illustration of the experimental set-up for dual-culture assays used to acquire *F. graminearum* chemotype 3-ADON or 15-ADON mycelial plugs for DNAs extraction. The sampling zone (S-zone) indicates the 0.5 x 1.5 cm^2 sample area situated approximately 0.2 cm behind the interaction zone (I-zone). The I-zone represents the interaction or contact zone between *F. graminearum* (Fgra) and *S. mycoparasitica* (SM);

Figure 24 is a chart showing real-time fluorescence curves of *F. graminearum* sequences amplified using Fgl6NF/R primer set from total DNA extracted from dual-culture assays of *F. graminearum* strains and pre-inoculated *Sphaerodes mycoparasitica* (SM), or singly grown *F. graminearum* cultures of chemotype 3 and 15;

Figure 25 is a chart showing comparisons between different concentrations of DNA from *F. graminearum* chemotype 3 and 15 amplified with *Tox5-l/2 (tri5* gene specific) and *Fgl6NF/R (F. graminearum-specific)* primer sets. Fungal DNAs were extracted from 5 d dual-culture assays pre-inoculated with *S. mycoparasitica* for 1 d. With T-test at $P = 0.05$, for the comparison between *S. mycoparasitica* treated and non-treated *F. graminearum* chemotype 3 and 15 for

Tox5-l/2 and *Fgl6NF/R* primer sets, respectively. (Log 10 transformed for DNA amplified with *Tox5-l/2* primers);

Figure 26 are micrographs of *Fusarium graminearum* mycelia at the contact zone with biological and chemical agents: (A) No visible cell changes with *Sphaerodes mycoparasitica* biotrophic mycoparasite; (B) Cell abruption with *Trichoderma harzianum* necrotrophic mycoparasite; (C) 3-ADON chlamydospores formation in chains when challenged with fungicide; (D) 15-ADON chlamydospores formation in clusters when challenged with fungicide. Scale bars indicate: (A) = 5 μm and (B) - (D) = 10 μm;

Figure 27 are charts showing gene expression of different *Tri* genes for *F. graminearum* chemotype 3 (3-ADON producer) and *F. graminearum* chemotype 15 (15-ADON producer) in *in vitro* assays with three separate treatments: (A) *Tri 4* gene; (B) *Tri5* gene; (C) *Tri6* gene; and (D) *Tri10* gene. Legends: B3 = *S. mycoparasitica* + 3-ADON producing *F. graminearum;* F3 = Folicur + 3-ADON producing *F. graminearum;* T3 = *T. harzianum* + 3-ADON producing *F. graminearum;* B15 = *S. mycoparasitica* + 15-ADON producing *F. graminearum;* F15 = Folicur + 15-ADON producing *F. graminearum;* and T15 = *T. harzianum* + 15-ADON producing *F. graminearum.* Means for three different treatments in *F. graminearum* chemotype 3 and 15 were analyzed separately with LSD test at $P = 0.05$. Values are means ± SE of three samples;

Figure 28 are charts showing gene expression of *PKS* genes for *F. graminearum* chemotype 3 (3-ADON producer) and F. *graminearum* chemotype 15 (15-ADON producer) in *in vitro* assays with three separate treatments: (A) *PKS4* and (B) *PKSB.* Legends: B3 = *S. mycoparasitica* + 3-ADON producing *F. graminearum;* F3 = Folicur + 3-ADON producing *F. graminearum;* T3 = *T. harzianum* + 3-ADON producing *F. graminearum;* B15 = *S. mycoparasitica* + 15-ADON producing *F. graminearum;* F15 = Folicur + 15-ADON producing F. *graminearum;* and T15 = *T. harzianum* + 15-ADON producing *F. graminearum.* Means for three different treatments in F. *graminearum* chemotype 3 and 15 were analyzed separately with LSD test at $P = 0.05$. Values are means ± SE of three samples;

Figure 29 is a micrograph of thin liquid chromatography (TLC) analysis for zearalenone (ZEA) extracted from six separate treatments. Legends: B3 = *S. mycoparasitica* + 3-ADON producing F. *graminearum;* F3 = Folicur + 3-ADON producing F. *graminearum;* T3 = *T.*

harzianum + 3-ADON producing *F. graminearum;* Fl5 = Folicur + 15-ADON producing *F. graminearum;* Tl5 = *T. harzianum* + 15-ADON producing *F. graminearum,* Bl5 = *S. mycoparasitica* + 15-ADON producing *F. graminearum.* and ZEA = Standard of zearalenone;

Figure 30 is a chart showing the ratio of *F. graminearum* chemotype challenged with Folicur fungicide to *F. graminearum* alone control produced for all four different mycotoxins - ZEA, DON, 3ADON and 15ADON. Legends indicate *F. graminearum* 3-ADON chemotype with Folicur (■) and *F. graminearum* 15-ADON chemotype with Folicur (D);

Figure 31 is a chart showing relative AUR gene expression in Fusarium strains after co-culturing with biological and chemical agents. Legend: 15ADON - F. graminearum 15-*acetyl*-deoxynivalenol chemotype; 3ADON - F. graminearum 15-oce(y/-deoxynivalenol chemotype; F.cul. - *F. culmorum;* F.ave. - *F. avenaceum* ; B - *Sphaerodes mycoparasitica* (biotrophic mycoparasite); Tricho - *Trichoderma harzianum* (necrotrophic mycoparasite); FoI - *Folicur* (tebuconazole) fungicide;

Figure 32 is a chart showing changes in AUR gene expression evidenced by the color of fungal hyphae. Legend: Ds #71232B: Red and highly virulent, tolerant at 80^0C for 4 hours; Es #4E040B: Moderately red and moderately virulent, tolerant 40^0C for 4 hours; Bs #A86608: White and non virulent, susceptible 40^0C for 4 hours;

Figure 33 are charts showing the effects of inoculating a *Fusari*wrø-susceptible barley cultivar with *F. graminearum, S. mycoparasitica,* and treating *F. graminearum-infected* barley with different concentrations of *S. mycoparasitica* on: (A) height of the plants; (b) average number of spikes per plant; and (C) the average weight of 5 spikes;

Figure 34 are charts showing the effects of inoculating a *Fusarium-susceptible* wheat cultivar with *F. graminearum, S. mycoparasitica,* and treating *F. graminearum-infected* wheat with different concentrations of *S. mycoparasitica* on: (A) height of the plants; (B) average number of spikes per plant; and (C) the average weight of 5 spikes;

Figure 35 is a chart comparing the biocontrol effects of *S. mycoparasitica* on the severity of Fusarium head blight symptoms with the protection provided by a commercial fungicide;

Figure 36 are charts showing standard curves of *Fusarium graminearum* chemotype 3 genomic DNA concentration standards versus cycle threshold (Ct) with PCR reactions performed in triplicate using primer sets: (A) *Tox5-l/2,* with genomic DNA ranging from 270 ng (Logio = 2.90) to 0.27 ng (Logio = -0.60), readings at 0.005 fluorescence line; and (B) *Fgl6NF/R,* with DNA template ranging from 270 ng (Log_{10} = 2.43) to 0.027 ng (Log_{10} = -1.57); in 10-fold dilution series, readings at 0.025 fluorescence line;

Figure 37 is a chart showing the effects of *S. mycoparasitica* (B) and Folicur fungicide (FoI) treatments on *F. graminearum* chemotype 3-ADON genomic DNA detected in barley spikes employing RT-PCR. Treatments were: Fus - *F. graminearum;* B-Fus - *S. mycoparasitica* with *F. graminearum;* Fol-Fus - Folicur fungicide with *F. graminearum;*

Figure 38 are micrographs showing *Sphaerodes mycoparasitica-Fusarium* spp. mycoparasitism assays: (A-a). Hook-shaped contact structures (arrows); (B-b). Clamp-like clasping cells (arrows). "a" and "b" are diagrammatic drawings for (A) and (B) respectively. Scale bars = 5μm;

Figure 39 are micrographs showing intracellular parasitism, hyphal inhibition response, and anamorphic stages during the *Sphaerodes mycoparasitica-Fusarium* spp. interactions: (A) Intracellular parasitism by *S. mycoparasitica* in *F. equiseti* (arrow); (B) *Fusarium* hyphal inhibition response when challenged with *S. mycoparasitica;* deformation of hyphae into rosette-like shapes (arrow); (C) Hyaline *S. mycoparasitica* anamorphic stages; (D) *Sphaerodes mycoparasitica* anamorphic stages with adsorption of red pigments from *F. culmorum.* Scale bars A, C, D = 5μm; B = 20μm;

Figures 4OA and 4OB are micrographs of intracellular parasitism by *Sphaerodes* inside *F. equiseti* (arrows), and 4OC and 4OD are micrographs of intracellular hyphae produced by *Sphaerodes* inside F. *equiseti* with hook-shaped contact structure (arrows). 4OA and 4OB were captured under light microscopy; whereas in 4OC and 4OD hyphae were stained with lactofuchsin and images were captured under fluorescent and confocal laser microscopy, respectively. Scale bars = 5μm; and

Figure 41 is a chart showing average hyphal diameters of parasitized and non-parasitized *F. equiseti* cells (■) and *F. culmorum* (D) on 1-week slide-cultures with *Sphaerodes mycoparasitica* biotrophic mycoparasite. Data are means and standard deviations. Same lowercase letters indicate no significant difference between parasitized and non-parasitized hyphae at $P = 0.05$, with T-test.

DETAILED DESCRIPTION OF THE INVENTION

Sphaerodes mycoparasitica (Ascomycetes, Melanosporales), has been isolated from isolates of *Fusarium avenaceum, Fusarium graminearum* and *Fusarium oxysporum* originating from wheat or asparagus fields. The species is characterized by a unique combination of ascospore size, shape (fusiform and triangular) and wall ornamentation (reticulate and smooth). Also, conidia are produced from simple phialides on the surface of ascoma peridial wall, on ascoma surrounding hyphae, and on irregularly branched conidiophores arising from hyphae. *S. mycoparasitica* has a phialidic anamorph and produces simple phialides on the surface of ascoma peridial wall or scattered irregularly on ascoma surrounding the hyphae, and on conidiosphores. *S. mycoparasitica* forms hook-like structures parasitizing living hyphae of *Fusarium.*

The 1266bp DNA sequence from the large subunit ribosomal RNA gene (LSU) of *S. mycoparasitica* is given in SEQ ID NO: 1.

SEQ ID NO: 1.

1 atagggagaa gaagcactgc gattgcccta gtaacggcga gtgaagcggc agcagcccag
61 atttggaatc tggtcctttt ggggcccgag ttgtaatctg cagaggaagc gtctggtgcg
121 gtgccggcct agttccctgg aacgggacgc cgtagagggt gacagccccg tacggtcggc
181 caccaaacct gtgtgtcgct ccttcgaaga gtcgcgtagt ttgggaatgc tgcgtaaagt
241 gggaggtatg ctcctcctaa ggctaaatac cggccagaga ccgatagcgc acaagtagag
301 tgatcgaaag atgaaaagca ccttgaaaat ggggttaaaa agtacgtgaa attgccaaag
361 gggaagcgct cgtggccaga ctcgtgcctt atggatcatc cggctatttc gccggtgcac
421 tccattaggc tcgggccagc gtcggtcggc gccggtacta aaagacagcg cgaacgtggc
481 tctcttcggg gagtgttata gcgcgctgtg taatgtgctg gcgccgtccg aggaccgcgc
541 atttatgcaa ggacgctggc gtaatggcca ctagcgaccc gtcttgaaac acggaccaag
601 gagtcgccca gagacgcgag tgtgcgggtg acaaacccct gcgcgaagtg aaagcgaacg
661 ctggtgggaa ccctcacggg tgcaccaccg accgatcctg atgtcttcgg atggatttga
721 gtatgagcgt ttctggtcgg acccgaaaga gggtgaacta tgcttgggta gggtgaagcc
781 agaggaaact ctggtggagg ccccgtttgg gttctgacgt gcaaatcgat ccataaacct
841 gggcatagcg gcgaaagact aatcgaacct tctagtagct ggttcgcatt ctctctctcg
901 cacgagagag agaaaacctc tgtgatatca cgattatcag tgaaaaccac accgagaccc
961 aacggagttc ttctggattt cctcatgctt caattaccac gcctagtgga cctacctgga
1021 gcgctacaat aaagtcatac gaaaatctcg aagatcgggg tgacggtgag ggatcctaag
1081 gttctctcgt tgagtgcgtt ggacgggcat ggccgtcagc gatctggggc gaccgttgcc
1141 ggatcataag ggctttagtg cttaggctat tggtattgag gggtctgaag acggtaatct
1201 gaaaccaaag gctttattct aaacccgcgc agcatgggcg tagtaggaag agacagcgaa
1261 gtctag

The 1233bp DNA sequence from the small subunit ribosomal RNA gene (SSU) of *S. mycoparasitica* is given in SEQ ID NO: 2.

SEQ ID NO: 2

1 agtgcggcat gttgtagcct aagcaattat acagcgaaac tgcgaatggc tcattatata
61 agttatcgtt tatttgatag tgccttacta cttggataac cgtggtaatt ctagagctaa
121 tacatgctga aagccccgac ttacggaggg gcgtatttat tagattaaaa accaatgccc
181 ttcggggctc tttggtgatt catgataact tctcgaatcg cacggccttg cgccggcgat
241 ggttcattca aatttcttcc ctatcaactt tcgatgtttg ggtagtggcc aaacatggtg
301 gcaacgggta acggagggtt agggctcgac cccggagaag gagcctgaga aacggctcct
361 acatccaagg aaggcagcag gcgcgcaaat tacccaatct caactcgagg aggtagtgac
421 ataaatacc gatgcagggc tctttagggt cttgcaattg gaatgagtac aatttaaatc
481 ccttaacgag gaacaattgg agggcaagtc tggtgccagc agccgcggta actccagctc
541 caatagcgta tattaaagtt gttgtggtta aaaagctcgt agttgaacct tgggcctggc
601 cggctggtcc gcctaacagc gtgcactggt gcggccgggt cttcccaccg cggagccgca
661 tgtccttcac tgggcgtgtc ggggaagcgg tacttttact gtgaaaaaat tagagtgctc
721 taagcaggcc tatgctcgaa tacattagca tggaataata gaataggaca gtcgttctat
781 tttgttggtt tctaggacgt ctgtaatgat taacagaaac aatcgggggc gtcagtattg
841 catcgtcaga ggtgaaattc ttagatcgat gcaagactaa ctactgcgaa agcattcgcc
901 aagggtgttt tcattaatca ggaacgaaag ttaggggatc gaagacgatc agataccgtc
961 gtagtcttaa ccataaacta tgccgactag ggatcgggcg gtgtaatttt gacccgctcg
1021 gcacttacga gaaatcttaa gtgcttgggc tccaggggag tatggtcgca aggctgaaac
1081 ttaaagaaat gacgaagggc accaccaagg gtgaacctgc ggcctagttg actcacacgg
1141 gaaactcacg aggtaggaat atgtagatga cggatggagc cttcagaata catatggg;'ca
1201 tgcgctctta ataccggtat tgaaaatggg cag

Sample cultures of *Sphaerodes mycoparasitica* have been deposited with Saskatchewan Microbial Collection and Database under the accession number SMCD2220-01, and in the

International Depositary Authority of Canada Collection (1015 Arlington Street, Winnipeg, Canada, R3E 3R2) under the accession number IDAC30 1008-01.

Sphaerodes mycoparasitica may be useful as an anti-fungal agent. *S. mycoparasitica* may be used to treat, ameliorate, or otherwise control infections of *Fusarium* fungi. *S. mycoparasitica* seems particularly useful for treating, ameliorating or otherwise control infections of *Fusarium avenaceum, Fusarium graminearum,* and/or *Fusarium oxysporum* and improving plant health or growth.

S. mycoparasitica may be used as a prophylactic agent to diminish the chance of a fungal infection, particularly a *Fusarium* infection, from occurring.

S. mycoparasitica may be used for treating plants affected by *Fusarium* Wilt Disease or *Fusarium* Head Blight. *S. mycoparasitica* may be used for hindering such conditions from spreading or as a prophylactic measure to diminish the risk of such conditions from occurring.

In the present invention, *S. mycoparasitica* may be isolated from suitable sources. For example, *S. mycoparasitica* may be isolated from *F. graminearum* or *F. avenaceum* isolates originating from wheat fields by the method described in Vujanovic V, *et. al.* Can. J. Microbiol. 48(9): 841-847 (2002). *S. mycoparasitica* is also available deposited with International Depositary Authority of Canada (IDAC30 1008-01).

S. mycoparasitica may be used to treat, ameliorate, or otherwise control infections of *Fusarium* fungi. *S. mycoparasitica* may be applied in any suitable manner to the organism in need of treatment. For example, *S. mycoparasitica* isolates may be used directly or they may be further processed into soil-applied granular-based, peat-based, seed-applied, or anthesis-applied liquid-based inoculants.

S. mycoparasitica may be produced via fermentation and formulated into a pesticide composition. A suitable fermentation process includes, selection of fermentation medium (solid state or submerged), concentration of fermentation constituents, oxygen transfer, incubation temperature, time of harvest and post harvest treatments. The aim is to establish the optimum conditions to ensure an abundant, stable, and efficacious mycoparasitic, microbial population. When formulating *S. mycoparasitica* into a pesticide composition, care should be taken to

selected ingredients that: (i) ensure stability during production, processing and storage, (ii) assist application, (iii) protect the pesticide from unfavourable environmental conditions, and (iv) promote pesticidal activity at the target. Exposure to inactivated host/pathogen or its compounds may improve the activity of *S. mycoparasitica.*

The formulations disclosed herein will usually comprise (i) an active ingredient, (ii) carriers - often an inert material used to support and deliver the densely populated active ingredient to the target, and optionally (iii) adjuvants - compounds that: promote and sustain the function of the active ingredient by protection from UV radiation, that ensure rain fastness on the target, and that retain moisture or protect against desiccation, or promote the spread and dispersal of the biopesticide using standard agriculture equipments such as those disclosed by Hynes and Boyetchko (2006, Soil Biology & Biochemistry 38: 845-84).

Internal Transcribed Spacer ribosomal DNA from *S. mycoparasitica* was sequenced. A 560bp DNA sequence from the ITS region is given in SEQ ID NO: 3.

SEQ ID NO: 3

1 ttcgtttctt atcgcattgg tgaccagcgg agggtcatta cgaatcggac catttatgtc
61 atggctctgc caaccctgtg aactttatac ttgtacgttg cctcggcgga acctgccttt
121 tcggcaggcc gccggccggc atatacgcaa acgctctgaa aaagctccgc gctctatctg
181 aataataaaa ctttaacgag taaaaacttt tggcaacgga tctcttggct ctggcatcga
241 tgaaaaacgc agcgaaatgc gatacgtaat gtgaattgca gaattcagtg aaccatcgaa
301 tctttgaacg caccttgcgg ccgccggtaa tccggcggcc atgcccgtcc gagcgtcgtt
361 tccaccctcg ggagttctcc tcctaagaaa atttctcccg gccttgggcc agcgcgttgc
421 gcggctgccc gaccaacggc ggcaggaccg gcgatgtcct ctgtgccctg catttatata
481 aaactcgcat tggtccccgg taaggcttgc cttgcaacca acttctttag gtcgacctca
541 gatcggatag ggatacccgc

The present invention provides a primer set, SmyITSF/R (SmylTSF is SEQ ID NO: 4 and SmyITSR is SEQ ID NO: 5), useful for identifying *S. mycoparasitica.:*

SEQ ID NO:4 5'-TCATGGCTCTGCCAACCCTGTAA-S'

SEQ ID NO: 5 5'-AATGCAGGGCACAGAGGACATCG-S'

The present primer is selective for *S. mycoparasitica* and can be used to assess and quantify the fungus in industrial products, plant materials, seed samples, and environmental samples by using PCR and RT-PCR technologies.

The SmyITSF/R primer set can be used for quantitative real-time PCR technology for analyzing gene expression, in fungal pathogens detection, and in quantification of fungi in living plants.

The SmyITSF/R primer set was tested with SMCD2220-01, seven *Fusarium* species, nine different ascomycetous fungal isolates, two zygomycete fungi, and three basidiomycetous fungal strains.

In PCR, this primer set only amplified SMCD2220-01, not the other fungi. Root biomass, total biomass, root length, total length, and seed germination of *F. graminearum* infected spring wheat were significantly increased with the treatments of *S. mycoparasitica,* as compared to inoculation with *F. graminearum.* In further RT-PCR studies, SmyITSF/R specific primer was used for *S. mycoparasitica* in combination with *F. graminearum-Fgl2NF/R* and *Trichoderma-TGP4-F/R* as a control, within which the primer showed high accuracy in assessing biocontrol-pathogen-plant interactions.

Total extracellular protein extracts from *Sphaerodes mycoparasitica* demonstrated significant inhibition of *Fusarium* spore germination. The present invention provides an antifungal agent comprising extracellular protein extracts from *S. mycoparasitica.* Two proteins were isolated from the total protein extract, one having a molecular weight of 13 kDa and one having a molecular weight of 50 kDa. The present invention provides an antifungal agent comprising one or both of these proteins.

Conventional molecular biological techniques may be used to isolate, characterize and produce the present proteins. For example, in order to identify the gene(s) for the present proteins, *S. mycoparasitica* could be challenged with *F. oxysporum* filtrates and upregulated mRNA isolated by standard Northern Blot. cDNA can be produced from the mRNA by Reverse Transcriptase PCR (RT-PCR). The cDNA can be amplified, purified and inserted into an appropriate vector. This vector may be inserted into an appropriate host cell.

Cloning and expression may focus on isolation of genes coding for antimicrobial proteins by designing primers for identified proteins. Isolated genes may be cloned in suitable expression vectors (yeast or bacteria) with suitable/efficient promoters in order to generate industrial strains for large-scale production of the concerned proteins. These vectors can be purchased from Promega, Invitrogen, Clontech, or other companies. The cloned genes may be tested for their protein expression, and the expressed proteins will be purified (using His-tag or other columns). Purified proteins may be tested against plant-pathogenic fungi for their antifungal activity by disc-plate assay and/or microtitre plate assay methods (e.g., Drummond and Waigh, 2000, Recent Research Developments in Phytochemistry.4:143-152). Then, rDNA technologies, involving gene mutation or addition of enhancers, introns or protein-specific promoters (GA inducible), or addition deletion may be used to enhance the production of proteins.

Selected genes may be transformed into wheat and barley to create a transgenic lines with antifungal activity against pathogenic fungi using techniques known to those skilled in these arts (e.g., Sanghyun et.al., 200,8 J. Expt. Botany, 59, 2371-2378; Dennis et. al., 2007. Plant Cell Reports. 26: 631-639).

S. mycoparasitica has demonstrated an ability to degrade the deoxynivalenol (DON) mycotoxin. The present invention provides a method of degrading acetyldeoxynivalenol, especially 3-acetyldeoxynivalenol.

S. mycoparasitica could be challenged with pure DON and upregulated mRNA isolated by standard Northern Blot. Reverse Transcriptase PCR could be used to identify the gene(s) for the upregulated proteins, an appropriate cDNA construct could then be inserted into an appropriate vector for protein production. Further, recombinant DNA Technology could be applied to create transgenetic plants with antimycotoxin properties.

The present invention provides antifungal compositions comprising *S. mycoparasitica,* isolates, cultures, or proteins thereof. The present invention also provides a method of controlling a fungal disease of a plant, and a method for mycotoxin detoxification, which methods comprise applying to the locus of the plant *S. mycoparasitica,* isolates, cultures, or proteins thereof, collectively hereinafter referred to as the 'antifungal agent'.

The compositions of the invention may, for example, be applied to the seeds of the plants, to the growth medium (e.g. soil or water), or to the foliage of the plants.

The present invention provides a composition comprising the antifungal agent and an agriculturally acceptable carrier or diluent, which will ensure stability and performance of the final product. Carrier or diluent should compatible with the active ingredient, agriculturally acceptable, have a good absorptive capacity and a suitable bulk density, allowing easy particle dispersion and attachment.

The compositions herein may be applied, as in aqueous sprays, granules and dust formulations in accordance with established practice in the art. An aqueous spray is usually prepared by mixing a wettable powder or emulsifiable concentrate formulation of a compound of the invention with a relatively large amount of water to form a dispersion.

Wettable powders may comprise a finely divided mixture of the antifungal agent, a solid carrier, and a surface-active agent. The solid carrier is usually chosen from among attapulgite clays, kaolin clays, montmorillonite clays, diatomaceous earths, finely divided silica, purified silicates, or combinations thereof. Surfactants which may be useful herein have wetting, penetrating, and/or dispersing ability. They are typically present in an amount of from about 0.5% to about 10% by weight. Surfactants herein may be chosen from, for example, alkylbenzenesulfonates, alkyl sulfates, naphthalenesulfonates and condensed naphthalenesulfonates, sulfonated lignins, and non-ionic surfactants.

Emulsifiable concentrates may comprise the antifungal agent of the invention in a liquid carrier, the carrier being a mixture of a water-immiscible solvent and a surfactant. Solvents that may be useful herein include aromatic hydrocarbon solvents such as the xylenes,

alkylnaphthalenes, petroleum distillates, terpene solvents, ether-alcohols, organic ester solvents or suitable combinations thereof.

When a composition of the invention is to be applied to plant debris or litter, in order to control of the source of contamination and inoculant dispersion, or to the soil, as for pre-emergence protection, granular formulations or dusts are sometimes more convenient than sprays.

In one embodiment the antifungal agents herein are encapsulated into alginate pellets. The pellets may be prepared in any suitable manner. For example, one useful method is described in Harveson et. al., (2002, Plant Disease; Vol. 86, No. 9 1025-1030).

The composition may comprise other active substances useful in an antifungal agent. For example, the compositions herein may comprise other antifungal agents such as *Trichoderma*, sulfur, neem oil, rosemary oil, jojoba oil, *Bacillus subtilis*, allylamines (e.g. terbinafine, antimetabolites (e.g. flucytosine), azoles (e.g. ketoconazole, itraconazole), echinocandins (e.g. caspofungin), polyenes (e.g. amphotericin B), systemic agents (e.g. griseofluvin), or combinations thereof.

The compositions herein may contain from about 0.1% to about 95%, by weight, of the antifungal agent and from about 0.1% to about 95%, by weight, of the carrier and/or surfactant. The direct application to plant seeds prior to planting may be accomplished in some instances by mixing either a powdered solid compound of the invention or a dust formulation with seed to obtain a substantially uniform coating which is very thin and represents only one or two percent by weight or less, based on the weight of the seed. In some instances, however, a non-phytotoxic solvent such as methanol is conveniently employed as a carrier to facilitate the uniform distribution of the compound of the invention on the surface of the seed.

The compositions herein may be useful in the treatment of fungal infections in plants. Consequently, the compositions herein may comprise *S. mycoparasitica*, isolates, cultures, or proteins thereof and a pharmaceutically acceptable carrier.

S. mycoparasitica sporulates in the presence of *Fusarium. S. mycoparasitica* ascospores proved resistant to germination under different standard laboratory conditions (sterile distilled

water, on water agar and commercially available media) and heat or cold-shock treatments. In contrast, spore germination was obtained on general potato dextrose agar medium amended with *Fusarium-f úrtales.* Significant improvement in percentage of spore germinations were obtained for the spores suspended in *Fusarium-f úrtrates. F. avenaceum* and F. *oxysporum* filtrates induced the highest germination, whereas F. *sporotrichioides* and F. *proliferatum* triggered lower germination frequency. Filtrates of beneficial fungal inoculants: *Trichoderma harzianum* (RootShield® available from BioWorks Inc., Victor, NY, USA; RootShield is a registered trademark of BioWorks Inc.), *Penicillium bilaii* (JumpStart® available from Novozymes Biologicals Ltd., Saskatoon, SK, CA; JumpStart is a registered trademark of Philom Bios Inc.), and *Chaetomium globosum* had no impact on germination. Ascospores suspended in F. *avenaceum-f úrtratQ,* showed double-polar germination pattern. On the other hand, when suspended in F. *oxysporum-f úrtrate,* significant amount of spores demonstrated a single-polar germination pattern. *S. mycoparasitica* grown on *F oxysporum* kept the same mycoparasitic germination patterns in three offspring generations when transferred on F. *avenaceum* which indicate a stable genome-regulated expression.

The present invention provides a method of sporulating *S. mycoparasitica* by exposing them to F. *avenaceum* or F. *oxysporum,* or filtrates, extracts, or compositions thereof.

The present invention provides a method for producing an antifungal composition comprising *S. mycoparasitica* or isolates, cultures, genes or proteins thereof. The method comprises:

(a) inducing *S. mycoparasitica* sporulation;

(b) culturing *S. mycoparasitica;*

(c) harvesting *S. mycoparasitica.*

The method may further comprise any of the following optional steps:

(d) storing/preserving *S. mycoparasitica;*

(e) applying S. mycoparasitica;

(f) assessing S. mycoparasitica;

(g) producing *S. mycoparasitica* proteins;

(h) harvesting *S. mycoparasitica* proteins;

(i) fractionating *S. mycoparasitica* proteins;

(j) separating *S. mycoparasitica* proteins;

(k) storing/preserving *S. mycoparasitica* proteins;

(l) applying *S. mycoparasitica* proteins;

(m) assessing *S. mycoparasitica* proteins.

EXAMPLES

Example 1

Sampling, fungal growth and microscopy

Myclobutanil-agar (MBA) medium was used for selective isolation of various *Fusarium* taxa and associated biotrophic mycoparasites from Canadian agriculture fields using the method described in Vujanovic V, *et. al.* (2002, Can. J. Microbiol. 48(9): 841-847). *Sphaerodes* was recovered occasionally from *F. graminearum* and abundantly from *F. avenaceum* isolates originating from wheat fields in Saskatchewan; it was also isolated from *Fusarium oxysporum* from asparagus fields in Quebec, Canada. A monosporal, single culture of the mycoparasite was obtained from each *Fusarium* species according to the method proposed by Harveson & Kimbrough (2001, Int. J Plant Sci. 162(2):403-410). Single ascospore isolates were maintained on Potato dextrose agar (PDA) (Difco, BD Biosciences, Mississauga, ON, CA) supplemented with antibiotics (100 μg I^{-1} streptomycin sulphate and 13 μg l^{-1} neomycin sulphate; Sigma-Aldrich Canada Ltd., Oakville, ON, CA) and stored at -80°C in Saskatchewan Microbial Collection and Database (SMCD2220-01) and in the International Depositary Authority of Canada (IDAC301008-01) collections. Fungal growth was assessed on modified Leonian's agar (MLA) and Potato dextrose agar (PDA) media. Biotrophic interactions between *Sphaerodes* and *Fusarium* strains were examined with the slide culture method proposed by Jordan & Barnett (1978, *Mycologia* 70(2):300-312). Morphological studies of ascomata, ascospores, mycelia, and anamorphic structures were performed after two weeks of incubation (21° C - 22° C) under a Carl Zeiss Axioskop2 with a Carl Zeiss AxioCam ICcI camera. Fungal materials for microscopic observation were mounted in lactofuchsin and lactophenol cotton blue dyes.

DNA extraction, amplification and sequencing

Three *Sphaerodes* strains: SMCD 2220-01 on *F. avenaceum* from wheat, SMCD 2220-02 on F. *graminearum* from wheat, and SMCD 2220-03 on F. *oxysporum* from asparagus were cultured on PDA medium at 21^{0}C for a week prior to DNA extraction. Genomic DNA was extracted with the DNeasy® Plant Mini Kit (Qiagen Inc., Mississauga, ON, CA; DNEasy is a registered trademark of Qiagen GmbH Corp, Hilden, Fed. Rep. Germany). LSU (large subunit) rDNA fragments were amplified using primer sets NS1/NS6 using techniques known to those skilled in these arts (e.g., Gardes & Bruns, 1993, *Molecular Ecology* 2: 113-118; White *et ah* 1990, PCR *Protocols: a guide to methods and application:* 315-322. Academic Press, New York) and LS1/LR5 (e.g., Hausner *et al.* 1993, *Canadian Journal of Botany* 71: 52-63; Rehner & Samuels, 1995, *Canadian Journal of Botany* 73 (Suppl. 1): S816-S823; Zhang & Blackwell, 2002, *Mycological Research* 106: 148-155). Target regions of fungal genomic DNA samples were amplified using polymerase chain reaction (PCR) in a 25µl reaction mixture containing 2.5µl of 10X buffer, 5µl of Q buffer, 0.5µl 10mM dNTPs, 1µl of each primer, 0.13µl of 0.625 unit of Taq DNA Polymerase, 2µl of extracted fungal DNA, and 12.87µl of sterilized ultra-pure Millipore water. The Qiagen Taq PCR core kits were purchased from Qiagen Inc., Mississauga, ON, CA. Purified DNA PCR products were sequenced.

Sequence alignment and phylogenetic analyses

Sequences of LSU from this study and sequences retrieved from GenBank were aligned using Clustal X software (version 1.82) (Thompson *et al.* 1997, *Nucleic Acids Research* 24: 4876-4882), and edited in Bioedit (Hall, 1999, *Nucleic Acids Symposium Series* 41: 95-98). Distance trees were produced with PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 software (Swofford 2000) using a neighbor-joining approach, and validated using bootstrap analyses with 1,000 repetitions. A fungal distance tree was prepared with sequences showing bootstrap values higher than 50%. Trees were rooted with sequences *Xylaria hypoxylon* U47841.

Taxonomy: *Sphaerodes mycoparasitica* Vujanovic, sp. nov. (Figures 1-5) [MycoBank no: MB 515144], in the International Depositary Authority of Canada as *Sphaerodes mycoparasitica* strain IDAC 301008-01.

Coloniae in agaro potato dextrosum lentior crescents, 4.0 cm ad 7d, floccose, pallido-brunneis. *Hyphis* septatis, ramosis, anastomosantibus, laevibus, palide fulvis, 2.5-5.0 μm diam compositum. *Ascomata* superficialia vel immersa, pyriformia vel globosa, ostiolata, flavo-brunnea, 250-300 μm longa, 200-280μm diam. *Collum* nul, conicum vel cylindricum, 30-75μm longum, (0-) 50-70 μm latum ad basim. *Peridium* membranaceum, cellulis 8-15 μm, e 3-6 stratis, 8-15 μm crassum, textura angulari compositum. *Setae* rectae vel parum curvae, hyalinae vel dilute flavae, crassitunicatae, 10-40 μm longae, septatae. *Asci* 8-spori, ovoidei vel clavati, 50-75 X 17-25 μm, superne late rotundati, brevistipitati, tenuitunicati, evanescentes. *Paraphysis* nullis. *Ascosporae* unicellulares, irregulariter biseriatae, primum hyalinae, deinde brunneae vel atrobrunneae, crassitunicatae, fusiformes, 18-24 X 9-12 μm, reticulatae, costis protrudentibus, e polo visae polygonales, utrinque umbonatae, foramine germinali praeditae. *Phialidis* hyalinis, status conidialis.

Culture characteristics: Colonies of *Sphaerodes mycoparasitica* strain IDAC 301008-01 cultured on MLA grew more rapidly than on PDA, 1.1 cm versus 0.6 cm per day (21-22^{0}C), consisting of slightly submerged mycelium and aerial hyphae, granulose due to production of ample number of ascomata. On MLA (Figures IA and 1C) and PDA Figures IB and ID), the cultures produced a woolly mycelium, yellowish to pinky-brownish on both sides. At 37^{0}C, no growth was registered. *Hyphae* were white to pale yellow, 2.5-5.0 μm diam., septate, anastomosis occurred soon after ascospore germination (Figure 2). Colonies on Potato dextrose agar (PDA) spread with abundant, white to pale yellow aerial mycelium and low number of ascomata. Ascomata, perithecial or cleistotecial, scattered or aggregated in small groups, superficial, pyriform to globose, ostiolate (when mature), light to dark yellowish brown, translucent, appearing black due to mass of mature ascospores, 250-300 μm high, 200-280 μm diam. *Neck* absent to short conical or cylindrical 25-75 μm long, 20-70 gm wide at the base, sometimes surrounded with a crown of short, upright setae, 10-40 μm long. *Peridium* membranaceous, 3-6-layered, 8-10 μm thick, translucent, pale yellow to light brown, composed by cells of 8-15 μm diam. disposed in *textura angularis.*. *Asci* 8-spored, clavate, 50-75 X 17-25 μm, rounded at apex, without apical structures, thin-walled and evanescent when mature. *Paraphyses* absent. *Ascospores* irregularly arranged inside the asci, at first hyaline but becoming brown to dark brown, thick-walled, single-celled, fusiform to rarely triangular, 18-24 X 9-12 μm,

reticulate to rarely smooth, with irregular transverse sections, and with a strongly umbonate germ pore at each end. *Phialides* hyaline, ampulliform produced directly on ascomata or on hyphae surrounding ascomata, and on irregularly branched conidiophores.

S. mycoparasitica strain IDAC 301008-01 has a unique combination of features shown in Figure 3. The ascomata height is generally less than 250 μm with a conical to cylindrical neck (Figure 3A). The setae length is generally less than 40 μm (Figure 3B) and the spore length is generally less than 23 μm (Figure 3C). The spores show a conspicuous wall ornamentation and prominent irregular longitudinal ribs indicated by the arrows in Figure 4A. The spores of *Sphaerodes quadrangularis* are shown in Figure 4B for comparison. *S. mycoparasitica* strain IDAC 301008-01 spores occaisonally show a triangular shape (Figures 3D and 3E). The formation of starting and mature ascoma of *S. mycoparasitica* strain IDAC 301008-01 are shown in Figure 3H. This strain produces simple phialides on the surface of ascoma peridial walls or alternatively, the phialides may be scattered irregularly on ascoma surrounding the hyphae, and on conidiosphores with a distinctive branching pattern (Figures 3F and 3G). *S. mycoparasitica* strain IDAC 301008-01 forms hook-like structures for parasitizing living hyphae of Fusarium (Figures 31 and 3J).

Example 2

Sphaerodes mycoparasitica (SMCD 2220-01) 21^{0}C isolates were cultured in potato dextrose broth (PDB) culture media. About 3 ml of culture were transferred to 250 ml Ehrlenmeyer flasks containing 50 ml PDB growth medium. The flasks were incubated for 7 days on a rotary shaker (150 rpm) at room temperature.

Extracellular protein extraction: Young mycelia were filtered through Whatman® No.l filter paper (Whatman is a registered trademark of Whatman International Ltd., Kent, UK). Filtered culture medium, containing extracellular proteins, and were concentrated by Amicon ultrafiltration centrifuge tube with a 3000 Dalton cut-off membrane by centrifugation at 4000 rpm at 4°C.

Disc diffusion assay: Antifungal activities of extracellular protein extracts were tested under sterile conditions by radial disc plate diffusion assay as described by Roberts & Selitrennikoff

(1986, *Biochim. Biophys. Acta,* 880: 161-170). The assay of the isolated protein for antifungal activity toward *F. oxysporum* and *F. graminearum* was carried out in petri plates containing potato dextrose agar. Mycelial plugs from actively growing fungal plates were placed in the center of the petri plates and sterile filter paper discs (5-mm diameter of Whatman® filter paper no. 1) were placed on the agar surface at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (60 µL) containing 2.5 µg of extracellular protein was added to a disk. Sterile distilled water and buffer served as controls. The plates were then incubated at room temperature for 4 days and examined for inhibition. The area of the mycelial colony was measured and the inhibition of fungal growth was determined by calculating the % reduction in area of mycelial colony with the controls (Figure 5A). After 4th day of germination, about 30% inhibition of the hyphal extension of *F. oxysporum* and 35% inhibition of hyphal extension of *F. graminearum* were observed (Figure 5B)

Fast protein liquid chromatography (FPLC) of extracellular proteins: Proteins were fractionated through Superdex 75 GL 10/30 column using FPLC AKTA® purifier system (GE Healthcare, Biosciences AB, CA: AKTA is a registered trademark of GE Healthcare Bio-Sciences AB Ltd., Uppsala, Sweden) according to the manufacturer's instructions. The column was previously equilibrated with sterile water and with 50 niM sodium phosphate buffer, pH 7.0 containing 0.15 M NaCl, followed by protein injection (about 500 µL) and elution of proteins with the same buffer with flow rate of 1.0 ml/min. Fractions of 0.8 ml were collected in each tube. Upon gel filtration on Superdex® 75 (Superdex is a registered trademark of GE Healthcare Bio-Sciences AB Ltd., Uppsala, Sweden), proteins were resolved into two distinct peaks (fl and f2) and few smaller peaks Figure 6). Samples from all the peak fractions were pooled, precipitated and tested for their antifungal activity.

Sodium dodecyl sulfate-polyactylamide gel electrophoresis (SDS-PAGE): SDS-PAGE (12%) of proteins recovered from FPLC fractions was performed according to the method of Laertunli (1970, *Nature 227:* 680-685). All peaks giving FPLC fractions were pooled and recovered by precipitation in 1:4 volume of chilled acetone and kept at -20^{0}C overnight. After centrifugation at 12,000 g for 10 min, precipitated proteins (pellets) were dissolved in minimum amount (30 µL) of assay buffer. Proteins were analyzed by SDS-PAGE having 5% stacking gel (pH 6.8) and 12% separating acrylamide gel (pH 8.8) in Tris-glycine buffer (pH 8.3) and appropriate markers.

Prior to SDS-electrophoresis, the protein was mixed with an equal volume of sample buffer (60 mM Tris-HCl buffer, 4% SDS, pH 6.8) containing 5% β-mercaptoethanol. A mixture of standard marker proteins (Bio-Rad protein markers, Bio-Rad Laboratories Inc., Mississauga, ON, CA) was used. V All samples were heated for 5 min at 95^0C and cooled to room temperature before loading on gel. Proteins were visualized by silver staining method (Bio-Rad Silver staining kit, Bio-Rad Laboratories Inc., Mississauga, ON, CA). Proteins molecular masses were estimated by comparison with the mobilities of standard molecular mass markers. Protein bands of molecular weight 13 kDa and 50 kDa were detected in f1 and f2 peaks respectively (Figure 7).

Microtitre plate assay: Percentage inhibition of spore germination was performed by microtitre plate assay method (Ghosh, 2006, *Ann. Bot. 98: 1145-1153;* Yadav *et al.* 2007, *J. Med. Microbiol. 56: 637-644)* to test the antifungal activity of proteins recovered from FPLC fractions. The possible toxicity of the fractionated proteins was tested by a percentage growth inhibition assay using the *F. oxysporum* and *F. graminearum.* The *in vitro* antifungal activities of fractionated proteins were determined in 96-well microtiter plates. In microplate wells, 10 µl of potato dextrose broth (PDB; BD Biosciences, Mississauga, ON, CA) was mixed with 3 µl of spore suspensions of *F. oxysporum* and *F. graminearum.* An aliquot of 7 µL of different peak containing proteins fractions were added to suspensions in microtitre plates (12-8 wells). Water 25 and buffer were used as negative controls. The microtitre plate was then incubated at room temperature in dark. Observations were made for inhibition in spore germination in both untreated and treated wells after 24 h using inverted and fluorescent microscope. The number of germinated and non-germinated spores and percentage of area covered by mycelia in microscope were used to determine the percentage of growth inhibition. The f1 and f2 protein-containing peaks had inhibitory effects on spore germination after 24 hrs compared to the control treatment.

The discharge of spores from sporangia was inhibited by these protein fractions. Secondary branching (mycelia formation) was observed in control treated spores of *F. oxysporum* (Figure 8A-c) and *F. graminearum* (Figure 8B-c). No branching was observed in f1 and f2 protein-treated spores of *F. oxysporum* (Figures 8A-a-b) and *F. graminearum* (Figure 8B-a-b). The size of germ tubes (hyphae) from control-treated spores was more than 100 µm compared to 10-15 µm for protein-treated spores (Figures 8A-a-b; 8B-a-b).

Example 3

Active growing mycelia of *S. mycoparasitica* (SMCD 2220-01) were cultivated on Potato Dextrose Broth (PDB) in a shaking flask at 21^{0}C for 3 days and then washed. Wet mycelium (0.1 g) were resuspended in 1 ml of PDA medium supplemented 100 µg/mL 3-ADON (Sigma-Aldrich Canada Ltd., Oakville, ON, CA) and incubated for 10 days.

A 0.2cm^2 plug *S. mycoparasitica* was incubated in ImI PDB containing 3-ADON at a concentration of 100 µg at RT for 7 days. The sample was analysed for DON degradation by TLC and HPLC assays.

Extraction of 3-ADON:

3-ADON was extracted by the method disclosed by Vasavada and Hsieh (1987, Appl. Micro. Biotechnol. 26: 517-521). The spent medium in sampled flasks was filtered through the Whatman® filter paper avoiding the mycelia. 3-ADON was extracted from the medium by three 10 ml volumes of ethyl acetate. The mixture of the medium and solvent was vigorously shaken and allowed to stand for 5 min for separation of phases. The organic phase was siphoned off and passed through sodium sulfate to remove residual water. The solvent was allowed to evaporate at room temperature. The residue was redissolved in acetonitrile for analysis.

Analysis of DON by TLC:

Thin layer chromatography (TLC) was used to analyze the DON. TLC was performed by the method disclosed by Andrea *et.al.* (2004, J. Basic Microbiol. 44: 147-156). Dried residues were dissolved in 75 µl and loaded onto silica gel TLC plates coated with fluorescent indicator (60 F_{254}, 0.2 mm layer; Merck Frosst Canada Ltd, Kirkland, PQ, CA). The spots were focused in the solvent system with ethyl acetate-toluene (3:1) for 40 5 min. The gel plates were sprayed with 20% aluminum chloride in 95% ethanol. Trichothecenes were visualized as dark spots under short wavelength UV light (254 nm). Chromatograms were photographed using gel documentation system. A known standard control of pure 3-ADON (Sigma-Aldrich Canada Ltd., Oakville, ON, CA) was employed to compare with other treatments. The sample containing *S. mycoparasitica* showed significantly less 3-ADON than the control sample.

High Performance liquid chromatography (HPLC)

A Water's HPLC with : 250 x 4.60 mm id Prodigy 5µ ODS (3) 10OA, 5µ micron Ci_8 column (Phenomenex Inc., Torrance, CA USA) and a photodiode-array (PDA) detector was used with a gradient solvent system (water-acetonitrile containing 0.005% (v/v) trifluoracetic acid). The PDA detector measured the UV spectrum (200-600 nm). Samples were dissolved in acetonitrile and 20 µl was loaded onto the column using an automatic injector. The DON was eluted with solvent as a mobile phase at a rate of 1 ml min^{-1} 3-ADON obtained from Sigma-Aldrich (Sigma-Aldrich Canada Ltd., Oakville, ON, CA) was used as a standard. The results are shown in Figure 9. The sample with *S. mycoparasitica* contained a much reduced level of 3-ADON.

Example 4

Potato dextrose agar (PDA), Potato dextrose broth (PDB), Yeast extract, Malt extract agar (MEA), Agar, and Peptone were purchased from BD Biosciences (Mississauga, ON, CA) . Streptomycin sulphate, Neomycin sulphate, and other reagents of analytic grade were from Sigma-Aldrich Canada Ltd. (Oakville, ON, CA) .

Fungal strains and growth conditions

Four *phytopathogenic Fusarium* strains *(F. avenaceum, F. sporotrichioides,* F. *oxysporum,* and *F. proliferatum);* three beneficial fungal inoculants *(Trichoderma harzianum* (RootShield ®), *Penicillium bilaii* (JumpStart ®), and *Chaetomium globosum;* and one mycoparasitic fungal strain *(Sphaerodes mycoparasitica* SMCD 2220-01) isolated from F. *oxysporum* host, were maintained on PDA amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate) and used throughout this study. *S. mycoparasitica* mycoparasitic fungal isolate was separated from their F. *oxysporum* host, and monosporium cultures were achieved according to methods described by Harveson RM, Kimbrough JW, (2001, Int. J. Plant Sci. 162: 403-410) with few modifications. Mature perithecia were picked up, suspended, and shaked in 2-ml sterilized water blanks. The spores-suspension was then spread on PDA plates. After few minutes, with the assistance of a Carl Zeiss Stemi 1000 dissecting microscope, individual spores were removed and transferred to PDA supplemented with 100

ml/1 of *Fusarium* filtrate. All fungal isolates were maintained in the culture collection of the Saskatchewan Microbial Collection and Database, Canada (SMCD).

Spores production

Mycelium plugs from the margin of the active growing monosporial Sphaerodes culture were cut and placed on Modified Leonian's Agar (MLA: Maltose, 6.25g; Malt extract, 6.25g; KH2PO4, 1.25g; Yeast extract, 1.Og; $MgSO_4$J H_2O, 0.625g; Peptone, 0.625g; Agar, 2Og, and IL of dH_2O. Inoculated MLA plates were incubated at room temperature (23^0C) in dark condition for a month before collecting the spores for germination assays. With the assistance of dissecting microscope, mature spores exuded from the ascomata and located on the ostiolar opening were harvested by picking up carefully with sterile needles and added to 2 ml tubes in 1 ml sterilized distillated water. The suspension was then filtered through four thin layers of cheesecloth to remove the remaining vegetative cells or mycelia. The density of the spore suspension was counted with a haemocytometer and adjusted to approximately $5\text{-}6xlO^5$ spores per ml in sterilized water. Freshly prepared aqueous spore suspensions were used.

Preparation of the fungal filtrates

Four pathogenic *Fusarium* strains and three beneficial fungal strains were grown in shake cultures for 7 days at room temperature (23°C) in 500 ml flasks, each with 100 ml PDB medium.

After incubation of 7 days in PDB medium, mycelia were removed by filtering through filter paper and the filtrates were then filter-sterilized. Freshly preparations fungal filtrates were employed for the spore germination tests.

Sphaerodes mycoparasitica strain SMCD 2220-01 was found to sporulate on or when inoculated together with *Fusarium* species, such as F. *avenaceum* and *F. oxysporum. S. mycoparasitica* was observed to produce approximately the same amount of ascomata on both *F. oxysporum* and *F. avenaceum. S. mycoparasitica* was not found to produce fruiting bodies on other *Fusarium* or fungal strains such as *F. proliferatum, F. sporotrichioides, P. bilaai, T. harzianum,* and *C. globosum.* In the contact zone between these biotrophic mycoparasites and *Fusarium* species, hook-shaped contact structures were formed.

Effects of heat and cold treatments on Sphaerodes spore germination

Aqueous ascospore suspensions were heat-shocked at 60°C and 65°C for 20 min, and cold-treated at 4°C, -20°C, and -70°C for 5 min and 20 min. The heat- and cold-shocked spores were then transferred and inoculated onto WA and PDA for 1 day and 3 days. The readings of spore germination were checked daily. Spores not subjected to heat and cold treatments were used as control.

There was no germination observed in any of the heat and cold treatment groups for 3 days. Additional observations were continued up to 7 days and no spore germination was observed.

Germination on various media

Aqueous spore suspensions were transferred and inoculated onto the surface of the following media:

- 1.5% water agar (WA), PDA, MLA, MEA, Carnation leaves agar (CLA);
- 1.5% water agar plus 100 ml/1 of *Fusarium* filtrate;
- PDA with 100 ml/1 of *Fusarium* filtrate.

Fusarium strains utilized were *F. avenaceum, F. oxysporum, F.* proliferatum, and *F. sporotrichioides.* Ascospore-inoculated plates were then incubated at room temperature (23°C) for 3 days. Spore germination was examined daily.

The results are summarized in Table 1. Each incubation day for Sphaerodes was analyzed separately. Numbers in each column represent the mean of ascospore germination (in %) ± standard deviation. Means within each column for each medium treatment followed by the same letter are not significantly different at $P \leq 0.05$ after Mann-Whitney U test.

Table 1:: Sporulation of *S. mycoparasitica* on various media

Medium	*S. mycoparasitica*	
	Day 1	Day 3
Water agar (WA)	0 d	0 d
F. avenaceum-WA	11.1 ± 1.7 b	35.2 ± 2.6 b
F. oxysporum-WA	1.6 ± 1.3 c	18 ± 1.4 c
F. proliferatum-W A	0 d	0 d
F. sporotrichioides-WA	0 d	0 d
Potato dextrose agar (PDA)	0 d	0 d
F. avenaceum-PDA	21.5 ± 1.4 a	63.2 ± 2.4 a
F. oxysporum-PDA	2.5± 2 c	37.5 ± 2 b
F. proliferatum-PDA	0 d	0 d
F. sporotrichioides-PDA	0 d	0 d

Effects of fungal filtrates on spore germination

The aqueous spore suspensions were suspended in the filtrates of four separate pathogenic *Fusarium* and three beneficial fungal strains for 1 day and 3 day in the ratio of 1:2 (1 part of aqueous spore suspension: 2 parts of fungal filtrate), and the filtrate-suspended spores were then transferred and inoculated onto PDA for additional 1 day. Control treatments were suspended with sterilized distilled water or PDB.

Spore germination

Microscopic assessments of ascospore germination were conducted after incubation for 1 day and 3 days. Percentage of germinated spores was obtained by scoring the spores on the Petri dish through utilizing the 20Ox and 40Ox objectives of the Carl Zeiss Axioskop2 microscope and systematically choosing 50 spores, starting at the top right corner and continuing to count till 50. Each drop of ascospore suspensions on a medium plate was considered as a subunit, and there were three subunits per plate. Each medium plate for each treatment was replicated, there were three replicas per treatments. The experiments were repeated twice. An ascospore was only considered as germinated spore when the germ tube was visibly noticeable. Germinated ascospores were counted and recorded as a percentage of the total ascospore number.

The results are summarized in Table 2. Numbers in each column represented mean of ascospore germination (in %) ± standard deviation. Each incubation day for *Sphaerodes* was analyzed separately. Means within each column of *Sphaerodes* for each filtrate-suspension treatment followed by the same letter are not significantly different at $P \leq 0.05$ after Mann-Whitney U test.

Table 2: Effects of fungal filtrates on S. *mycoparasitica* spore germination

***Shpaerodes* sp.**	**Spore germination (in %)**			
Treatments	**ld suspension**	**ld sus + ld PDA**	**3d suspension**	**3d sus + ld PDA**
Water	0 c	0 e	0 e	2 ± 1.2 c
PDB	0 c	0 e	0 e	2.8 ± 1.9 c
F. avenaceum-filtrate	89.2 ± 6.2 a	91 ± 8.4 a	93.2 ± 6.7 a	94.2 ± 7.9 a
F. oxysporum-filtrate	0 c	81.8 ± 11 b	85± 7.9 b	91 ± 10 a
F. proliferatum-filtrate	2.6 ± 3.6 b	3.2 ± 4.6 d	10 ± 7.4 c	11.2 ± 8 b
F. sporotrichioides-filtrate	0 c	3 ±2.8 d	3.4 ± 3.3d	10 ± 5.6 b
P. bilaii-filtrate	0 c	0 e	0 e	0 c
T harzianum-filtrate	0 c	9 ± 1.1 c	1.6 ± 1.6 d	10.4 ± 1.5 b
C. globosum-filtrate	0 c	0 e	0 e	0 c

Example 5

Since *F. graminearum* 3-ADON is the most pathogenic and mycotoxigenic in wheat, 3-ADON was used to quantifying mycoparasite-Fusarium/wm-wheat root interactions under Phytotron controlled conditions.

Fungal strains and growth

Fusarium strains: *F. graminearum* 3-ADON strain SMCD2243, biotrophic mycoparasite *Sphaerodes mycoparasitica* SMCD2220-01, and Trichoderma harzianum T-22 (RootShield-'cornmercial product) as a control strain with mycoparasitic properties were maintained on PDA amended with antibiotics.

Taxon specific primers

Specific primer sets for: *F. graminearum-¥g* 2NFiR (Nicholson et al. 1998. *Plant Pathology* 53: 17-37.); *Trichoderma-TG?4-F* IR (Kim and Knudsen 2008. *Applied Soil Ecology* 40: 100-108); and *S. mycoparasitica* SMCD2220-01-SmyITSF/R were used in this study.

Mycoparasite/Fusarium/plant interactions

Wheat CDC-TEAL 2001 plants were inoculated with mycotoxigenic and pathogenic *Fusarium* as well as *Trichoderma* (control mycoparasite) or *Sphaerodes* SMCD2220-01, and were subjected to RT-PCR quantification to assess the amount of fungal DNAs in roots of wheat under controlled conditions after 14 days of incubation in Phytotron conditions.

Growth conditions andfungal inoculation

Five mycelial plugs from *S. mycoparasitica, T. harzianum,* and *F. graminearum* were cut, transferred and grown in three separate shake cultures for 14 d at room temperature (23°C) in 500 mL tlasks each with 100 mL of PDB (potato dextrose broth). After incubation of 14 d in PDB medium, the fungal cultures were filtered through a Whatman® No. 1 filter paper to remove the liquid medium. The mycelia were then transferred to 50 mL sterile Falcon tubes with 20 sterile glass beads and 40 mL of autoclaved distillated water. The Falcon tubes filled with mycelia materials were then vortex vigorously for 1 min to separate the mycelia into smaller pieces. Mycelial suspension was filtered through 2 layers of cheesecloth to remove the glassbeads and bigger mycelial clumps. The flow-through was then used as mycelial suspension stock (10°) for serial dilution. S-tock of mycelial suspension was further diluted into a series spanning from 10^{-2}, 10^{-3}, and 10^{-4}. This dilution series were plated on PDA using the pour plate method. The number of CFU (colony forming units) was counted and recorded. Mycelial suspensions were adjusted with sterile water to about 10^{-5} - 10^{-6} CFU/mL for *S. mycoparasitica* and *T. harzianum,* and to about 10^{-4} - 10^{-5} CFU/mL for *F. graminearum.*

Quantification of interactions between mycoparasite-pathogen-wheat roots was conducted on the spring wheat CDC-TEAL 2001. Wheat plants were grown in containers (4 x 4 x 16 cm) with 10 g of different layers of soil-less growing mix (Figure 10). All the seeds were surface-sterilized prior to sowing. The containers 10 were lined with filter paper and packed with

6 g of Pro-Mix® soil-less mix (Sun Gro Horticulture Canada Ltd., Delta, BC, CA; Pro-Mix is a registered trademark of Premier Horticulture Ltd., Riviere-du-Loop, PQ, CA) which comprised the first layer 20. This layer 20 was then overlayed with a second layer 30 (Ig) of either Pro-Mix" mix amended and homogenized with ~5 to 6 x 10^4 CFU of *F. graminearum* mycelial suspension or alternatively with Pro-Mix® mixwith water only. The second layer 30 was followed by a third layer 40 (Ig) of either Sunshine® peat moss (Sunshine is a registered trademark of Sun Gro Horticulture Canada Ltd., Seba Beach, AB, CA) supplemented and homogenized with ~ 5 to 6 x 10^5 CFU of S. *mycoparasite* or alternatively with *T. harzianum* mycelial suspension or alternatively with water only. Six spring wheat seeds were then sowed on top of the third layer 40 and topped with a fifth layer 60 of 2 g of Sunshine® peat moss (Figure 10). Spring wheat seeds were germinated and grown under a 16-h photoperiod (22°C day/15°C night) with light intensity of 250 µmol m^{-2} s^{-1}, watered every 2 d, and fertilized every 14 d using 1300 ppm of NPK (20-20-20) fertilizer. All treatments in the experiment were in three replicates and the experiment was repeated twice.

At the mid-seedling growth, corresponding to Zadok's growth stage 13 (Zadoks et al. 1974, Weed Research 14:415-421), wheat plants with their roots were removed from the pots and washed under running tap water to remove all the soil particles. Washed roots were dried with filter papers. Number of germinated seeds, total biomass, root biomass, total length, and root length were counted and measured. Percentage of seed germination was calculated with the following formula: (Number of germinated seeds in particular treatment/number of germinated seeds in control treatment) x 100%. The roots were then subjected to total DNA extraction with DNeasy® Plant Mini Kit (Qiagen Inc., Mississauga, ON, CA). Extracted total DNAs from roots of different treatments were employed in real-time PCR quantification.

Statistical analyses

Root biomass (g), total biomass (g), root length (cm), total length (cm), seed germination (%); and *S. mycoparasitica, T harzianum,* and *F. graminearum* genomic DNA quantification from the roots of Spring wheat plants were analyzed by using analysis of variance (ANOVA). Log^{10} transformations were carried out whenever required to meet the ANOVA requirements.

Multiple comparisons for more than two samples were analyzed by utilizing Tukey's studentized range test at $P = 0.05$ (SPSS 1990).

Wheat growth and fungal inoculation

Root biomass, total biomass, root length, total length, and seed germination of *F. graminearum* infected spring wheat were significantly increased with the treatments of *S. mycoparasitica* compared to inoculation with *F. graminearum* alone (Table 3).

Table 3: Effects of SMCD2220-01 (SM) and *F. graminearum* 3-ADON (Fgra) inoculation treatments on root biomass (g), total biomass (g), root length (cm), total length (cm) and seed germination (%) of spring wheat plants

Treatment	**Root biomass (g)**	**Total biomass (g)**	**Root length (cm)**	**Total length (cm)**	**Seed germination** (%)
Control	0.27 ± 0.06 **b**	0.56 ± 0.56 **b**	9.63 ± 0.74 **b**	31.88 ± 2.39 **c**	NA*
SM	0.32 ± 0.03 ab	0.69 **±** 0.69 a	13.88± 1.1 1 a	38.13 ± 2.76 ab	101 ± 2 a
Fgra	0.18 ± 0.03 c	0.38 ± **0.48 c**	7.88 ± 1.89 c	23.75 ± 1.98 d	31.25 ± 1.4 c
SM-Fgra	0.27 + 0.03 **b**	0.54 ± 0.54 **b**	12.13 ± 1.35 ab	32.13 ± 3.91 be	87.25 ± 2 **b**

* Values expressed are means of six replicates ± standard deviation of the mean. Values followed by same letters within each column are not significantly different using Tukey's range test ($P < 0.05$).

Table 3, the mycoparasite *S. mycoparasitica* demonstrates both biomass stimulation and bioprotection or biocontrol.

Conformation of the Sphaerodes-specific primer set

The SmyITSF/R primer set was tested with *S. mycoparasitica*, seven *Fusarium* species, night different ascomycetous fungal isolates two zygomycete fungi, and three basidiomycetous fungal strains. This primer set only amplified *S. mycoparasitica.*

Figure 11 shows SmylTSF/R primers amplified PCR products for *S. mycoparasitica* (SM), five *Fusarium* strains (Fa = *F. avenaceum,* Fo = *F. oxysporum,* Fs = *F. sporotrichioides,* Fg3 = *F. graminearum* chemotype 3, and Fg15 = *F. graminearum* chemotype 15), two *Trichoderma* species (T22 = *T. harzianum* T22 and Tv = *T. viride),* two *Cladosporium* species

(CC = *C. cladosporioides* and CM = *C. minourae)*, and *Penicillium aurantiogriseum* (PA) were electrophoresed on 1% agarose gel at 100 V for 20 minutes. The size of the band is around 300 to 400 bp.

Standard curves

The standard curves based on known diluted concentrations of DNAs from *S. mycoparasitica, F. graminearum,* and *T. harzianum* were constructed. Standard curves were achieved using a series of 10-fold diluted DNA spanning from 3.8 x 10^2 to 3.8 x 10^{-2} ng for *S. mycoparasitica,* 2.7 x 10^3 to 2.7 x 10^{-1} ng for *F. graminearum* chemotype 3, and 7.0 x 10^2 to 7.0 x 10^{-2} ng for *T. harzianum.* Quantification demonstrated linear relation (r^2 = 0.999 for *S. mycoparasitica,*, r^2 = 0.998 for *F. graminearum,* and r^2 = 0.996 for *T. harzianum)* between logio of fungal genomic DNA (in ng/µl) and real-time PCR threshold cycles (Ct) (threshold fluorescence signal of 0.025 was used for all three fungal isolates) (Figures 12A, 12B, and 12C).

Figure 13 shows RT-PCR sigmoidal coloured curves for *Sphaerodes mycoparasitica* (SMCD 2220-01), with 0.025 fluorescence line, showing the range of 3.8 x 102 to 3.8 x 10-2 ng in a ten-hold decreasing manner.

RT-PCR confirmation of the Sphaerodes-biocontrol effects

Real-time PCR, which evaluated quantity of *S. mycoparasitica* SMCD2220-01 and *F. graminearum,* confirmed that amounts of *F. graminearum* DNA detected in treatments with *S. mycoparasitica* SMCD2220-01 and *T harzianum* were significantly reduced (Figure 14A). Previously, treatments with mycoparasitic *Sphaerodes retispora* had been observed to show significant suppression of F. *oxysporum* in watermelon plants (Harveson et al. 2002, Plant Dis. 86: 1025-1030). The amount of *S. mycoparasitica* DNA detected was not significantly different between wheat inoculated with *F. graminearum* or without *Fusarium* (Fig. 14B). The amount of *T. harzianum* DNA detected in the treatment inoculated with *F. graminearum* was observed to be significantly reduced, as compared to *non-Fusarium* treatment (Fig. 14C).

In conclusion, during in vitro culture-based studies, only *S. mycoparasitica* SMCD2220-01 was observed to enhance wheat seed germination and formation of secondary roots, whereas T-22 induced post-emergence damping-off symptoms. Under controlled

conditions in pytotron, *S. mycoparasitica* SMCD2220-01 was able to reduce the quantity of *F. graminearum* in spring wheat root, as well as improving the survival and growth of the spring wheat seedlings. In contrast to *T. harzianum,* the amount of *S. mycoparasitica* SMCD2220-01 DNA detected was not significantly different between wheat inoculated with *F. graminearum* and without *Fusarium.* Hence, *S. mycoparasitica* SMCD2220-01 could be a better biocontrol candidate for the *F. graminearum* pathogen in wheat.

Example 6

Quantitative RT-PCR results from testing Mycoparasite-FMs*arium* interaction on Wheat host using growing conditions disclosed herein also confirmed the efficiency of *S. mycoparasitica* SMCD2220-01 for significantly decreasing an accumulation of genes implicated in *Fusarium* DON-mycotoxin production.

Strain used: F. *graminearum* 3-ADON SMCD2243, *F. graminearum* 15-ADON SMCD2244 and *S. mycoparasitica* SMCD2220-01 strains.

Primersets used: Tox5-l/2 for *Fusarium* (Wu *et al.* 2002. J. Environ Monit. 4:377-382) and SmyITSF/R (disclosed herein) for *S. mycoparasitica.*

Results are summarized in the Figure 15. Real-time fluorescence curves of tri5 gene sequences amplified by using Tox5-l/2 primer set from total DNA extracted from dual-culture assays of *Fusarium graminearum* strains and pre-inoculated *Sphaerodes mycoparasitica* SMCD2220-01 (SM) or singly grown *F. graminearum* 3-ADON and 15-ADON chemotypes.

Example 7

The effects of filtrates collected from different *Fusarium sp.* on the germination of *Sphaerodes* ascospores were assessed in this study. Also assessed were the ascospore germination patterns.

Media, Reagents, and chemicals: Potato dextrose agar (PDA), potato dextrose broth (PDB), yeast extract, malt extract agar (MEA), agar, and peptone were purchased from Difco (Becton

Dickinson Diagnostics, Sparks, Maryland). Streptomycin sulphate, Neomycin sulphate, and other reagents of analytic grade were from Sigma-Aldrich (Oakville, ON, CA) .

Fungal strains and growth conditions: Four phytopathogenic *Fusarium* strains *(F. avenaceum* SMCD 2241, *F. oxysporum* SMCD 2242, *F. proliferatum* (Matsush.) Nirenberg SMCD 2244, and *F. sporotrichioides* Sherb SMCD 2243), three beneficial fungal inoculants: *T. harzianum* (RootShield ®), P. *bilaii* ([JumpStart ®), and *Chaetomium globosum* Kunze; and one mycoparasitic fungal strain, *S. mycoparasitica* were maintained on PDA amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate) and used throughout this study. An isolate of the mycoparasitic fungus, *S. mycoparasitica,* was separated from its *F. oxysporum* host on myclobutanil agar (MBA) selective medium as previously described and a monosporic culture was achieved by picking up mature perithecia which were then suspended and shaken in 2-mL sterile distilled water blanks to encourage release of ascospores. The ascospore-suspension was then spread on PDA plates. After a few minutes, a Carl Zeiss Stemi 1000 dissecting microscope was used to identify individual ascospores, which were removed and transferred to PDA supplemented with 100 mL/L of *Fusarium* filtrate *(F. avenaceum* and *F. oxysporum).* All fungal isolates were maintained in the culture collection of the Saskatchewan Microbial Collection and Database, Canada (SMCD).

Ascospore production: Mycelium plugs from the margin of the actively growing monosporic-derived *S. mycoparasitica* culture were cut and placed on Modified Leonian's Agar (MLA: maltose, 6.25g; malt extract, 6.25g; KH_2PO_4, 1.25g; yeast extract, l.Og; $MgSO_4$-$7H_2O$, 0.625g; peptone, 0.625g; agar, 2Og, and IL of dH_20) (Malloch and Cain 1971). Inoculated Modified Leonian's Agar (MLA) plates were incubated at room temperature (23° C) under darkness conditions for a month before collecting spores for germination assays. Mature ascospores were collected according Goh and Vujanovic (2010).

Preparation of the fungal filtrates: Four pathogenic *Fusarium* strains and three beneficial fungal strains were grown in shake cultures for 7 d at room temperature (23 °C) (with 250 rpm) in 500 mL flasks, each with 100 mL PDB medium prior to fungal filtrates extraction. After incubation, the mycelia were removed by filtering through filter paper (Whatman ® Grade No. 1) and the filtrates were then filter-sterilized with 0.02-μm-pore-size nitrocellulose filter (Fisher Scientific

Ltd., Nepean, ON, CA). Only fresh preparations of fungal filtrates were employed for the spore germination test in this study.

Effect of heat and cold treatments on S. mycoparasitica spore germination: Heat activation and cold-shock treatments were performed to investigate ascospore germination in *S. mycoparasitica.* Aqueous ascospore suspensions were heat-shocked at 60 and 65 °C for 20 min, and cold-treated at 4, -20, and -70 °C for 5 min and 20 min. The heat- and cold-shocked spores (10 µL) were then transferred and inoculated onto water agar (WA) and PDA for 1 d and 3 d. Spore germination was checked daily. Spores not subjected to heat and cold treatments were used as controls. All treatments were in three replicates and experiment was repeated twice.

No germination was observed in all the heat and cold treatments over a 7 d period.

Germination on various media: Aqueous spore suspensions (10 µL) of *S. mycoparasitica* were transferred and inoculated onto the surface of the following media: 1.5% water agar (WA), PDA, MLA, MEA, Carnation leaf agar (CLA) (Tschanz et al. 1976), 1.5% water agar plus 100 mL/L of *Fusarium* filtrate, and PDA with 100 mL/L of the *Fusarium* filtrate. *Fusarium* filtrates were created using *F. avenaceum, F. oxysporum, F. proliferatum,* and *F. sporotrichioides. S. mycoparasitica* ascospore-inoculated plates were then incubated at room temperature (23 °C) for 3 d, and spore germination was examined daily. All treatments were in three replicates and experiment was repeated twice.

There was no spore germination on 1.5% WA, PDA, MLA, MEA, and CLA. The effects of 1.5% WA and PDA media amended with 100 mL/L of *Fusarium- filtrates* on spore germination were examined and are summarized in Table 4.

No germination was recorded on day 3 initially and observations were continued up to 7 d on WA or PDA alone and WA or PDA with either *F. proliferatum* or *F. sporotrichioides.* However, when either F. *avenaceum* or *F. oxysporum* filtrates were added to WA and PDA, the percentage of ascospore germination in *S. mycoparasitica* drastically increased after 3 d incubation. On day 3, germination increased on the PDA amended with *Fusarium-f útratQ,* compared to growth on WA supplemented with *Fusorium-f útrate,* for *S. mycoparasitica*

Table 4: Percentage germination of *Sphaerodes mycoparasitica* ascospore on various types of *Fusarium-filtrate* supplemented media, including water agar and potato dextrose agar checks

S. mycoparasitica **Medium**	**Spore germination (%)*** Day 1**	Day 3**
Water agar (WA)	0 [d]	0 [α]
F. avenaceum-WA	11.1 ± 1.7 [b]	35.2 ± 2.6 [b]
F. oxysporum-WA	1.6 ± 1.3 [C]	18 ± 1.4 [C]
F. proliferatum-WA	0 [d]	0 [d]
F. sporotrichioides-WA	0 [d]	0 [d]
Potato dextrose agar (PDA)	0 [d]	0 [d]
F. avenaceum-PDA	21.5 ± 1.4 [a]	63.2 ± 2.4 [a]
F. oxysporum-PDA	2.5 ± 2 [C]	37.5 ± 2 [b]
F. proliferatum-PDA	0 [d]	0 [d]
F. sporotrichioides-PDA	0 [d]	0 [d]

* Numbers in each column represented mean of ascospore germination (in %) ± standard deviation.

** Each incubation day for *Sphaerodes* was analyzed separately. Means within each column for each medium treatment followed by the same letter are not significantly different at $P \leq 0.05$ after Kruskal-Wallis test.

Effects of fungal filtrates on spore germination: Fungal filtrates from four different phytopathogenic *Fusarium* species and three beneficial fungal isolates were employed to study spore germination of *S. mycoparasitica,* and host specificity response. Aqueous spore suspensions of *S. mycoparasitica* were suspended in the filtrates of four separate pathogenic *Fusarium* spp. and three beneficial fungal strains for 1 d and 3 d in the ratio of 1:2 (1 part of aqueous spore suspension: 2 parts of fungal filtrate). Filtrate-suspended spores (10 μL) were then transferred and inoculated onto PDA for an additional day (spore germination was counted on PDA plate). Spore germination was count according to Goh and Vujanovic (2010) after an additional day of inoculation on PDA medium. Control treatments were suspended with sterilized distilled water or PDB. Abbreviations for different treatments at four separate chronosequences throughout the experiment: Id Sus, Id Sus + Id PDA, 3d Sus, and 3d Sus + Id PDA represent 1 day *Fusarium-filtrate* suspension, 1 day filtrate suspension with an additional day incubation on PDA medium, 3 day suspension, and 3 day filtrate suspension with an additional day incubation on PDA, respectively.

No spore germination was observed in the treatments with *P. bilaii* and *C. globosum* fungal filtrates. Both water and PDB suspension controls appeared to trigger approximately 1.8-3.8% spore germination for *S. mycoparasitica,* on day 3 with an additional day on PDA only (Table 5). Ascospores of *S. mycoparasitica* suspended in filtrates of pathogenic *F. sporotrichioides* and beneficial *T. harzianum* showed no germination on day 1 in suspension, but low number of ascospore germination was observed after 1 day in suspension followed by an additional day on PDA, and in the other incubation treatments. Ascospores suspended in F. *proliferatum-f úratQ* were observed to germinate in low abundance for 1 d, 1 d with an additional day on PDA, 3 d, and 3 d plus an additional day on PDA. *Sphœrodes* ascospores showed the highest number of germinated ascospores in the F. œvenœceum-f úrate suspension. In the day 1 suspension treatments, ascospore germination of *S. mycoparasitica* in *F. avenaceum-f úrate* was significantly higher (89.2%) than in other treatments. When ascospores of the *S. mycoparasitica* were suspended in *F. oxysporum-f úrate,* the amount of spore germination was increased compared to control. There was no germination recorded for the 1 d suspension in the *F. oxysporum-f λWratQ* treatment. However, a low number of ascospores were stimulated in *F. oxysporum-f úτate* suspension on the second day. Based on the ascospore germination rate and response, *S. mycoparasitica* showed high specificity to F. *avenaceiim* and *F. oxysporum.*

Table 5: Percentage spore germination of *Sphaerodes mycoparasitica* ascospores suspended in different *Fusarium* and biocontrol fungi filtrates assesed throughout four chronosequences. Control treatments were suspended with sterilized distilled water (SDW) or potato dextrose broth (PDB).

S. mycoparasitica	**Spore germination (%)***			
Treatment**	1d Suspension	1d Sus + 1d PDA	3d Suspension	3d Sus + 1d PDA
SDW	0 [c]	0 [e]	0 [e]	2 ± 1.2 [c]
PDB	0 [c]	0 [e]	0 [e]	2.8 ± 1.9 [c]
F. avenaceum-filtrate	89.2 ± 6.2 [a]	91 ± 8.4 [a]	93.2 ± 6.7 [a]	94.2 ± 7.9 [a]
F. oxysporum-filtrate	0 [c]	81.8 ± 11 [b]	85 ± 7.9 [b]	91 ± 10 [a]
F. proliferatum-filtrate	2.6 ± 3.6 [b]	3.2 ± 4.6 [d]	10 ± 7.4 [c]	11.2 ± 8 [b]
F. sporotrichioides-filtrate	0 [c]	3 ± 2.8 [d]	3.4 ± 3.3 [d]	10 ± 5.6 [b]
P. biloxi-filtrate	0 [c]	0 [e]	0 [e]	0 [c]
T. horzianum-filtraie	0 [c]	9 ± 1.1 [c]	1.6 ± 1.6 [d]	10.4 ± 1.5 [b]
C. globosum-filtrate	0 [c]	0 [e]	0 [e]	0 [c]

* Numbers in each column represented mean of ascospore germination (in %) ± standard deviation.

** Each incubation day for *S. mycoparasitica* was analyzed separately. Means within each column of *S. mycoparasitica* for each filtrate-suspension treatment followed by the same letter (in superscript) are not significantly different at $P \leq 0.05$ after Kruskal-Wallis test.

Spore germination assessments: Microscopic assessments of ascospore germination were conducted after incubation for 1 d and 3 d (spore germination was counted on suspensions). Percentage of germinated spores was obtained by scoring the spores on a Petri dish while observing them through the 20Ox and 40Ox objectives of the Carl Zeiss Axioskop2 microscope and systematically choosing 50 spores, starting at the top right corner and continuing to count until 50. Each drop of the ascospore suspension on a growth medium plate was considered as a subunit, and there were three subunits or replicates per plate. The experiments were repeated twice. An ascospore was only considered germinated when the germ tubes exceeded the width of the ascospore (approximately 12μm). Germinated ascospores were counted and recorded as a percentage of the total ascospore number that was counted.

Ascospores from *S. mycoparasitica* showed two kinds of germination patterns. Single polar germination was more prevalent in the *F. oxysporum-fúrate* suspension (Figures 16A, 16B, 16D, 16E). A small number of spores from *S. mycoparasitica* found to produce shorter double-polar germination in the treatment with *F. oxyporum-fúrate* at 3 d suspension with additional 1 d on PDA (Figure 16F). In the F. *αvenαceum-fúrate* suspension, ascospores of *S. mycoparasitica* showed higher preference for two-polar germination (Figures 16G). Single polar germination was also found in *F. avenaceum-fúrats* suspension; however it was lower than in *F. oxysporum-fúrtatQ* treatment. Commonly, these single-polar germinated spores produced larger web-like organizations and longer hyphal formation (in *F. αvenαceum-fúrtate* suspension), which was rarely found in F. *oxysporum-fúrate* suspended spores (Figures 16H, 161). The majority of the germinated ascospores in *S. mycoparasitica,* which was activated through suspension in F. *oxysporum-fúrvate* were detected to be either at an angle of 90° (Figures 16E, 16F) or between 90° and 180° (Figure 16F) at the tip of the polar germ pores. However, very few spores germinated at an angle of 180° (Figure 16D). Most activated spores (with F. *αvenαceum-fúrate* treatment) showed germination at an angle of 180° (Figure 16G).

Example 8

The host specificity of *S. mycoparasitica* to F. *graminaerum* 3-ADON and 15-ADON strains were assessed in this study.

Media, reagents and chemicals: Potato dextrose agar (PDA), potato dextrose broth (PDB), yeast extract, malt extract agar (MEA), agar, and peptone were purchased from Difco (Becton Dickinson Diagnostics). Streptomycin sulphate, Neomycin sulphate, and other reagents of analytical grade were from Sigma-Aldrich.(Oakville, ON, CA) IQ SYBR Green Supermix for real-time PCR reactions was acquired from Bio-Rad Laboratories (Mississauga, ON, CA).

Fungal strains and growth: All phytopathogenic *Fusarium* strains: *Fusarium graminearum* 3-ADON (Fgra3) SMCD 2243, and 15-ADON (Fgral5) SMCD 2244 chemotypes, *F. avenaceum* (Fave) SMCD 2241, *F. oxysporum* (Foxy) SMCD 2242, *F. proliferatum* (Fpro) SMCD 2244, *F. sporotrichioides* (Fspo) SMCD 224; and one mycoparasitic *Sphaerodes mycoparasitica* SMCD 2220 strain were retrieved from Saskatchewan Collection and Database (SMCD), maintained on PDA amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate) and used throughout this study.

Spore production and germination assays: Ascospores of *S. mycoparasitica* were produced on Modified Leonian's agar (MLA), harvested and prepared as previously described. Also, *Fusarium* spp. filtrates were prepared, *S. mycoparasitica* spore germination assays in six different *Fusarium* filtrates were carried out as previously described.

Sphaerodes mycoparasitica spore germination suspended in both *F. graminearum* chemotype 3-ADON and 15-ADON filtrates was lower compared to *F. avenaceum* for the first incubation day, and compared to both *F. avenaceum* and *F. oxysporum* for the remaining incubation days ($P \leq 0.05$; with Mann-Whitney Test) (Figure 17). No significant differences in germination between *F. graminearum, F. proliferatum,* and *F. sporotrichioides* filtrate treatments were observed for the first two incubation days. However, treatments with *F. graminearum* filtrates showed significantly higher germination rate of *S. mycoparasitica* compared to *F. sporotrichioides* filtrate treatment during later incubation time points (Figure 18).

Dual-culture assays: Dual-culture assays for examining the degree of hyphal reduction/inhibition or damage to *F. graminearum* chemotypes were assessed as disclosed in Goh and Vujanovic (2009, Mycologia DOI: 10.3852/69-171). *S. mycoparasitica* is slow-growing fungus as

compared to *F. graminearumus* 3 and 15 strains. Therefore, *S. mycoparasitica* was pre-inoculated onto the PDA plates for I d, at 21 ^{0}C in darkness, prior to inoculating *Fusarium* mycelial plugs. Linear mycelial growth of *Fusarium* strains for both treatments indicated above was measured and recorded daily for 5 days. Around 0.5 x 1.5 cm^2 (sampling zones) located approximately 0.2 cm behind the contact zone between *F. graminearum* and *S. mycoparasitica* was excised and subjected to DNA extraction. Each treatment was with three replicates and the experiment was repeated twice. The PDA plate inoculated with *F. graminearum* only was the positive control. Total genomic DNA was extracted using a DNeasy® Plant Mini Kit. The DNA was eluted once in 50 µl of buffer AE and stored at -2O^{0}C until real-time PCR quantification assays (as described below).

Since *S. mycoparasitica* demonstrated slower mycelial growth (0.56 cm per day; n = 9) compared to *F. graminearum* 3-ADON (0.74 cm per day; n=6) and 15-ADON (0.68 cm per day; n=6) chemotypes, the linear growth of *F. graminearum* mycelia in dual-culture was assessed using the pre-inoculation method. *S. mycoparasitica* was pre-inoculated on PDA for 1 d followed by *F. graminearum* inoculation. The pre-inoculation approach demonstrated significant differences (starting day 3) in linear growth suppression of *F. graminearum* chemotype 3 and 15 compared to the co-inoculation approach (Figures 19A and 19B).

Establishment of mycoparasitism: Fusion biotrophic mycoparasitic interactions between *S. mycoparasitica* and both *F. graminearum* chemotype strains, and intracellular parasitism interactions were examined and assessed on slide cultures according the methods described in Goh & Vujanovic (2009).

On day 3 of inoculation on PDA with *F. graminearum* 3-ADON and 15-ADON, no clamp-like or hook-like structures were formed by *Sphaerodes mycoparasitica* on *Fusarium* strains. On day 5 of inoculation, clamp-like and hook-like contact structures as well as *Fusarium* hyphal cells penetration (with haustoria) were observed (Figures 2OE to 201). Furthermore, on day 3, *S. mycoparasitica* removed red pigment from the mycelia of *F. graminearum* 3-ADON on the slide culture (Figures 2OA to 20D). As a result, S. *mycoparasitica* mycelia adopted reddish color (Figure 20C). Between day 4 and 5, formation of red crystal-like pellets was detected on the surface of mycoparasite hyphae (Figure 20D). The mechanism behind the color changes

observed remains unknown. For *F. graminearum* chemotype 15-ADON, no uptake of red complex or release of red crystal-like structures by *S. mycoparasitica* hyphae were noted. Nevertheless, flower-like hyphal structures appeared which could indicate possible growth inhibition of 15-ADON *F. graminearum* (Figure 20J). Significant differences in diameters of infected and non-infected hyphae were seen for both *F. graminearum* chemotypes (Figure 21).

Primers and standard curves: F. graminearum-specific (Fgl6NF/R) and trichothecene *Tri5* gene-specific (Tox5-l/2) primer sets were used in this study. Standard curves for *F. graminearum*- and *Tri5* gene-primer sets were generated, based on threshold cycles (Ct), by using a series of 10-fold diluted genomic DNAs from F. *graminearum* (spanning from 2.7 x 10^2 to 2.7 x 10^{-2} ng/µl for *F. graminearum-specific* primer set, from 2.7 x 10^2 to 2.7 x 10^{-1} ng/µl of 3-ADON strain and 3.0 x 10^1 to 3.0 x 10^{-2} ng/µl of 15-ADON strain DNAs for *Tox5-J/2* primer set). Ct values were recorded and obtained by the Opticon Monitor software version 3.1 (Bio-Rad Laboratories Inc., Mississauga, ON, CA). Standard curves for different primer sets were constructed by plotting the threshold cycles (Ct) value versus the logarithm (logio) of the concentration of 10-fold serial diluted *F. graminearum* DNAs as described above. Amplifications with different primer sets on the genomic DNAs of two *F. graminearum* chemotypes were run in triplicates to obtain the mean and standard deviation of each 10-fold serial dilution.

Real-time PCR quantification: Real-time PCR amplifications on total genomic DNA extracted from the sampling zones (as described above) were performed using MiniOpticon (Bio-Rad Laboratories Inc., Mississauga, ON, CA). All the real-time PCR reactions were performed by utilizing the real-time PCR MJ white tubes (Bio-Rad Laboratories Inc., Mississauga, ON, CA) in a total volume of 25 µl. The reaction mixture for all real-time PCR assays were: 12.5 µl of IQ Supermix (Bio-Rad Laboratories Inc., Mississauga, ON, CA), 1 µl of each 10 µM forward/reverse primers (Invitrogen), 9.5 µl of sterilized UltraPure Millipore water, and 1 µl of DNA template. Real-time PCR conditions for Fgl6NF/R primer set used were outlined in Nicholson *et al.* (1998, Physiol. MoI. Plant Pathol. 53: 17-37) with melting curve analysis at 60 to 95^{0}C. Parameters for Tox5-l/2 primer set as described in Schnerr *et al.* (2001, Int. J. Food microbiol. 71: 53-61).

Standard curves for different primer sets with different *F. graminearum* DNA sources were constructed (Figure 22). Growth suppression or inhibition at the sampling zones (Figure 23) for *F. graminearum* chemotype 3 and 15 was further confirmed with real-time PCR amplifications with *F. graminearum*- and *tri5* gene-specific primer sets. Sigmoidal curves for the four different treatments *(F. graminearum* chemotype 3 or 15 only and *F. graminearum* chemotype 3 or 15 pre-inoculated with *S. mycoparasitica)* with Fgl6NF/R primer set were generated using Opticon Monitor software version 3.1 and illustrated in Figure 24.

Using Fgl6NF/R primer set, quantity of *F. graminearum* chemotype 3 DNA in the sampling zones significantly decreased when pre-inoculated with *S. mycoparasitica* compared to un-inoculated treatment ($P = 0.01$) (Figure 25). However, DNA of *F. graminearum* chemotype 15 did considerably but not significantly reduced ($P = 0.085$ using T-test). Using Tox5-l/2 primer set, amount of *tri5* gene fragments diminished appreciably in both *F. graminearum* chemotype 3 and 15 challenged with *S. mycoparasitica* ($P \leq 0.05$).

Example 9

The effects of *S. mycoparasitica* (biotrophic mycoparasite) and *T. harzianum* (necrotrophic mycoparasite) on expression of *Tri* and *PKS* genes by *F. graminaerum* 3-ADON and 15-ADON strains were assessed in this study.

Fungal strains and growth: Two *Fusarium graminearum* 3- ADON (SMCD 2243) and 15-ADON (SMCD 2244) chemotypes, *Trichoderma harzianum* necrotrophic (SMCD 2166) and *Sphaerodes mycoparasitica* biotrophic (SMCD 2220) mycoparasites were obtained from the Saskatchewan Microbial Collection and Database (SMCD). All strains were maintained on Potato dextrose agar (PDA, BD Biosciences, Mississauga, ON, CA) amended with antibiotics (100 mg/L streptomycin sulphate and 12 mg/L neomycin sulphate; Sigma-Aldrich Canada Ltd., Oakville, ON, CA) prior to study initiation.

Chemical fungicide control: A concentration of 100 µmol/L tebuconazole was prepared from Folicur® 432F (43.2% tebuconazole, Bayer CropScience Inc., Saskatoon, SK, CA; Folicur is a

registered trademark of Bayer Aktiengesellschaft, Leverkusen, Fed. Rep. Germany). This fungicide preparation was used throughout the study.

In vitro assay, sampling and RNA extraction: Dual-culture assay was carried out between *F. graminearum* and Folicur® (100 µmol/L tebuconazole) or biological *(T harzianum* or *S. mycoparasitica)* agents on Minimal medium as disclosed by Xue et al. (2009, Can. J. Plant Pathol. 31: 169-179). Inoculated dual-culture plates were incubated at room temperature (23C) in dark conditions for a week. Mycelia of *F. graminearum* were harvested after a week of inoculation with either chemical or biological agents. A 0.5-cm^2 mycelial plug was cut out approximately 0.5 cm away from the border of interaction and fungal cells were disrupted with liquid nitrogen. Total RNA from the samples were extracted using Aurum Total RNA mini kit and extracted RNA was treated with DNAse (Bio-Rad Laboratories Inc., Mississauga, ON, CA) according the manufacturer's recommendations. Samples were then stored at -70C till gene expression analysis.

During the *In vitro* studies, both *F. graminearum* 3-ADON and 15-ADON chemotypes changed mycelia morphology corresponding to each treatment (Figures 26A - 26B). However, the main diagnostic distinction was the tendency of *F. graminearum* chemotypes to abundantly produce chlamidospores in clusters or chains when exposed to tebuconasole fungicide compared to mycoparasites (Figures 26C - 26D). In the case of *F. graminearum* chemotype 15 (15-ADON producer), all four trichothecene genes - *Tri4, Tri5, Trio,* and *TriIO* in this *Fusarium* strain were found to be induced in high amounts when treated with chemical tebuconazole compared to treatments that co-inoculated with biological agents (either *S. mycoparasitica* or *T. harzianum)* (Figures 27A - 27D). *F. graminearum* chemotype 3 (3-ADON producer) was observed to demonstrate induction of *Tri4, Tri5* and *TriIO* genes when co-inoculated with necrotrophic mycoparasitic *Trichoderma harzianum* (Figures 27A, 27B, 27D). When *F. graminearum* chemotype 3 was treated with Folicur® fungicide, only *TriIO* gene was being induced (Figure 27D). Generally, all four *Tri* genes were repressed significantly when challenged with biotrophic mycoparasitic *Sphaerodes mycoparasitica* (Figure 27A - 27D).

Real-time Reverse-transcription PCR: Real-time RT-PCR was performed by using an IScript One-Step RT-PCR kit with SYBR Green on a MiniOpticon Cycler System (Bio-Rad

Laboratories Inc., Mississauga, ON, CA), according to the manufacturer's instruction. Primer sets used for amplification and gene expression were summarized in Table 6.

Table 6: Primer sets used to amplify *Tri4, Tri5, Tri6, TrilO, PKS4, PKSl 3,* and β-*tubulin* by Real-time PCR.

SEQ ID	Gene	Primer sequences	Reference
SEQ ID NO: 6/7	*Tri4*	Tri4-F: TAAACGCCCGCGAAGTTCACA Tri4-R: TGGTGATGGTTCGCTTCGAG	Jiao et al. 2008, FEMS Lett. 285: 212-219
SEQ ID NO: 8/9	*Tri5*	Tr5F: AGCGACTACAGGCTTCCCTC Tr5R: AAACC ATCC AGTTCTCC ATCTG	Doohan et al. 1999, Appl. Environ. Microbiol. 65: 3850-3854
SEQ ID NO: 10/1 1	*TM*	Tri6-1 TCTCTACCAACGGTGGATTCAACC Tri6-2 AGCCTTTGGTGCCGACTTCTTG	Pinson-Gadais et al. 2008, Mycopathol. 165: 51-59.
SEQ ID NO: 12/13	*TrilO*	TrilO-F: TCTGAAC AGGCGATGGTATGG A TrilO-R: CTGCGGCGAGTGAGTTTGACA	Jiao et al. 2008
SEQ ID NO: 14/15	*PKS4*	PKS4-PS.1 GTGGGCTTCGCTAGACCGTGAGTT PKS4-PS.2 ATGCCCTGATGAAGAGTTTGAT	Lysøe et al. 2006, Appl. Environ. Microbiol. 72:3924-3932
SEQ ID NO: 16/17	*PKSl 3*	PKS13-PS.1 CCCCCAACTCGACGTCAAATCTAT PKS13-PS.2 TTCTTCCCGCCGACTTCAAAACA	Lysøe et al. 2006
SEQ ID NO: 18/19	β-*tubulin*	FGtubf GGTCTCG ACAGC AATGGTGTT FGtubr GCTTGTGTTTTTCGTGGCAGT	Lysøe et al. 2006

RT-PCR sample (-25 μL) contained 3 μL of RNA template, 8.85 μL of nucleae-free water, 12.5 μL of RT-PCR reaction mixture (2x), 0.5 μL of IScript RT enzyme mix (50x) (Bio-Rad Laboratories Inc., Mississauga, ON, CA), 0.1 μL of 50 μM solutions of both forward and reverse targeted gene-specific primers (Invitrogen Corp., Carslbad, CA, USA). Real-time PCR conditions were performed as outlined by manufacturer's recommendations: 50C for lOmin, 95C for 5min, followed by 40 cycles of denaturing at 95C for 10s and annealing at 55C for 30s, 95C for lmin and 55C for 1 min. PCR reactions were checked for absence of any primer-dimer formation or non-specific PCR amplification by performing melting curve analysis. Contamination of RNA template with residual genomic DNA was eliminated because there was no amplification detected using reverse transcriptase free real-time RT-PCR reaction as template. Fold change in gene expression for each treatment was normalized to β-*tubulin* internal reference gene and relative to the expression for the control treatment *(Fusarium* alone on minimal medium), using the $2^{-\Delta\Delta CT}$ method proposed by Livak and Schmittgen (2001). $\Delta\Delta C_T = (C_{T,\,ta^{rg}_{et}}$-

gene - C_τ, Fgtub)treatment - (CT, target-gene - C_τ, $F_g$tub)controi, **where treatment** and control indicate ***F.*** *graminearum* challenged with chemical or biological agent and F. *graminearum* alone, respectively.

For gene expression analyses based on targeted genes *{PKS4* and *PKSl 3)* that responsible for zearalenone biosynthesis, these two polyketide synthase genes from both F. *graminearum* chemotypes were detected to be repressed in all chemical or biological treatments (Figures 28A - 28B). Repression of *F. graminearum* chemotype 15 *PKS4* and *PKSl 3* genes in the treatment with *S. mycoparasitica* biotrophic mycoparasite was illustrated to be significantly higher compared to treatments with *T. harzianum* and tebuconazole fungicide (Figures 28A - 28B).

Tri genes were observed to be more sensitive in *F. graminearum* chemotype 3 when co-inoculated with *Trichoderma* necrotrophic mycoparasite, however, in *F. graminearum* chemotype 15, *Tri* genes appeared to be more responsive towards treatment with chemical fungicide (Figures 27A-27D).

PKS4 gene for both *F. graminearum* chemotypes was monitored to be more sensitive towards treatments with *Trichoderma* necrotrophic mycoparasitic fungus compared to chemical and biotrophic mycoparasitic agents (Figure 28A). However, *PKS13* gene in *F. graminearum* chemotype 15 was found to demonstrate higher sensitivity towards chemical stimulus (Figure 28B).

Mycotoxins extraction and analyses: DON, ZEA, 3-ADON and 15-ADON mycotoxins were extracted from agar mycelial plugs ($0.5cm^2$) cut from the sampling zone located approximately 0.5 cm behind the contact zone between *F. graminearum* and *S. mycoparasitica.* The extraction was performed by three 10-ml volumes of ethyl acetate. The samples were sonicated on ice and shaken vigorously in ethyl acetate, and then they were allowed to stand for 5 min for separation of phases. The organic phase was siphoned off and passed through sodium sulfate to remove water. The solvent was allowed to evaporate at room temperature (23 °C) for 3 days. The residue was then re-dissolved in 2 ml of acetonitrile for thin liquid chromatography (TLC) (only ZEA) and high performance liquid chromatography (HPLC) (all mycotoxins) analyses. TLC was performed on Silica gel 60 plates (Merck Frosst Canada Ltd, Kirkland, PQ, CA) in the solvent

system with ethyl acetate-toluene (3:1) for 40 min. 3-ADON on the gel plates was sprayed with 20% aluminum chloride in 95% ethanol, detected and analyzed following the protocols disclosed by Vasavada and Hsieh (1987, Appl. Microbiol. Biotech. 26: 517-521). Known standard controls of pure DON, ZEA, 3-ADON and 15-ADON (Sigma- Aldrich Canada Ltd., Oakville, ON, CA) were employed to compare with other treatments. All four mycotoxins were quantified using a Water's 2695 HPLC system with: 250 x 4.60 mm, Luna 5µ micron C18 (2) IOOA column (Phenomenex Inc., Torrance, CA USA) and a photodiode-array (PDA) detector was used with an isocratic solvent system (methanol: water-methano containing 5% (v/v) (90:10) ratio). The PDA detector measured the UV spectrum (190-500 nm). Samples were dissolved in acetonitrile and 10 µl loaded onto the column using automatic injector and mycotoxins were eluted with solvent as a mobile phase at a rate of 0.75 ml min-1 for 25 mins. Peak height method was incorporated to determine the exact amount of DON, ZEA, 3-ADON and 15-ADON against a standard curves. Ratio between mycotoxins extracted from treatments of *F. graminearum* treated with Folicur and *F. graminearum* only was calculated for HPLC.

The amount of DON produced was between 70 to 90 ug/L. Consequently, it was too low to be detected in TLC analysis. ZEA was high enough to be analyzed with TLC and both *F. graminearum* chemotypes were observed to produce higher amounts of ZEA toxin under the treatment with Folicur® compared to other treatments (Figure 29). With HPLC, ZEA was found to be reduced when challenged with Folicur® compared to *F. graminearum* colony only (Figure 30). DON and 3-ADON was detected to increase for both *F. graminearum* when inoculated together with Folicur® (Figure 30). Folicur® was monitored to trigger highest amount of 15-ADON under *in vitro* assay (Figure 30).

Example 10

The effects of *S. mycoparasitica* (biotrophic mycoparasite) and *T. harzianum* (neurotrophic mycoparasite) on expression of aurofusarin gene by *F. graminaerum* 3-ADON and 15-ADON strains were assessed in this study.

Fungi, media, and culture conditions: Fungal strains were obtained from culture collections at University of Saskatchewan (Food and Bioproduct Sciences fungal collection). Strains of the following fungal species were used, plant pathogenic *Fusarium graminearum* , *F. graminearum*

(3ADON), *F. graminearum* (15ADON), *F. avenaceum, F. culmorum, , F. proliferation, F. oxysporum, F. arthrosporoides* and mycoparasitic *Spaeherodes mycoparasitica* and *Trichoderma harzianum.* Fungi were maintained on potato dextrose agar for 2 wks at 21^{0}C in darkness. Folicur® was also used in this study.

Experiments for the impact of red color nuances on aurofusarin gene expression was carried out using previously classified F. *avenaceum* isolates, based on VCGs and colony colours. *F. avenaceum* isolates color identification numbers (CIN) were generated with Hex Color Code Chart (http://wu/w.2crcatcawcbsite.com/build/ hex-colors.html#cotorgenerator). CINs are: Ds #71232B: Red and highly virulent, tolerant at 80^{0}C for 4 hours; Es #4E040B: Moderately red and moderately virulent, tolerant 40^{0}C for 4 hours; Bs #A86608: White and non virulent, susceptible 40^{0}C for 4 hours. Three 0.1 g mycelium samples from three replicates of each isolate was mixed and used for DNA extraction and RT-PCR analyses.

Fungal DNA Extraction and PCR: Fungal cultures were grown on 1.5 ml PD broth and centrifuged at 10,000 rpm for 5 min. Supernatant was discarded and, subsequently a total DNA of 2-week old cultures representative of each phenotype/VCG was extracted from the pellet with an Ultra Clean microbial DNA Isolation Kit (Qiagen Inc., Mississauga, ON, CA) following manufacture's instructions. The purified DNA was resuspended in 50 µl of elution buffer and stored at -20" C until further analyses. Full length genomic sequences of PKS region (7.2Kb) from *Fusarium graminearum* were obtained from NCBI Genbank. Six pairs of primers were designed (Table 7).

Table 7: PCR Primers designed from full-length genomic sequences of the PKS region of *F. graminearum*

SEQ ID	Primer	Sequence
SEQ ID NO: 20	PKSFl	TCGAGTTTCGTGTTGCGTGT
SEQ IS NO. 21	PKSRl	AGGTAGTTCGCCATACCCGT
SEQ ID NO: 22	PKSF2	AATTGTGCCCGAGGCAGTAC
SEQ ID NO: 23	PKSR2	CATTGGTTCCGCCCGCAATAG
SEQ ID NO: 24	PKSF3	TGACAACTTCGCTGGTTTGGA
SEQ ID NO: 25	PKSR3	CATAGCTTGGCCAGTGCCATC
SEQ ID NO: 26	PKSF4	GAAGTCATTCGGTGTTGAGC
SEQ ID NO: 27	PKSR4	GCTCTGGATTGGGTATCGCAC
SEQ ID NO: 28	PKSF5	ACTCGAGCATCCGTCGCAATG
SEQ ID NO: 29	PKSR5	AGCAACATCTCCGTCTGGAG
SEQ ID NO: 30	PKSF6	GTTGAACTGTCCATGGCTGA
SEQ ID NO: 31	PKSR6	GAATGAAGGCAATCTGCTGC

All the reactions were carried out in 25-µl volumes containing 2.5 µl of 1OX PCR buffer, 5 µl of Q solution, 1 µl of each primer (10 µM), 0.5 µl of 1OmM dNTPs mix, and 1.25 U of Taq polymerase (Qiagen Inc., Mississauga, ON, CA). Reaction mixtures were mixed gently and were given flash spin prior to PCR on a Eppendorf Master Cycler (ep gradient S). PCR amplicons were purified using the QIAquick® PCR purification kit (Qiagen Inc., Mississauga, ON, CA; QIAquick is a registered trademark of Qiagen GmbH Corp., Hilden, Fed. Rep. Germany) and commercially sequenced (Plant Biotechnology Institute, Saskatoon, SK).

Development of a real time RT-PCR assay for detection and quantification of the aur gene in Fusarium species: All sequences were sequenced, sequences were aligned, several primer sets were designed and tested. One set, named *Auro RT* (SEQ ID NO: 32) and *Auro RTR* (SEQ ID NO: 33) was selected based on efficiency to amplify aurofusarin gene.

SEQ ID NO: 32 ACCTCACTGGAATCAGAGCGCAGC

SEQ ID NO: 33 ATGACRACTTCCCGTGGRCC

The specificity of this set of primers was tested by conventional PCR assay using genomic DNA from *Fusarium* and mycoparasites using the amplification reactions volumes indicated above. The amplification protocol was 1 cycle of 120 s at 94 ^{0}C, 35 cycles of 30 s at 94 ^{0}C (denaturalization), 30 s at 56 ^{0}C (annealing), 45 s at 72 ^{0}C (extension), and 1 cycle of 10 min at 72 ^{0}C.

The pair of primers used to amplify the β-tubulin *(tub)* gene (the endogenous control gene used to normalize the results) were *FGtubf* (SEQ ID NO: 34) and *Fgtubr* (SEQ ID NO: 35).

SEQ ID NO: 34 GGTCTCGACAGCAATGGTGTT

SEQ ID NO: 35 GCTTGTGTTTTTCGTGGCAGT

The PCR efficiencies of the real time RT-PCR for both genes were checked by performing a 10-fold serial dilution of positive control template to generate a standard curve, and by plotting the Ct as a function of log [10] of template.

Presence of aurofusarin was tested and quantified by RT-PCR in pathogenic *Fusarium graminearum* , *F. graminearum* (3ADON), *F. graminearum* (15ADON), *F. avenaceum, F. culmorum,* Mycoparasite, F. *proliferatum, F. oxysporum, F. arthrosporoides* and mycoparasitic, *Sphaerodes mycoparasitica* and *Trichoderma harzianum.* Relative gene expression was studied when *Fusarium* isolates were co-cultured with Folicur®, *Sphaerodes* and *Trichoderma.*

The designed RT-PCR primer set was checked for its specificity by conventional PCR on tested fungal species. A single band 157 bp long was amplified in *F. graminearum* (3ADON), *F. graminearum* (15ADON), *F. avenaceum, F. culmorum* but not in other strains. One week old grown cultures tested for their aurofusarin relative gene expression and β-tublin gene was used as an internal control. *F. graminearum* which produces 15ADON showed highest level gene expression followed by F.*graminearum* (3AD0N) and similar kind of expression results were observed with F. *avenacum* and F. *culmorum.* Aurofusarin relative gene expression was quantified when each *Fusarium* species was co-cultured for a week with *Sphaerodes, Trichoderma* and Folicur® (Figure 31). Among all tested, *S. mycoparasitica* was most effective in reducing the aurofusarin relative gene expression followed by *Trichoderma* and Folicur®

(Figure 31). *S. mycoparasitica* was most affective on *F. graminearum* producing 15ADON, but also efficiently reduced the expression level in *F. culmorum* and *F. avenaceum* and *F.graminearum* producing 3ADON (Figure 31). Similarly, *Trichoderma* was able to somewhat affect *F. graminearum* producing 15ADON and *F. culmorum,* but showed no impact on *F. avenaceum,* whereas enhanced the expression in *F.graminearum* producing 3ADON (Figure 31). Finally, Folicur® could be able to provoke minor reduction in *F. graminearum* producing 15ADON *aur* gene expression, but with less impact on *F. culmorum* and *F. avenaceum.* Inversely, Folicur® increased expression almost 2-fold when compared to *F. graminearum* producing 3ADON (Figure 31).

RNA isolation, reverse transcription and real time RT-PCR: All the cultures were grown for a week under dark and used for expression studies. Fungal total RNA was isolated using the "Total Quick RNA Cells and Tissues" Kit (Bio-Rad Laboratories Inc., Mississauga, ON, CA), according to the manufacturer's instructions, and stored at -80 °C. DNAse I treatment to remove the chromosomal DNA contamination from the samples was performed using the "Deoxyribonuclease I, Amplification Grade" (Invitrogen Corp., Carlsbad, CA, USA). First strand cDNA was synthesized using the "Iscript RNA PCR Reagent Kit" (Bio-Rad Laboratories Inc., Mississauga, ON, CA). Relative Quantification of *aur* gene expression was performed in a MiniOpticon Sequence Detection System using the SYBR Green PCR Master Mix (Bio-Rad Laboratories Inc., Mississauga, ON, CA) and the primer pairs indicated above. The PCR thermal cycling conditions for both genes were as follows: an initial step at 95 ^{0}C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. SYBR green PCR master mix 12.5 µl (Bio-Rad Laboratories Inc., Mississauga, ON, CA) was used as the reaction mixture, with the addition of 6.5 µl of sterile milli-Q water, 1.0 µl of each primer (5 µM), and 5 µl of template cDNA, in a final volume of 25 µl. In all the experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each sample (triplicate) was amplified twice in every experiment. The results were normalized using the all fungi cDNA amplifications run on the same plate. The *tub2* gene is an endogenous control that was used to normalize quantitation of mRNA target for differences in the amount of total cDNA added to each reaction. Real Time RT-PCR analysis is based on the threshold cycle (CT), which is defined as the first amplification cycle at which the fluorescence

signal is greater than the minimal detection level, indicating that PCR products become detectable. Relative quantitation was the analytical method used in this study. A comparison within a sample is made with the gene of interest *(aur)* to that of the endogenous control gene *{tub!).* Quantitation is relative to the control gene by subtracting the CT of the control gene *(tub2)* from the CT of the gene of interest *(aur)* (ΔCT). Each ΔCT value (corresponding to each sample) was subtracted by the calibrator value (FpMMó-lC) to obtain the corresponding ΔΔCT values. ΔΔCT values were transformed to Iog2 (due to the doubling function of PCR) to generate the relative expression levels.

In total 9 isolates were subjected to RT-PCR analyses for quantitative gene expression analyses. Three kinds of colored samples were used in this study composed of 3 white colored, 3 moderately red colored and 3 dark red colored isolates. White colored isolate was used as control and their expression was normalized and taken as one. As reported moderately red colored isolates were able to tolerate 40^0C whereas; dark red isolates were able to tolerate 80^0C. When RT-PCR results observed dark colored isolates expressed almost more than 9 folds enhanced relative gene expression whereas, moderately red colored isolates expressed 4 folds enhanced expression (Figure 32).

Example 11

Greenhouse studies were performed to assess the effects of *S. mycoparasitica* inoculants on development of *Fusarium* head blight symptoms and the accumulation of tricothecene mycotoxin gene in wheat and barley spikes.

Fungal isolates and growth: S. mycoparasitica SMCD 2220, and *F. graminearum* 3-ADON chemotype SMCD 2243 were obtained from the Saskatchewan Microbial Collection and Database (SMCD). These fungal cultures were grown on potato dextrose agar (PDA, BD Biosciences, Mississauga, ON, CA) supplemented with antibiotics prior to the study. Mycelial suspensions of these fungal strains (10^4 for *F. graminearum* and 10^6 for both biotrophic mycoparasite and antagonistic fungus) were produced for greenhouse experiment, as follows.

F. graminearum 3-ADON chemotype strain was inoculated in potato dextrose broth (PDA, BD Biosciences, Mississauga, ON, CA), incubated at room temperature (-21 ^{0}C) in

darkness using shaker (lOOrpm), for a week prior to harvesting the mycelia for greenhouse application. Mycelia of *F. graminearnm* were transferred into sterilized commercial blender for cutting the mycelial inoculants into small debris and these mycelia were adjusted to concentration of 10^4 CFU/mL.

S. mycoparasitica was inoculated in the best preferment yeast peptone dextrose (YPD) (yeast, 5g; peptone, 1Og; dextrose, 1Og per 1 L of sterile-double distilled water) broth, incubated at room temp in darkness, using shaker at 100 rpm for a week prior to harvesting the mycelia. Mycelia of *S. mycoparasitica* were transferred into sterilized commercial blender for cutting the mycelial inoculants into small debris and the mycelia were adjusted to concentration of 10^4 CFU/mL for greenhouse application.

Greenhouse trials: The *Fusari*wm-susceptible wheat CDC-TEAL and barley BOLD cultivars were used to test the efficacy of biotrophic mycoparasite *S. mycoparasitica* and one potential *Fusarium* antagonistic fungal isolate as potential biocontrol agent for managing *Fusarium* head blight diseases. Surface-sterilized wheat and barley seeds were planted in 15-cm diameter pots containing Pro-Mix ® soil (Sun Gro Horticulture, Delta, BC, CA) and maintained at 23-25 ^{0}C during the day time and 18-20 °C during the night time in a greenhouse with light intensity of 360 µmol m^{-2} s^{-1}, watered every 2 d, and fertilized every 14 d using 1300 ppm of NPK (20-20-20) fertilizer. All treatments in the experiment were with three replicates and repeated twice.

At the anthesis stage, wheat and barley spikes were sprayed with mycelial suspension of *S. mycoparasitica* at a concentration of 10^6 CFU/mL, antagonistic fungal isolate at a concentration of 10^6 CFU/mL, 65 µmol/L tebuconazole, or sterile distilled water. The treatments were applied as outlined in Xue et al. (2009, Can. J. Plant Pathol. 31: 169-179) with slight modifications. Following the inoculation of potential biocontrol agents, plants were kept at room temperature and covered with sterile Whirl-Pak ® bags (Whirl-Pak is a registered trademark of Aristotle Corp., Stamford, CT, USA) for overnight to allow fungal growth prior to *F. graminearum* inoculation. Plants were then inoculated with mycelial suspension of *F. graminearum* at a concentration of 10^4 CFU/mL. *Fusarium graminearum* inoculum was sprayed on the spikes and covered with sterile Whirl-Pak ® bags for overnight. The inoculated plants were maintained for an additional 21 days prior to sampling. Percentage of infected spikelets (IS) per

spike, FHB index, FDK, and weight of 100 seeds were rated as outlined in Xue et al. (2009). The FHB visual severity scale was determined according to the methods disclosed by Stack and McMullen (http://www.ag.ndsu.edu/pubs/plantsci/smgrains/pplO95w.ht m).

The biocontrol effects of different concentrations of *S. mycoparasitica* of *Fusarium* head blight symptoms in barley are shown in Figure 33. The data in Figures 33A, 33B, and 33C show that inoculation of a susceptible barley cultivar with *F. graminearum* significantly reduced that height of the plants, average numbers of spikes form per plant, and the average weight of 5 spikes. However, inoculation of the barley cultivar with *S. mycoparasitica* did not affect growth and development. Treatment of *F. graminearum-infQctQd* barley with *S. mycoparasitica* at an inoculation level of 10^6 CFU/mL prevented the onset of any *Fusarium* head blight symptoms. Treatment of *F. graminearum-infected* barley with lower concentrations of *S. mycoparasitica,* i.e., 10^4 CFU/mL and 10^5 CFU/mL, did not protect against the occurrence of the disease symptoms (Figures 33A - 33C).

The biocontrol effects of different concentrations of *S. mycoparasitica* of *Fusarium* head blight symptoms in wheat are shown in Figure 34. The data in Figures 34A, 34B, and 34C show that inoculation of a susceptible wheat cultivar with *F. graminearum* significantly reduced that height of the plants, average numbers of spikes form per plant, and the average weight of 5 spikes. However, inoculation of the wheat cultivar with *S. mycoparasitica* did not affect growth and development. Treatment of *F. graminearum-infected* wheat with *S. mycoparasitica* at an inoculation level of 10^6 CFU/mL prevented the onset of any *Fusarium* head blight symptoms. Treatment of *F. graminearum-infected* wheat with lower concentrations of *S. mycoparasitica,* i.e., 10^4 CFU/mL and 10^5 CFU/mL, did not protect against the occurrence of the disease symptoms (Figures 34A - 34C).

The data in Figure 35 and Table 8 show that treatment of *F. graminearum-infected* barley with *S. mycoparasitica* at an inoculation level of 10^6 CFU/mL provided a comparable level of protection against the occurrence of *Fusarium* head blight symptoms, as was provided by the commercial fungicide Folicur®.

Table 8: Effect of *S. mycoparasitica*, Folicur® fungicide and *F. graminearum* on percentage of infected spikelets, *Fusarium* head blight index, percentage of *Fusarium* damaged kernels, and weight of 100 seeds of the barley cultivar BOLD in greenhouse trials.

Treatment	Infected spikelets (%)	*Fusarium* head blight index (%)	*Fusarium* damaged kernels (%)	Weight of 100 seeds (g)
F. graminearum (Fg)	62.6±8.0 a	56.7±11.9 a	75±9.4 a	4.1±0.4 c
S. mycoparasitica – Fg	15.4±3.1 bc	4.1±1.0 b	19±6.5 b	5.4±0.6 ab
Folicur® – *F. g*	19.6±6.9 b	7.2±4.8 b	16±6.5 b	5.7±0.5 a

Real-time PCR quantification: Total DNA was extracted from the wheat and barly spices with DNeasy® Plant Mini Kit (Qiagen Inc., Mississauga, ON, CA). Extracted total DNAs from spikes of different treatments were employed in real-time PCR quantification. Two different primer sets and PCR conditions used in this study were described in Nicholson et al. (1998) for Fgl6NF/R primer set and Schnerr et al. (2001) for Tox5-l/2 primer set. Total DNA extracted from spring wheat and barley spikes harvested from greenhouse trials were carried out in a MiniOpticon (Bio-Rad Laboratories Inc., Mississauga, ON, CA). All real-time PCR reactions were performed using real-time PCR MJ white tubes (Bio-Rad Laboratories Inc., Mississauga, ON, CA) with a total volume of 25 µl of IQ supermix (Bio-Rad Laboratories Inc., Mississauga, ON, CA), 1 µl of each 10 µM forward/reverse primers (Invitrogen), 3.4 µl of BSA (Bovine Serum Albumin) (1.47 µg/µl) (Ishii and Loynachan 2004), 5.1 µl of sterilized UltraPure Millipore water, and 2 µl of DNA template.

Figure 36 shows standard curves of *F. graminearum* chemotype 3 genomic DNA concentration standards versus cycle threshold (Ct) with PCR reactions performed in triplicate using primer sets (A) *Tox5-l/2,* with genomic DNA ranging from 270 ng (Logio = 2.90) to 0.27 ng (Logio = -0.60), readings at 0.005 fluorescence line and (B) *Fgl6NF/R,* with DNA template ranging from 270 ng (Logio = 2.43) to 0.027 ng (Logio = -1-57); in 10-fold dilution series, readings at 0.025 fluorescence line. Error bars indicate standard deviation for the mean of *F. graminearum* chemotype 3 standard curves derived from *tri5* gene and *F. graminearum* specific primer sets. Figure 37 shows effects of *S. mycoparasitica* (B) and Folicur fungicide (FoI) treatments on *F. graminearum* chemotype 3-ADON genomic DNA detected in barley spikes employing RT-PCR. Treatments were: Fus - *F. graminearum;* B-Fus - *S. mycoparasitica* with *F. graminearum;* Fol-Fus - Folicur fungicide with *F. graminearum.* All values obtained were the means of six replicates. Error bars indicate standard deviation of the mean. Means of *F. graminearum* DNA with Tox 5 primer were log10 transformed prior to LSD test. Both primer sets were analyzed separately. Values followed by the same letters within each primer set are not significantly different using LSD test at $P < 0.05$.

Example 12

In addition to the known Fusarium spp. hosts for *S. mycoparasitica* (i.e., *F. avenaceiim, F. graminearum* and *F.* oxysporum), we surprisingly discovered that *F. culmorum* and *F. equiseti* are also mycoparasitised by *S. mycoparasitica* as disclosed in the following study.

Fungal isolates and growth: Biotrophic mycoparasites *S. mycoparasitica* SMCD2220, *Sphaerodes quadrangularis* strain CBSl 12764, *Sphaerodes retispora* var. *retispora* strain CBS 994.72, and pathogenic *Fusarium* strains *(F. arthrosporioides* SMCD2247, *F. culmorum* SMCD2248, *F. equiseti* SMCD2134, *F. flocciferum* SMCD2135, F. *poae* SMCD2136, and *F. torulosum* SMCD2139) were obtained from the Saskatchewan Microbial Collection and Database (SMCD), Saskatchewan, Canada. All fungal isolates were grown and maintained on potato dextrose agar (PDA, BD Biosciences, Mississauga, ON, CA) prior to the study.

Fungal-fungal interactions: For examination of the interaction between isolates of *Sphaerodes* and *Fusarium* species, both biotrophic mycoparasite and *Fusarium* isolates were inoculated and assessed using slide culture assays. Slides were maintained in a sterile humidity chamber and daily observations on the hyphal interactions at the meeting place (contact zone) were performed under a Carl Zeiss Axioskop2 equipped with Carl Zeiss AxioCam ICcI camera with 20x, 40x and 10Ox objectives. Formation of biotrophic mycoparasitic contact structures attaching *Sphaerodes* species to *Fusarium* hyphae were examined, recorded and compared to drawings from the literature (Jordan & Barnett, 1978, Mycologia 70: 300-312; Rakvidhyasastra & Butler, 1973, Mycologia 65: 580-593; Whaley & Barnett, 1963, Mycologia 55: 199-210). Diameters of both parasitized and non-parasitized *Fusarium* hyphal cells were measured under light microscopy with a 100χ objective lens. Each treatment used six replications consisted of *Sphaerodes* or *Fusarium* alone, and *Sphaerodes-Fusarium* co-inoculated. The experiment was repeated twice. In the slide-culture assay, *Fusarium* mycelia infected with *Sphaerodes* haustoria were stained with lactofuchsin. Stained hyphae of both *Fusarium* and *Sphaerodes* in slide-culture were then examined with a Carl Zeiss Axioskop2 fluorescent microscope attached to Carl Zeiss AxioCam ICcI with 40χ and 100χ objectives. Slide-culture assays were also subjected to Zeiss META 510 confocal laser scanning microscopy (CLSM) analysis to observe intracellular mycoparasitism under a C-Apochromat 63x N.A.1.2 phase-contrast water immersion objective

through Z-stacking mode to scan through the *Fusarium* hyphae with intracellular infection (CLSM with 514nm excitation - argon and LP585 emission filters).

Hyphae-hyphae interactions and contact structures in the contact zone were examined for seven days. On day three, *Sphaerodes mycoparasitica* was found to produce hook-shaped contact structures on *F. equiseti* and *F. culmorum* (Figure 39). On day five, more hook-shaped contact structures and intracellular penetration of *F. equiseti* were observed (Figures 39A, 39A-39D). The combination of lactofuchsin dye and fluorescent or confocal laser scanning microscopy revealed that the parasitized or penetrated *Fusarium* cells became empty (loss of cytoplasm = no fluorescence) or fluoresced with low intensity (very pale) (Figures 39A-39D) as compared to healthy *Fusarium* cells. During the seven days of observation, no *S. mycoparasitica* hyphae were observed within *F. culmorum* cells. *S. mycoparasitica* produced hook-shaped contact structures (Figure 38A, a) more frequently than clamp-like contact structures (Figure 38B, b) on both *F. equiseti* and *F. culmorum.* Diameters of *F. equiseti,* but not *F. culmorum,* hyphae parasitized by *S. mycoparasitica* were observed to be significantly reduced compared to non-parasitized *Fusarium* hyphae (with T-test, $P = 0.001$ and $P > 0.05$, respectively) (Figure 41).

None of the *Fusarium* taxa tested appeared to be suitable hosts for mycoparasitic *S. quadrangularis* and *S. retispora* even after 10 days of co-inoculation on slide cultures. No contact biotrophic parasitic structures or intracellular parasitism by *S. quadrangularis* and *S. retispora* on the tested *Fusarium* strains were observed at the interaction or contact zone. Also, *F. arthrosporioides, F. flocciferum, F. poae,* and *F. torulosum* did not appear to be suitable hosts for *S. mycoparasitica.* Around five days after inoculation on slide culture assays, mycelia of *F. arthrosporioides* were inhibited by *S. mycoparasitica. F. arthrosporioides* started to form rosette-like mycelia at the contact zone with *S. mycoparasitica* (Figure 39B).

On the fifth and seventh days after inoculation, anamorphic structures were produced by *S. mycoparasitica* more abundantly in the zone of contact with *F. culmorum* (Figures 4OC and 40D). Anamorphic structures or asexual organs in close proximity to *F. culmorum* mycelia were red-colored (Figure 40D), whereas the organs at a distance were not (Figure 40C).

CLAIMS

1. A biologically pure culture of *Sphaerodes mycoparasitia* strain IDAC 301008-01 .

2. A biologically pure culture of *Sphaerodes mycoparasitia* strain SMCD2220-01 .

3. An isolated nucleic acid molecule from a biologically pure culture of *Sphaerodes mycoparasitia*, comprising a nucleotide sequence set forth in any of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO:3.

4. A nucleotide construct comprising a nucleic acid molecule according to claim 3, wherein the nucleic acid molecule is operably linked to a promoter that drives expression in a cell.

5. A microbial cell having stably incorporated into its genome at least one nucleotide construct comprising a nucleic acid operably linked to a promoter that drives expression of said nucleic acid in said microbial cell, wherein said nucleic acid comprises the nucleotide sequence set forth in one of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO:3.

6. Use of a culture of *Sphaerodes mycoparasitia* strain IDAC 301008-01 or strain SMCD2220-01 for controlling disease symptoms caused by *Fusarium* spp. in plants.

7. Use of a culture of the microbial cell of claim 5 for controlling disease symptoms caused by *Fusarium* spp. in plants.

8. Use of a culture according to claim 6 or 7, for modulating synthesis of one of a *Fusarium* trichothecene mycotoxin deoxynivalenol (DON), mycotoxin 3-ADON, mycotoxin 15-ADON, mycotoxin zerelanone, and mycotoxin aurofusarin.

9. A composition for controlling disease symptoms caused by *Fusarium* spp. in plants, the composition comprising a culture of *Sphaerodes mycoparasitia* strain IDAC 301008-01 and/or strain SMCD2220-01 and a carrier therefor.

10. A composition for controlling disease symptoms caused by *Fusarium* spp. in plants, the composition comprising a microbial culture of a microbial cell according to claim 5 and a carrier therefor.

11. A composition according to claim 9 or 10, for modulating synthesis of one of a *Fusarium* trichothecene mycotoxin deoxynivalenol (DON), mycotoxin 3-ADON, mycotoxin 15-ADON, mycotoxin zerelanone, and mycotoxin aurofusarin.

12. A method for controlling disease symptoms caused by *Fusarium* spp. in plants, the method comprising treating a batch of seeds with the composition of claim 9 or 10, and then culturing the treated seeds into plants.

13. A method for controlling disease symptoms caused by *Fusarium* spp. in plants, the method comprising treating a batch of seeds with the culture of claim 6 or 7, and then culturing the treated seeds into plants.

14. A method for controlling disease symptoms caused by *Fusarium* spp. in plants, the method comprising treating the plants with the composition of claim 9 or 10.

15. A method for controlling disease symptoms caused by *Fusarium* spp. in plants, the method comprising treating the plants with the culture of claim 6 or 7.

16. A method according to any of claims 12 to 15, wherein synthesis of one of a *Fusarium* mycotoxin 3-ADON, mycotoxin 15-ADON, mycotoxin zerelanone, and mycotoxin aurofusarin, is modulated.

17. An exocellular protein recoverable from a culture of *Sphaerodes mycoparasitia* strain IDAC 301008-01, said exocellular protein having a molecular weight of 13 kDa.

18. An exocellular protein recoverable from a culture of *Sphaerodes mycoparasitia* strain IDAC 301008-01, said exocellular protein having a molecular weight of 50 kDa.

19. Use of the exocellular protein of claim 17 or 18 for controlling disease symptoms caused by *Fusarium* spp. in plants.

20. A composition for controlling disease symptoms caused by *Fusarium* spp. in plants, the composition comprising the exocellular protein of claim 17 or 18, and a carrier therefor.

21. A method for testing a sample of plant seeds for the presence therein of aurofusarin, the method comprising:

processing a portion of the sample of plant seeds to produce a DNA sample therefrom; and

processing the DNA sample with a PCR primer set comprising a set of gene sequences set forth in SEQ ID NO: 32 and SEQ ID NO: 33 to detect the presence and/or expression therein of a gene or gene sequence coding for aurofusarin.

22. An isolated nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 32 or SEQ ID NO: 33.

23. A nucleotide construct comprising a nucleic acid molecule according to claim 22.

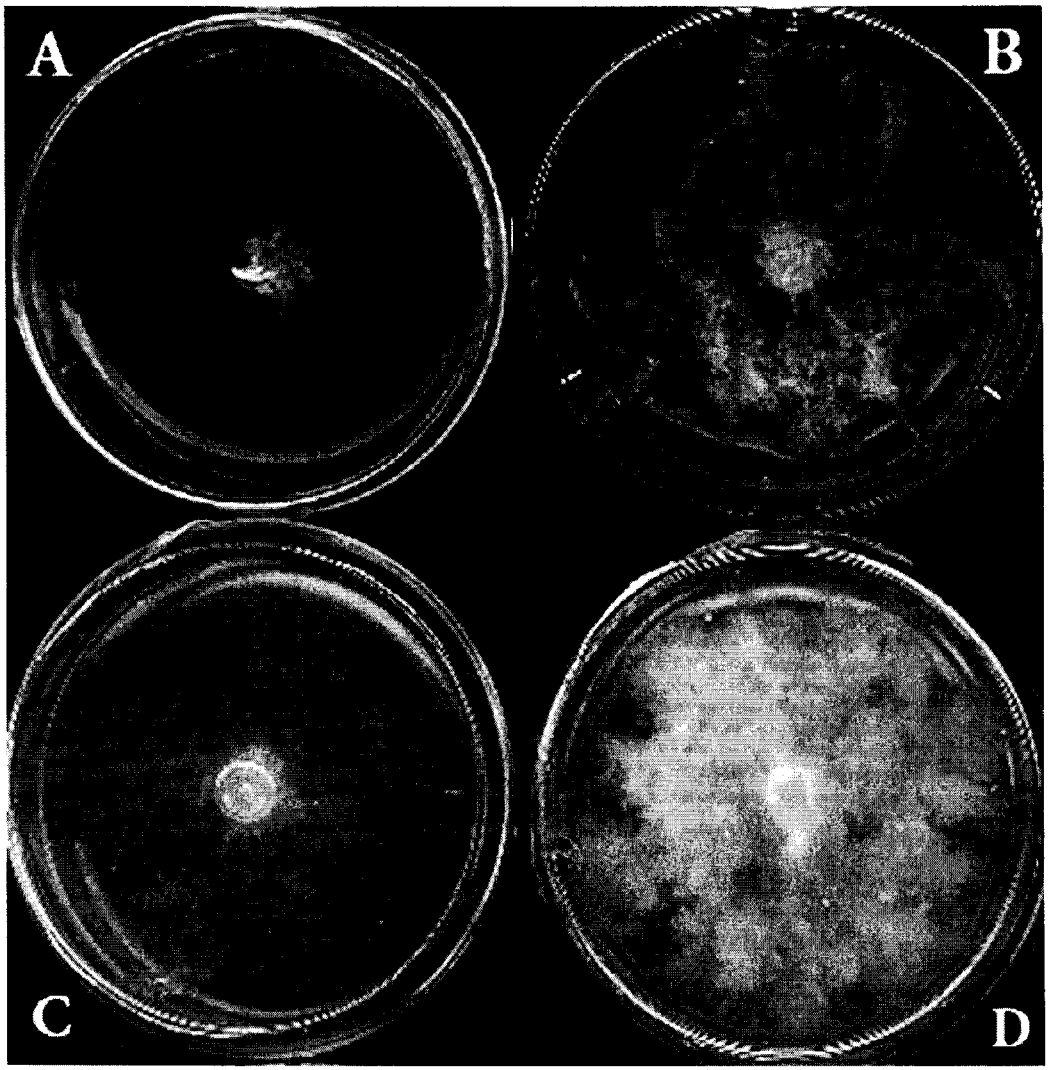


Figure 1

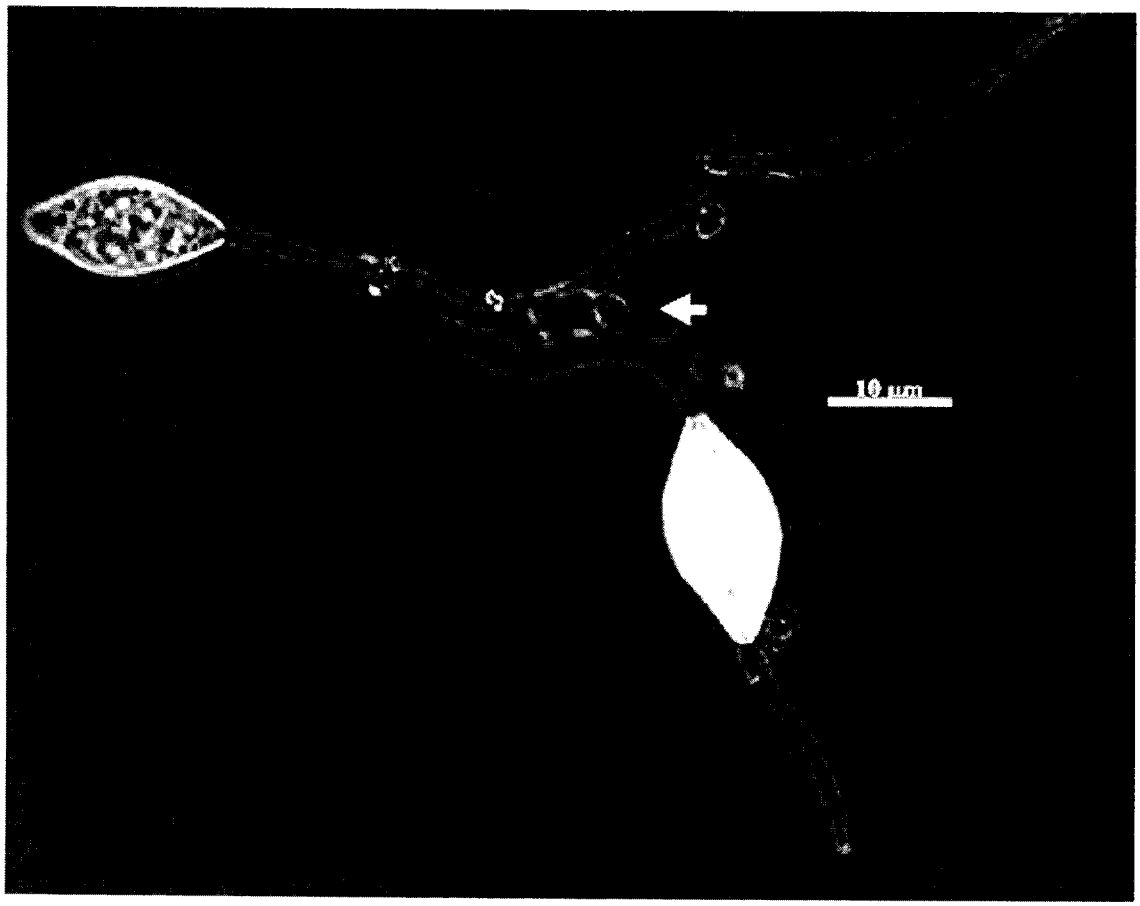


Figure 2

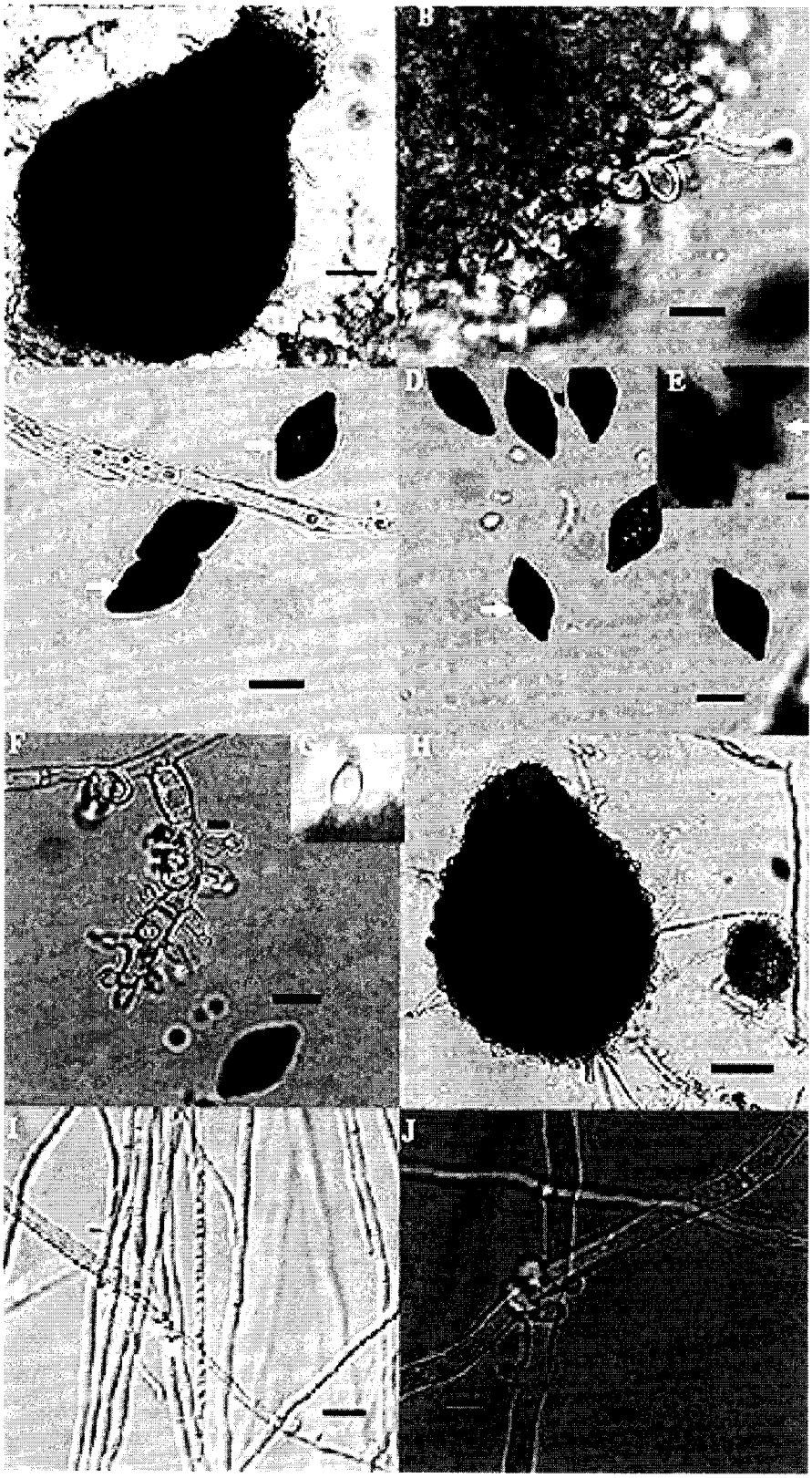


Figure 3

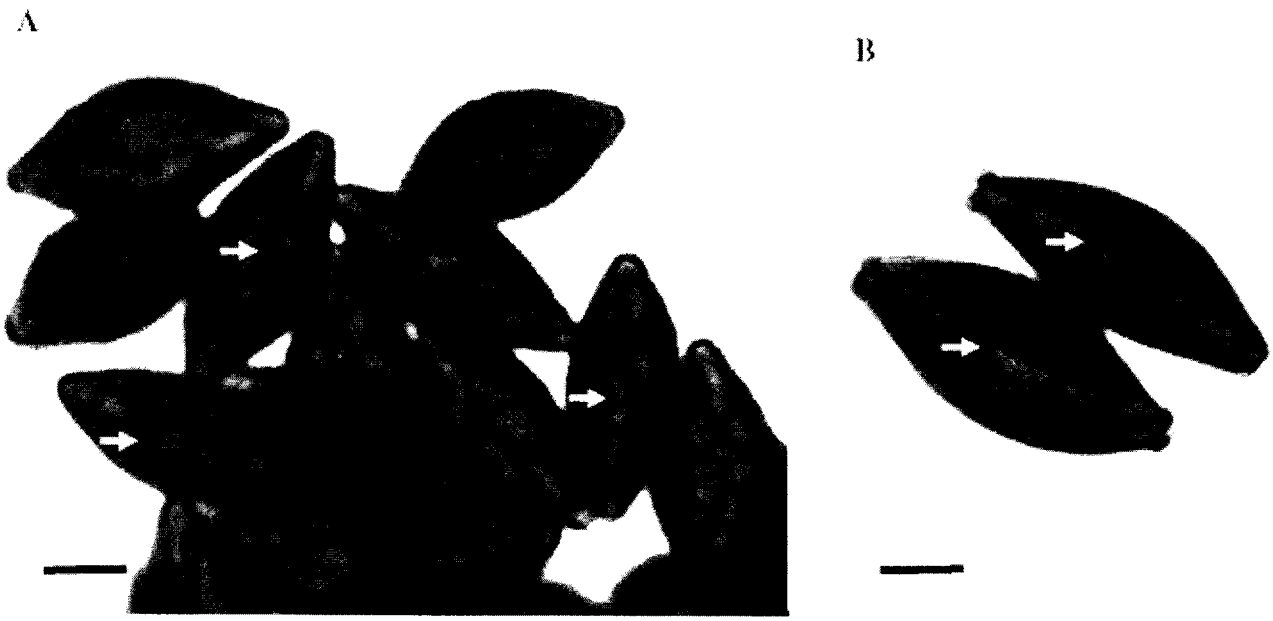


Figure 4

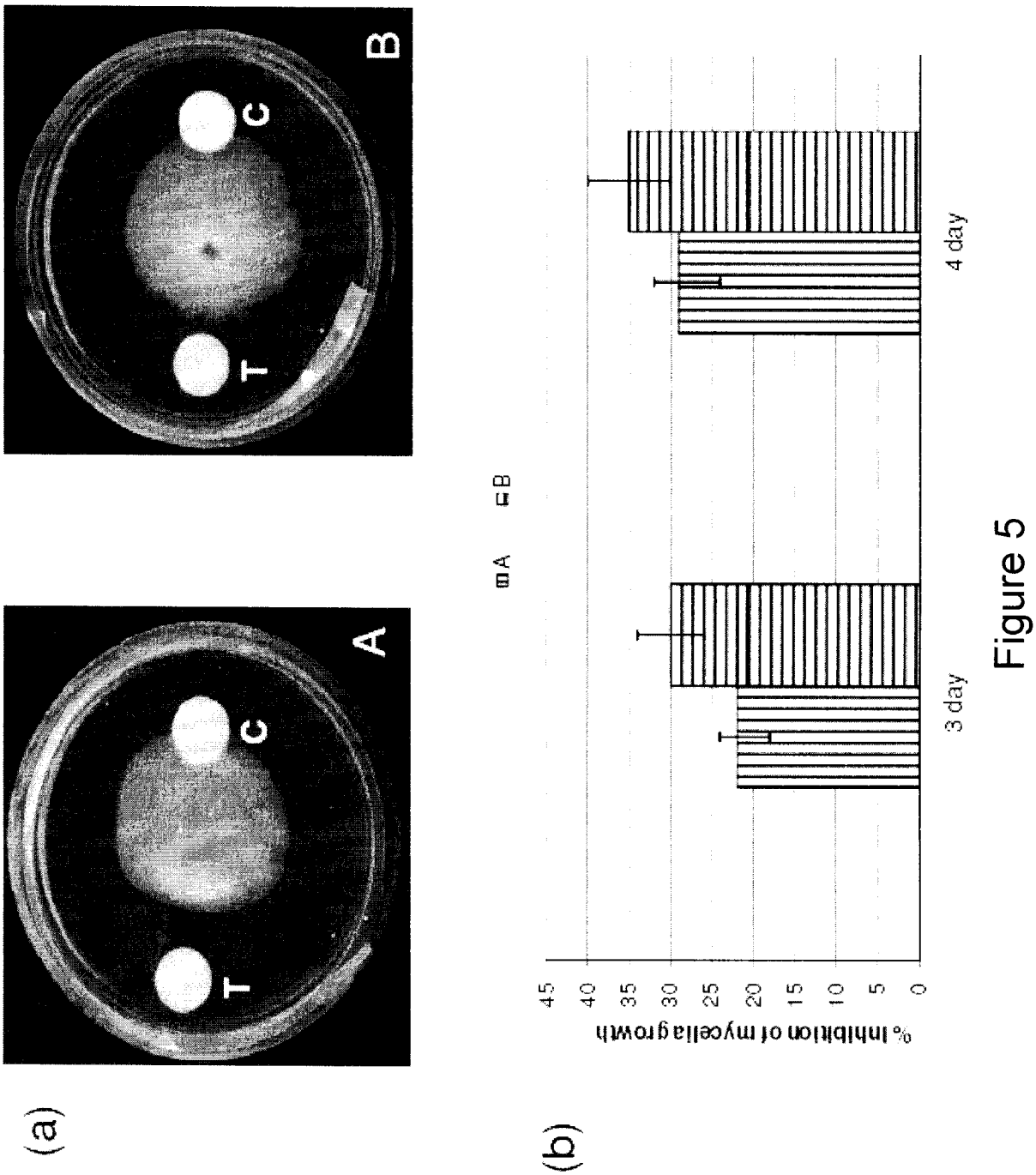

(a)
A
C
T
B
C
T
(b)
A
B
% inhibition of mycelia growth
0
5
10
15
20
25
30
35
40
45
3 day
4 day

Figure 5

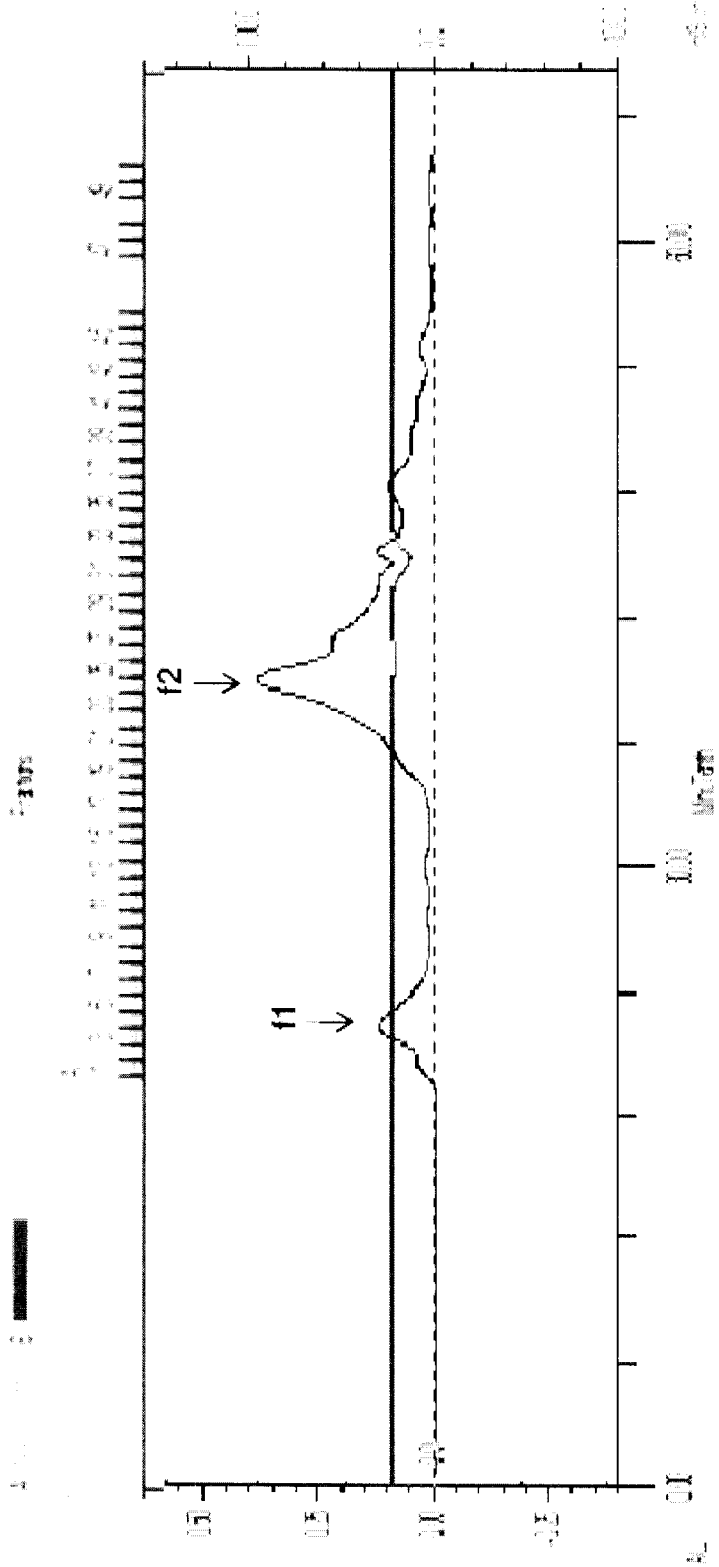


Figure 6

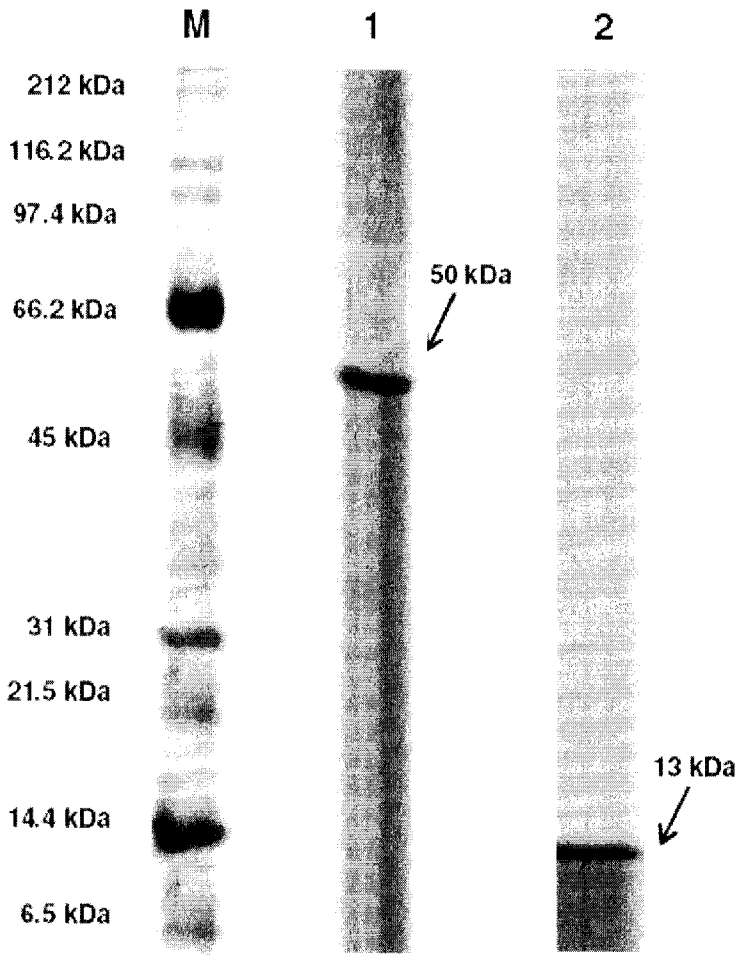

M
1
2
212 kDa
116.2 kDa
97.4 kDa
66.2 kDa
45 kDa
31 kDa
21.5 kDa
14.4 kDa
6.5 kDa
50 kDa
13 kDa

Figure 7

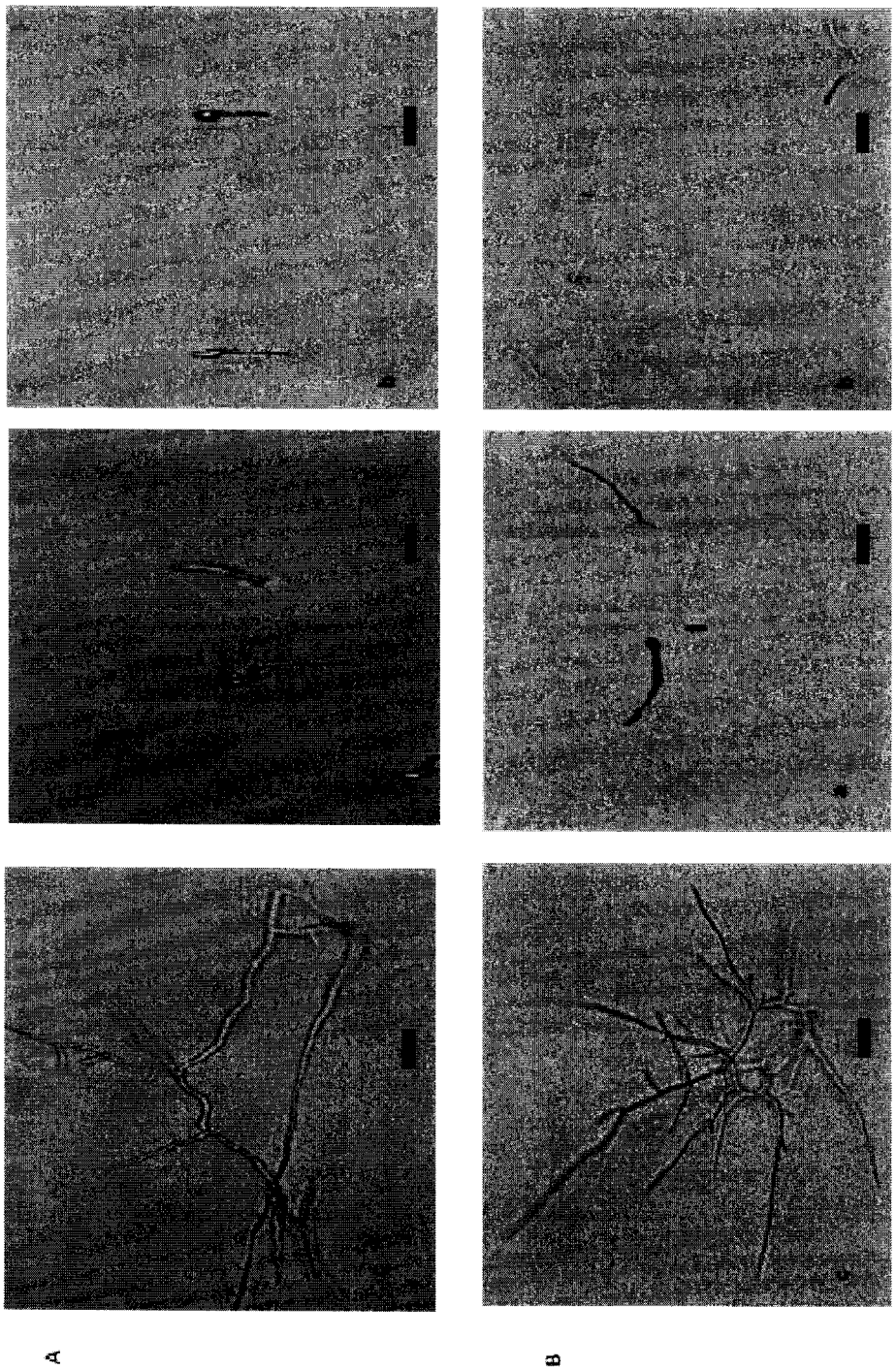


Figure 8

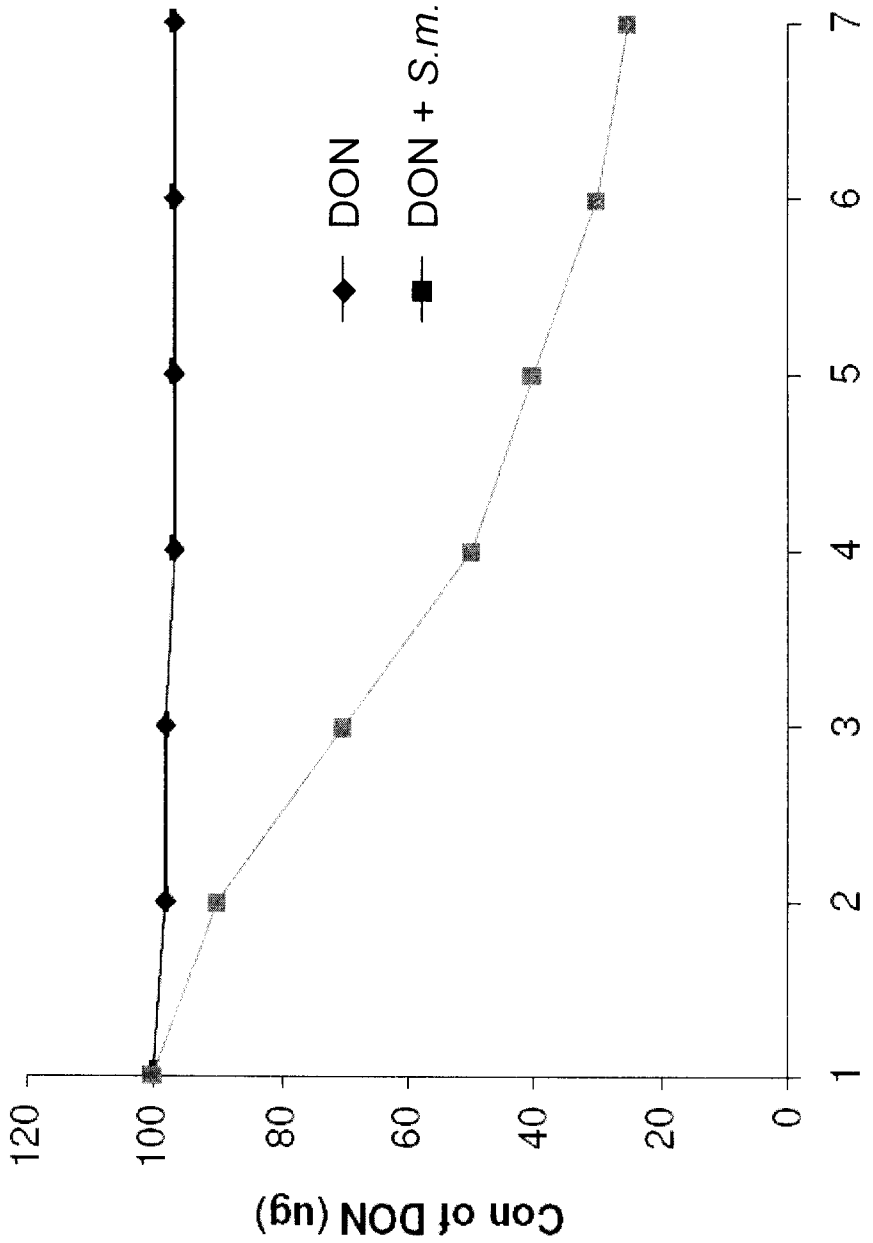


Figure 9

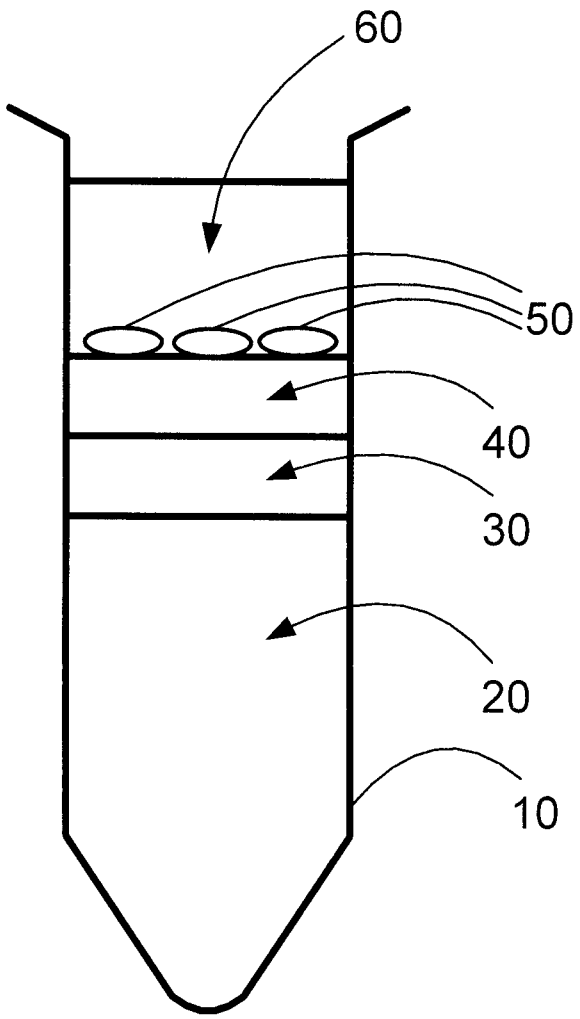


Figure 10

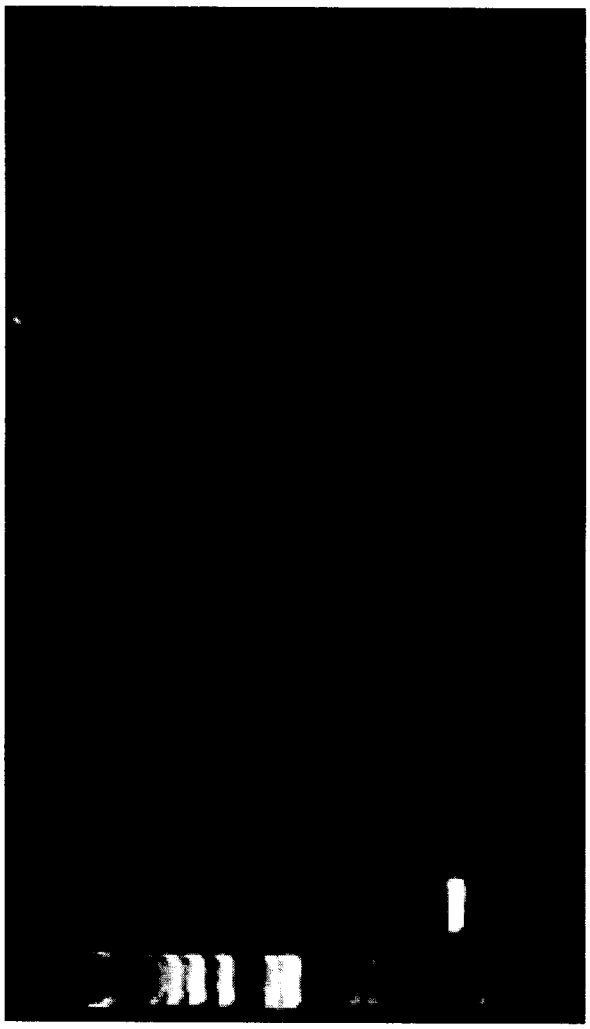


Figure 11

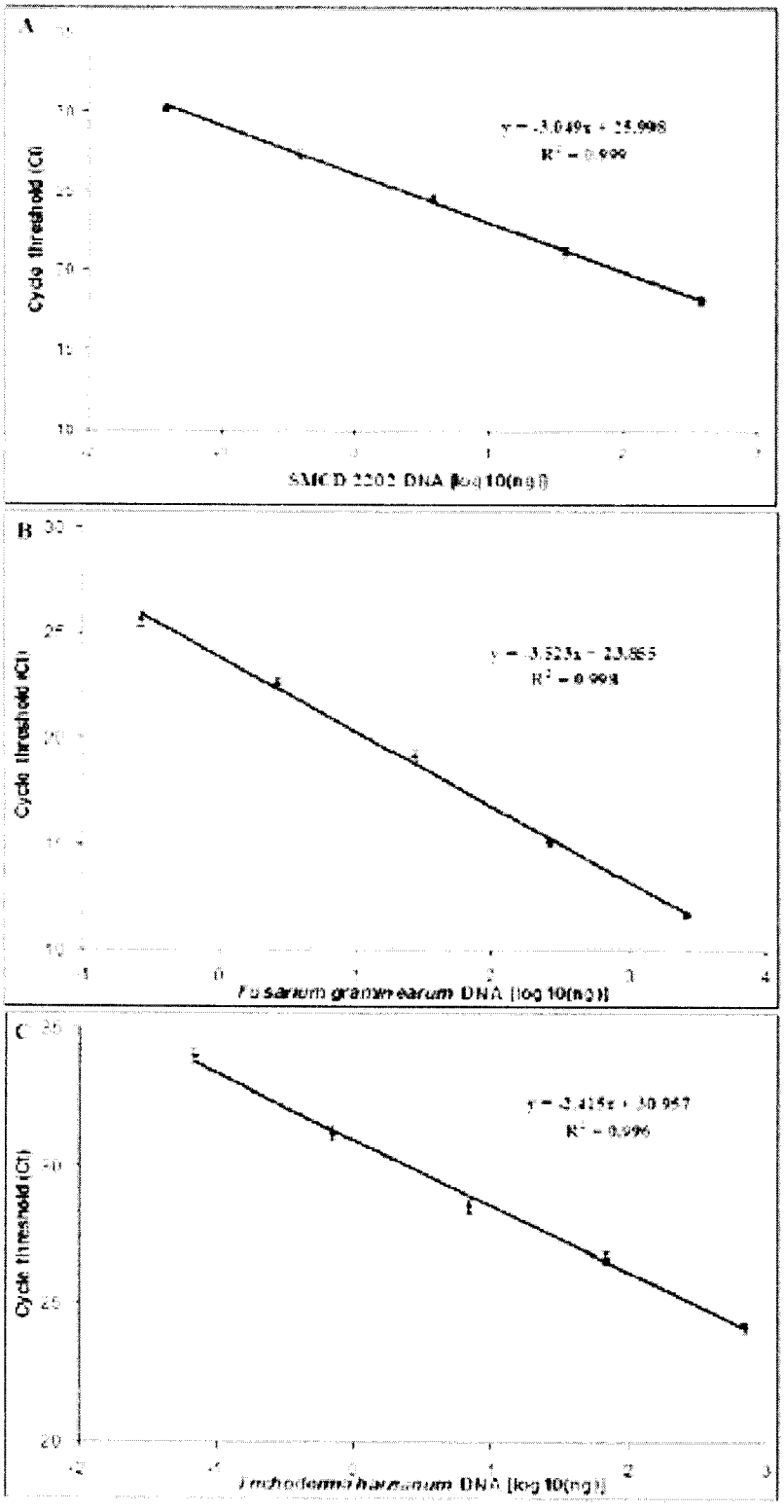

A
Cycle threshold (Ct)
B
Cycle threshold (Ct)
Fusarium graminearum DNA (log10(ng))
C
Cycle threshold (Ct)

Figure 12

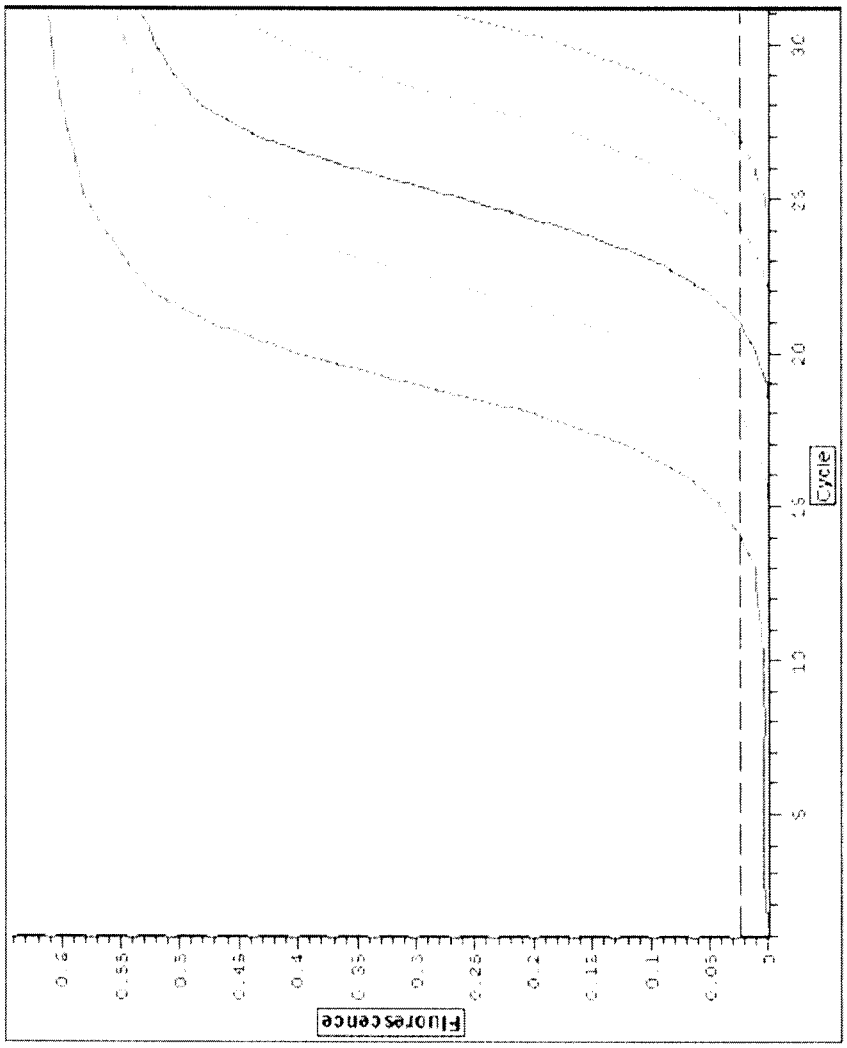

Fluorescence
Cycle
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
5
10
15
20
25
30

Figure 13

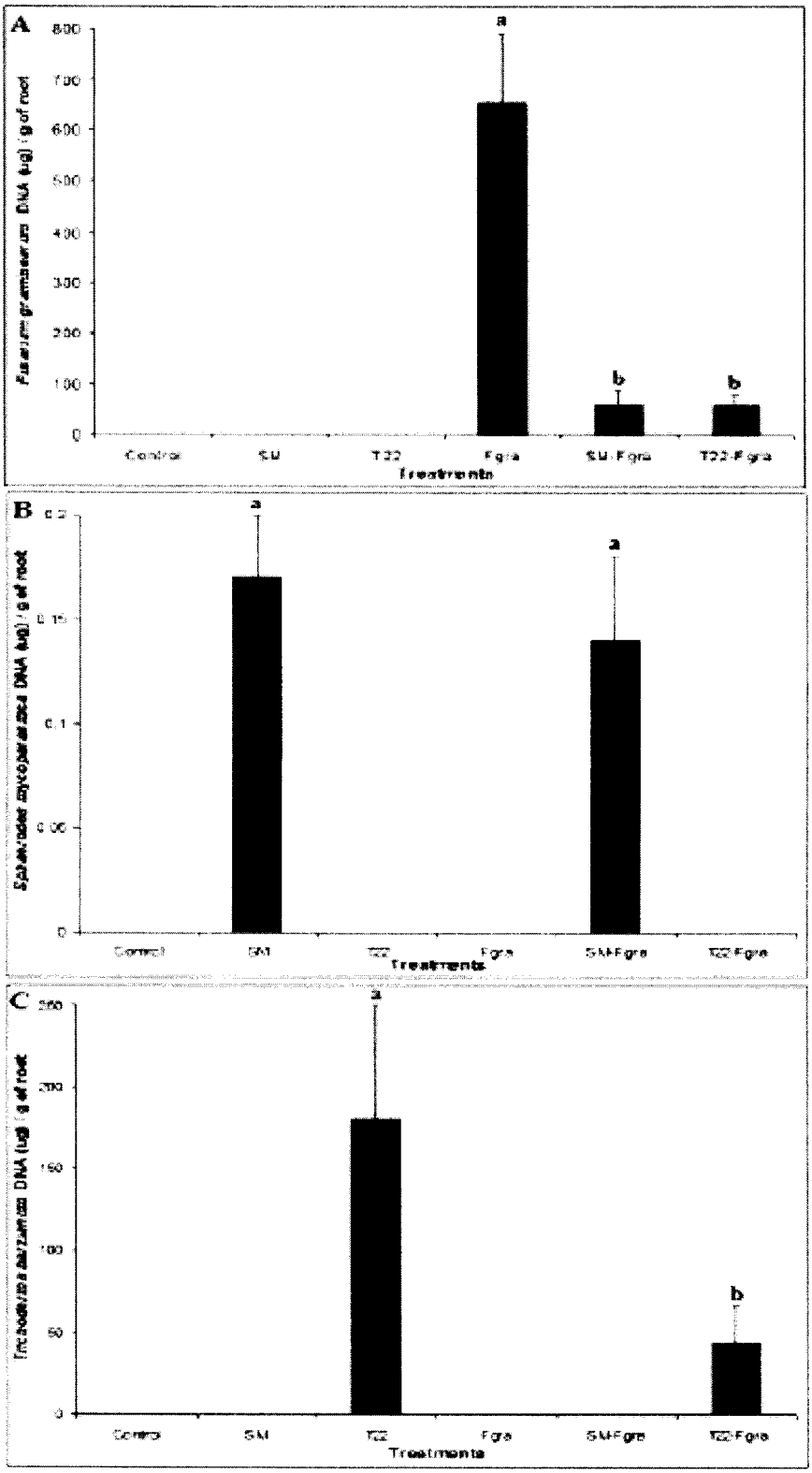


Figure 14

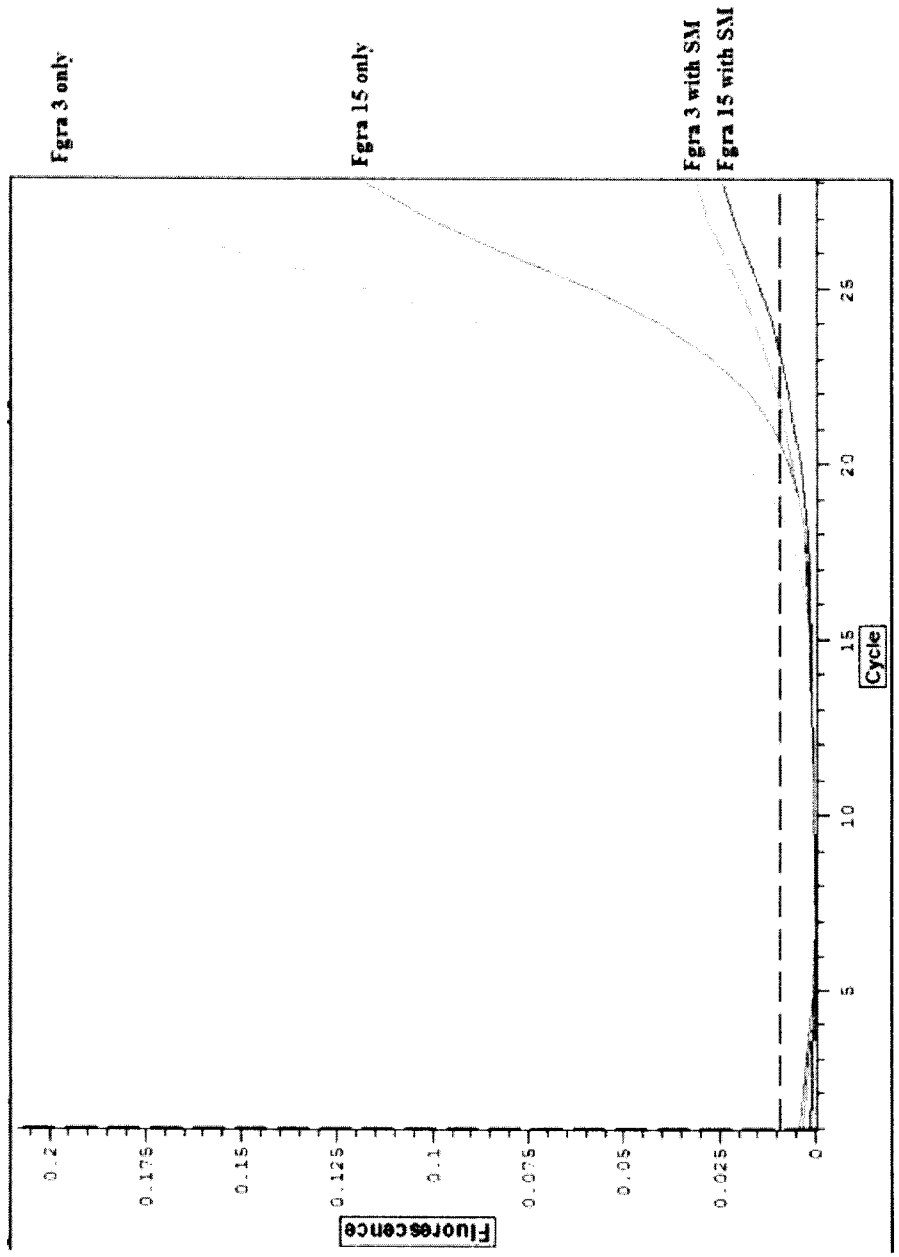

Fgra 3 only
Fgra 15 only
Fgra 3 with SM
Fgra 15 with SM
Fluorescence
Cycle
0.2
0.175
0.15
0.125
0.1
0.075
0.05
0.025
0
5
10
15
20
25

Figure 15

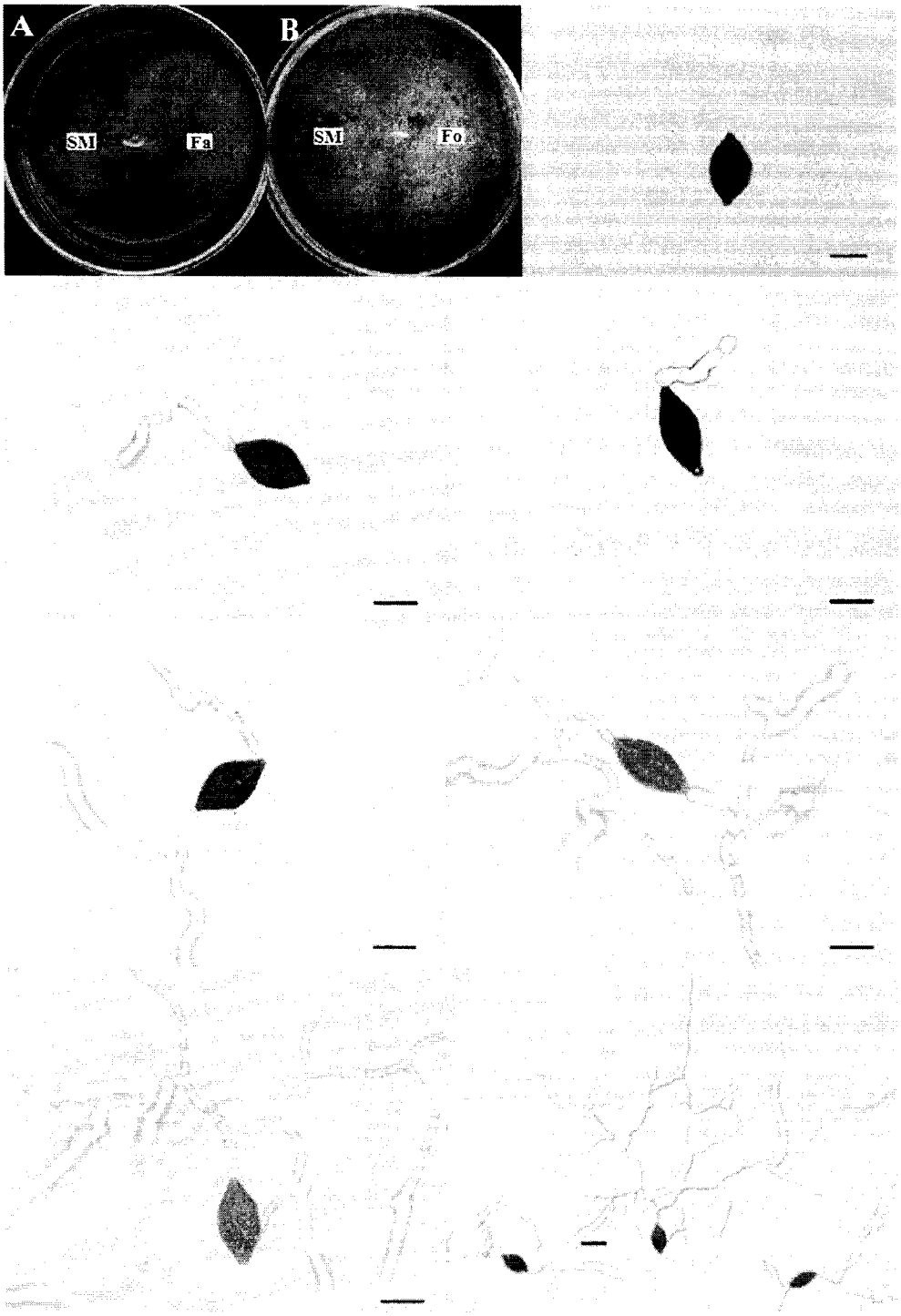


Figure 16

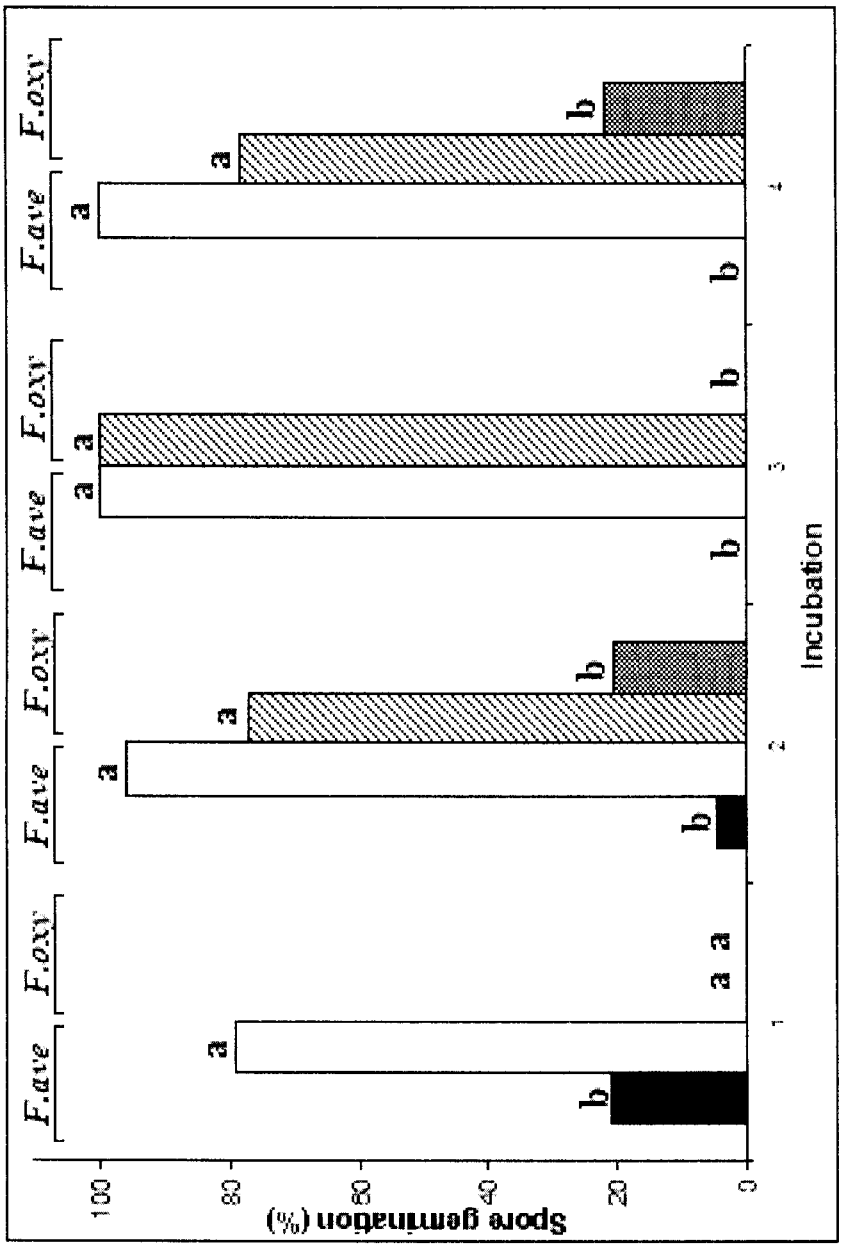

Spore germination (%)
100
80
60
40
20
0
F.ave
F.oxy
a
b
1
2
3
4
Incubation

Figure 17

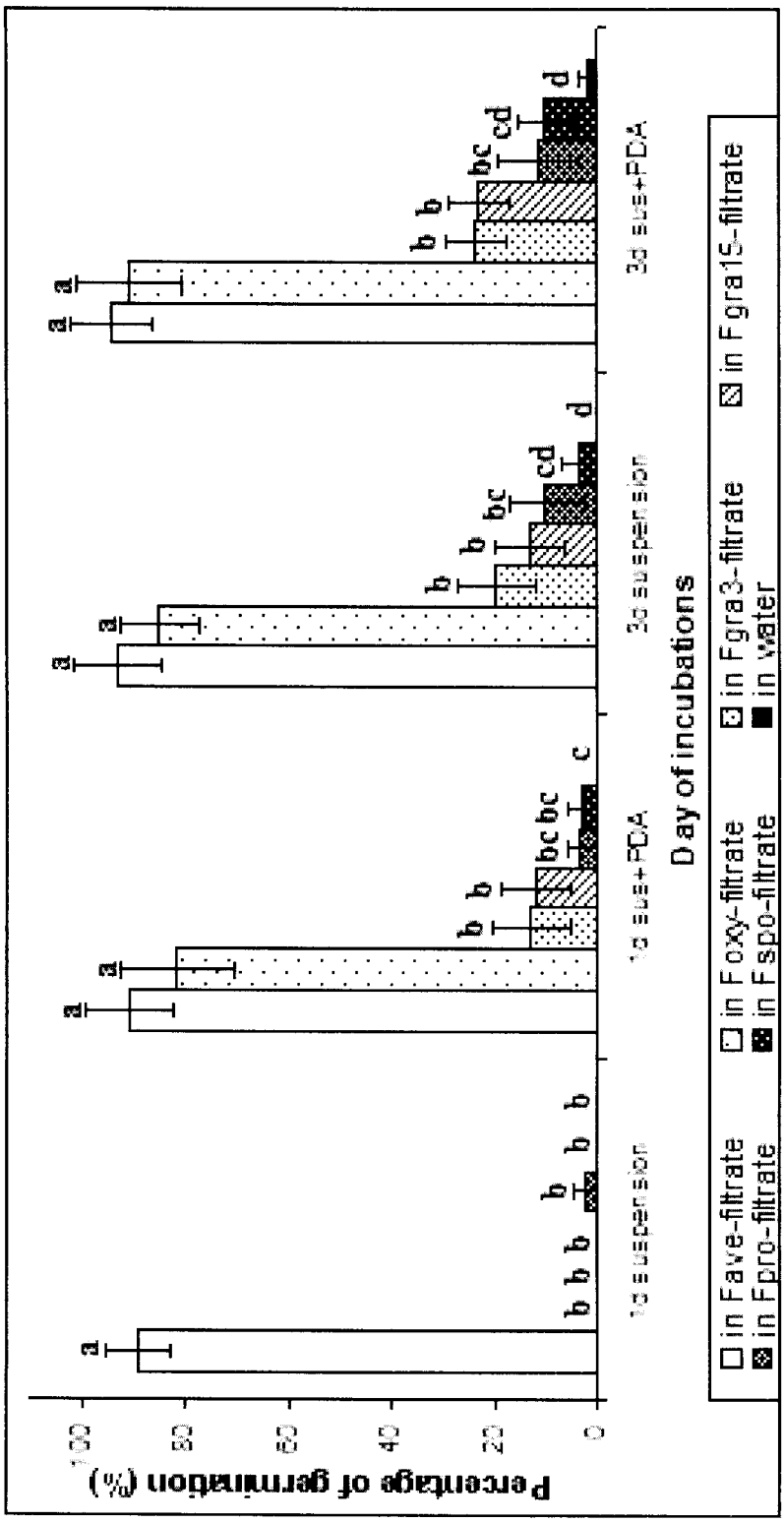

Percentage of germination (%)
100
80
60
40
20
0
Day of incubations
in Fave-filtrate
in Fpro-filtrate
in Foxy-filtrate
in Fspo-filtrate
in Fgra3-filtrate
in water
in Fgra15-filtrate

Figure 18

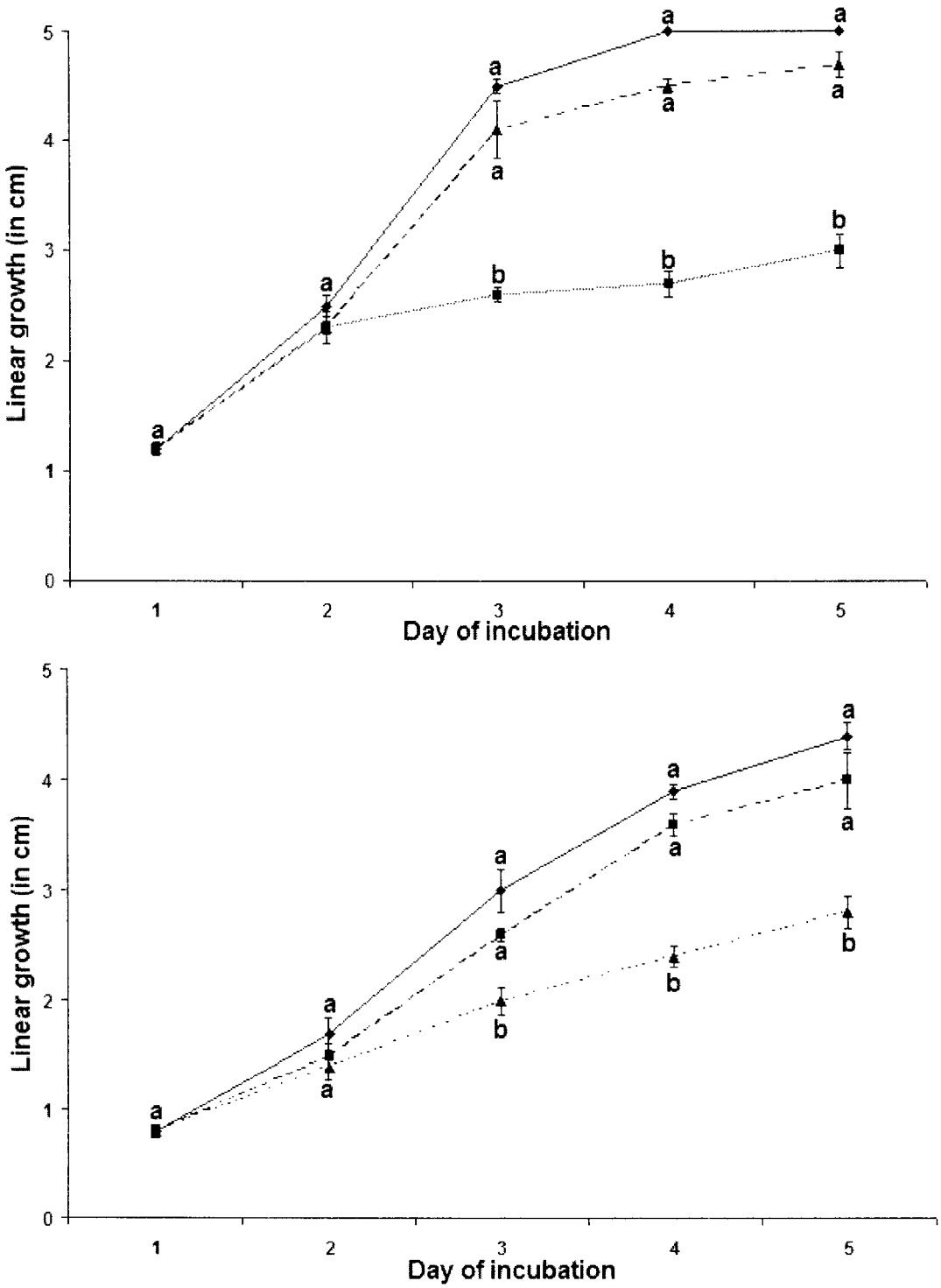


Figure 19

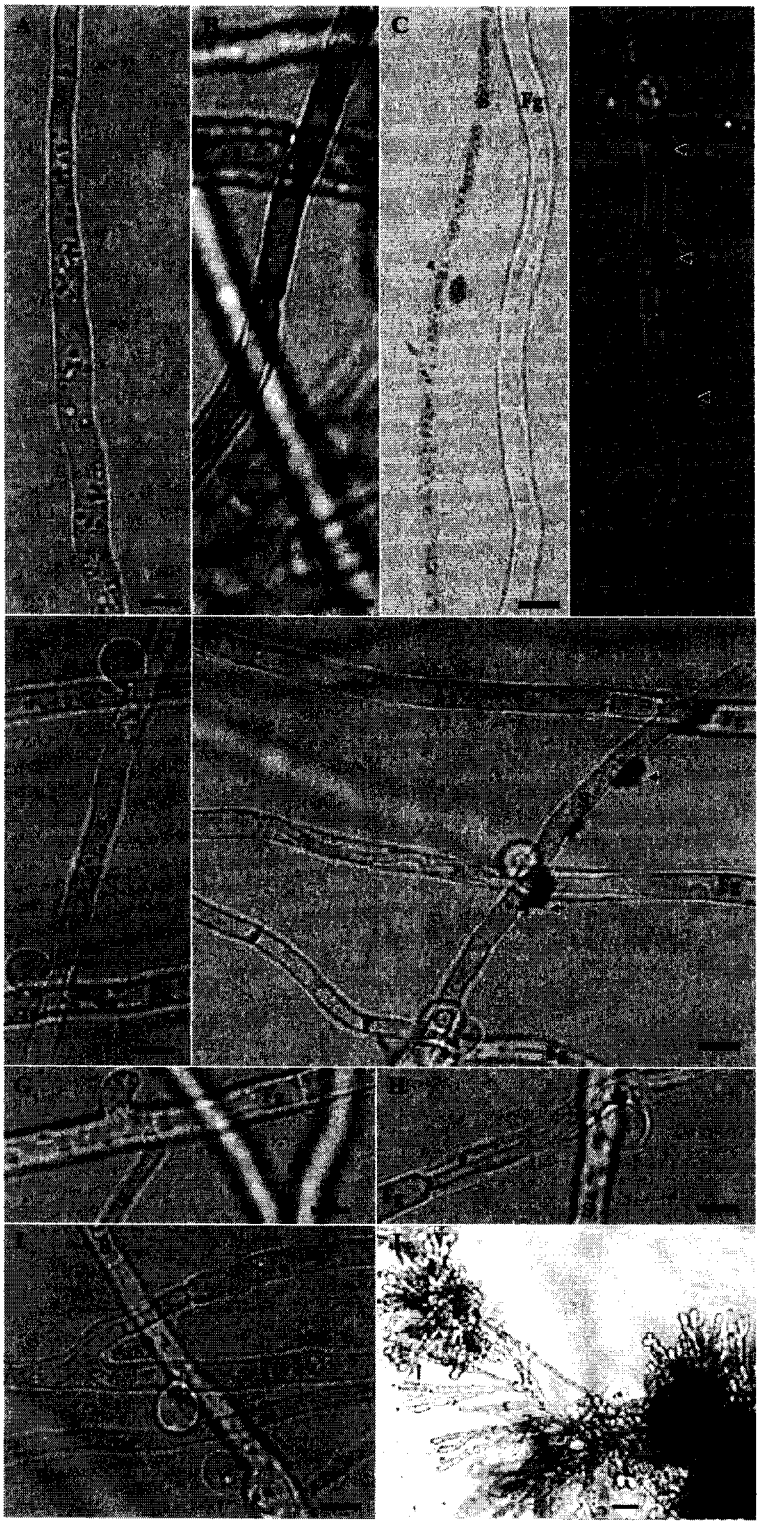


Figure 20

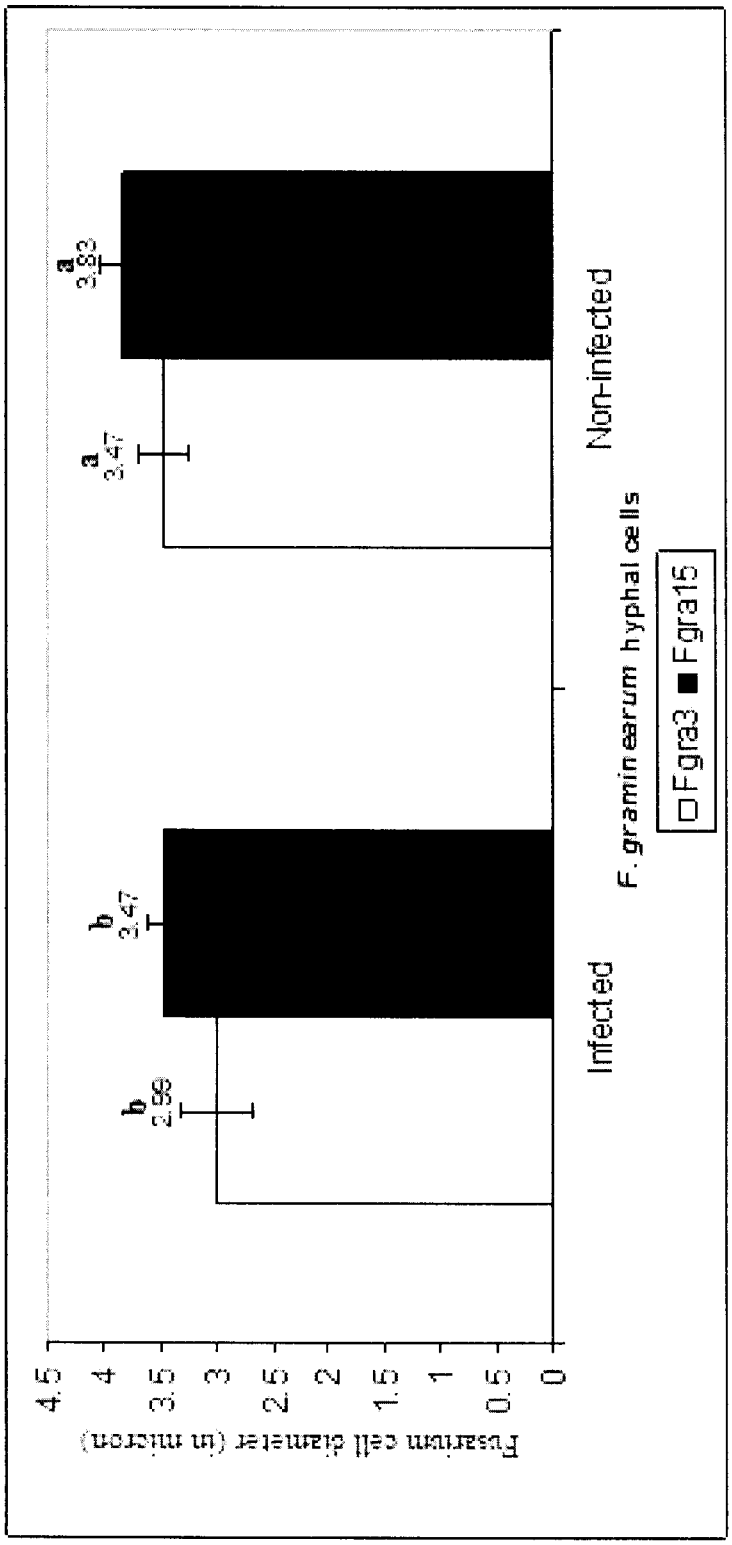

Fusarium cell diameter (in micron)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a 3.83
a 3.47
b 3.47
b 2.98
Non-infected
Infected
F. graminearum hyphal cells
Fgra3
Fgra15

Figure 21

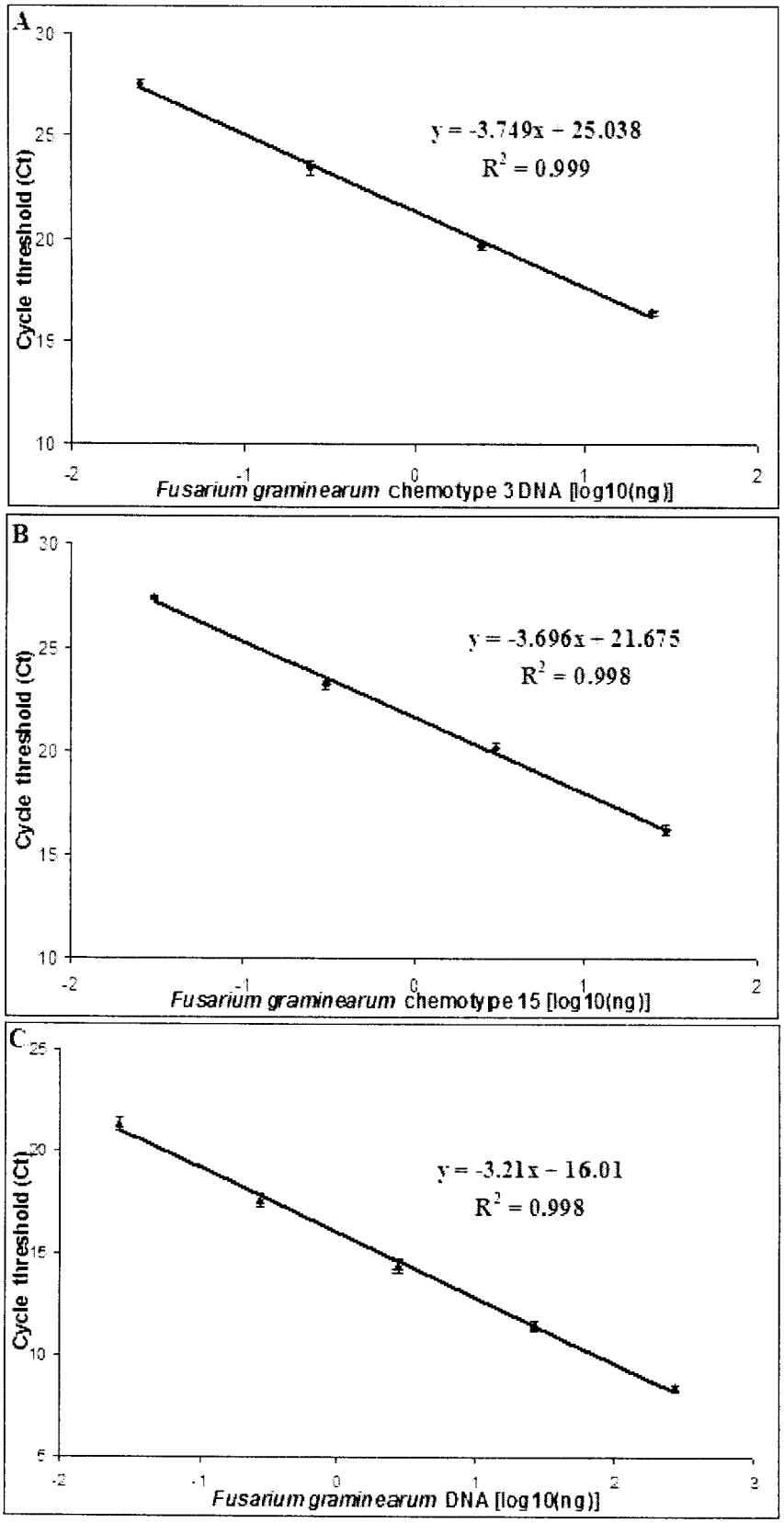

A
y = -3.749x - 25.038
R² = 0.999
Cycle threshold (Ct)
Fusarium graminearum chemotype 3 DNA [log10(ng)]
B
y = -3.696x - 21.675
R² = 0.998
Cycle threshold (Ct)
Fusarium graminearum chemotype 15 [log10(ng)]
C
y = -3.21x - 16.01
R² = 0.998
Cycle threshold (Ct)
Fusarium graminearum DNA [log10(ng)]

Figure 22

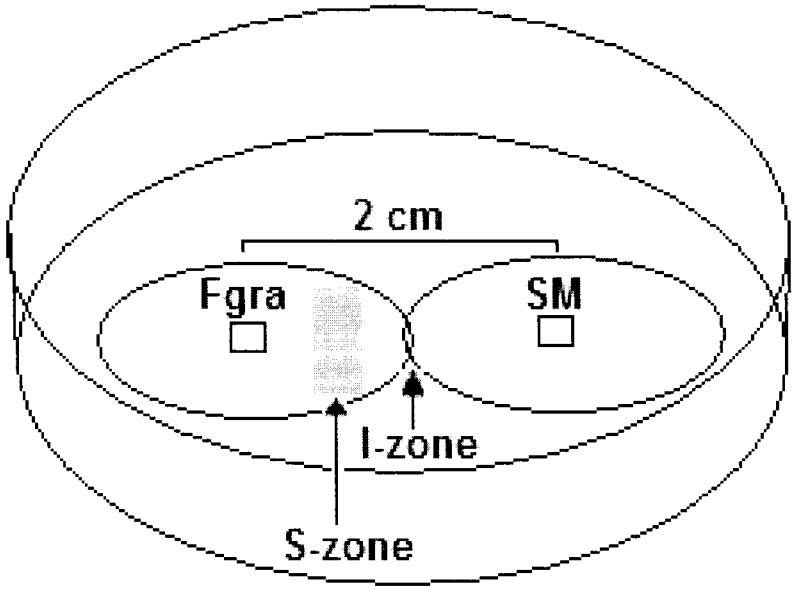


Figure 23

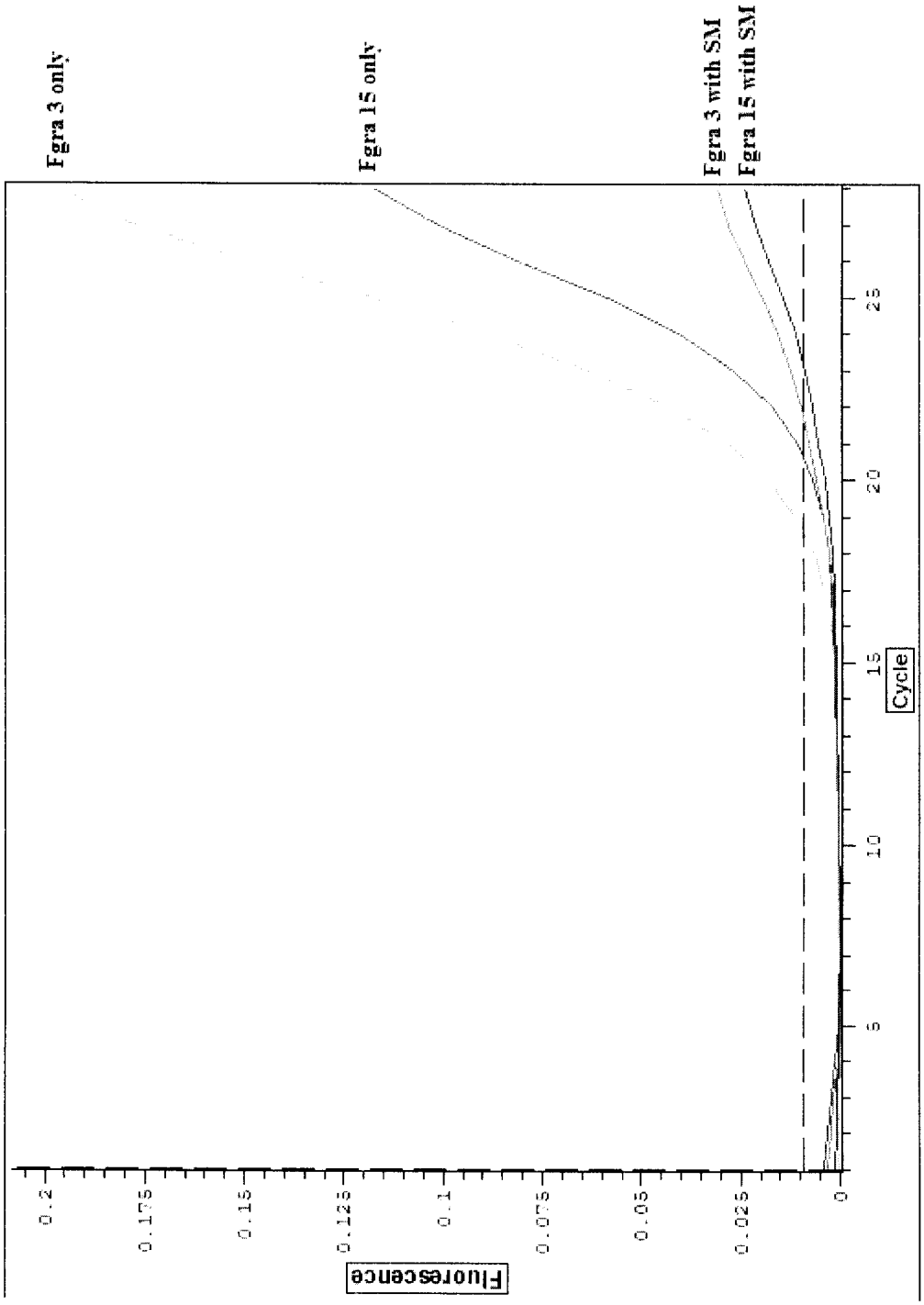

Fgra 3 only
Fgra 15 only
Fgra 3 with SM
Fgra 15 with SM
Fluorescence
0.2
0.175
0.15
0.125
0.1
0.075
0.05
0.025
0
5
10
15
20
25
Cycle

Figure 24

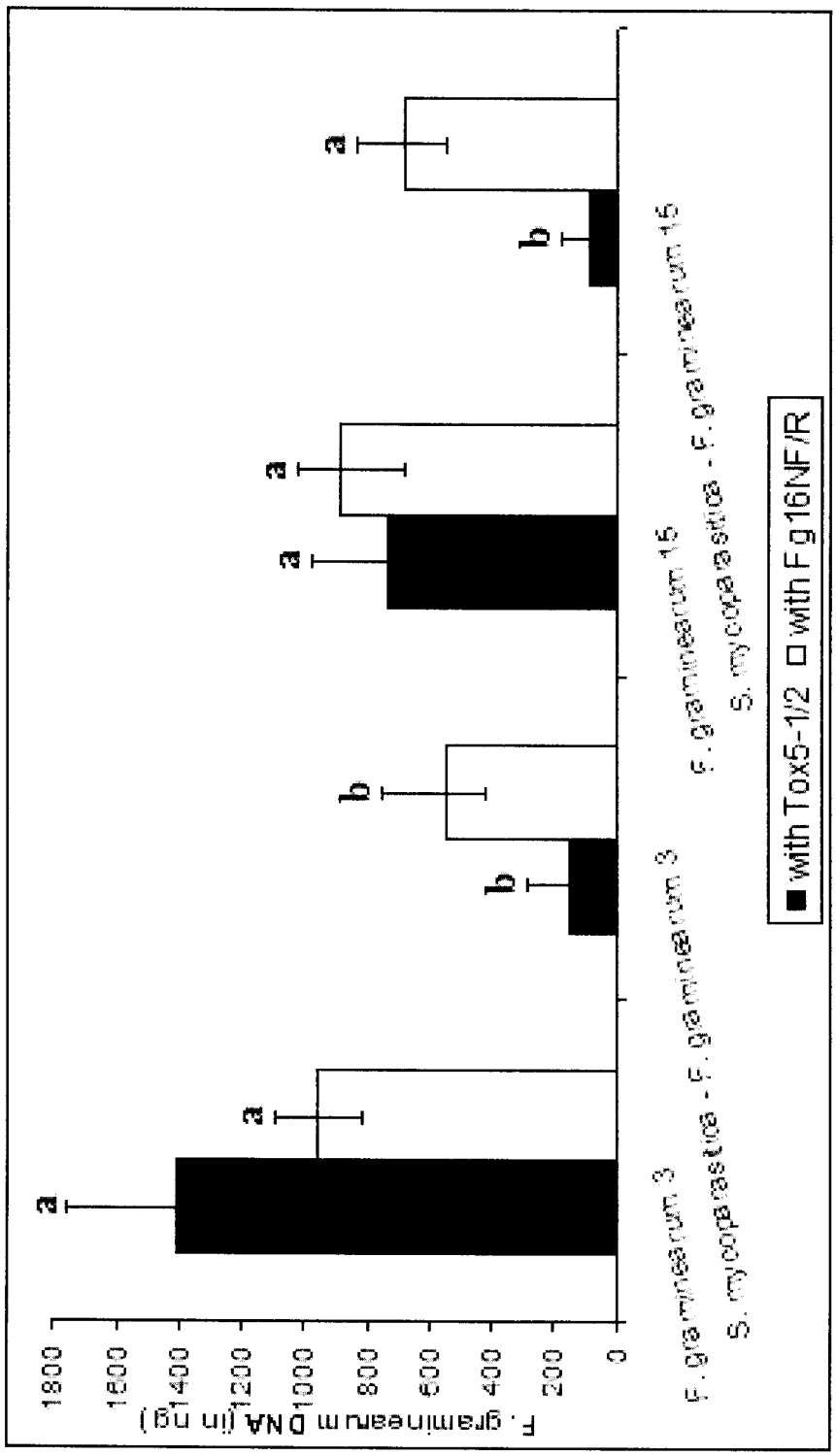

F. graminearum DNA (in ng)
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
b
b
a
a
b
a
F. graminearum 3
S. mycoparasitica - F. graminearum 3
F. graminearum 15
S. mycoparasitica - F. graminearum 15
with Tox5-1/2
with Fg16NF/R

Figure 25

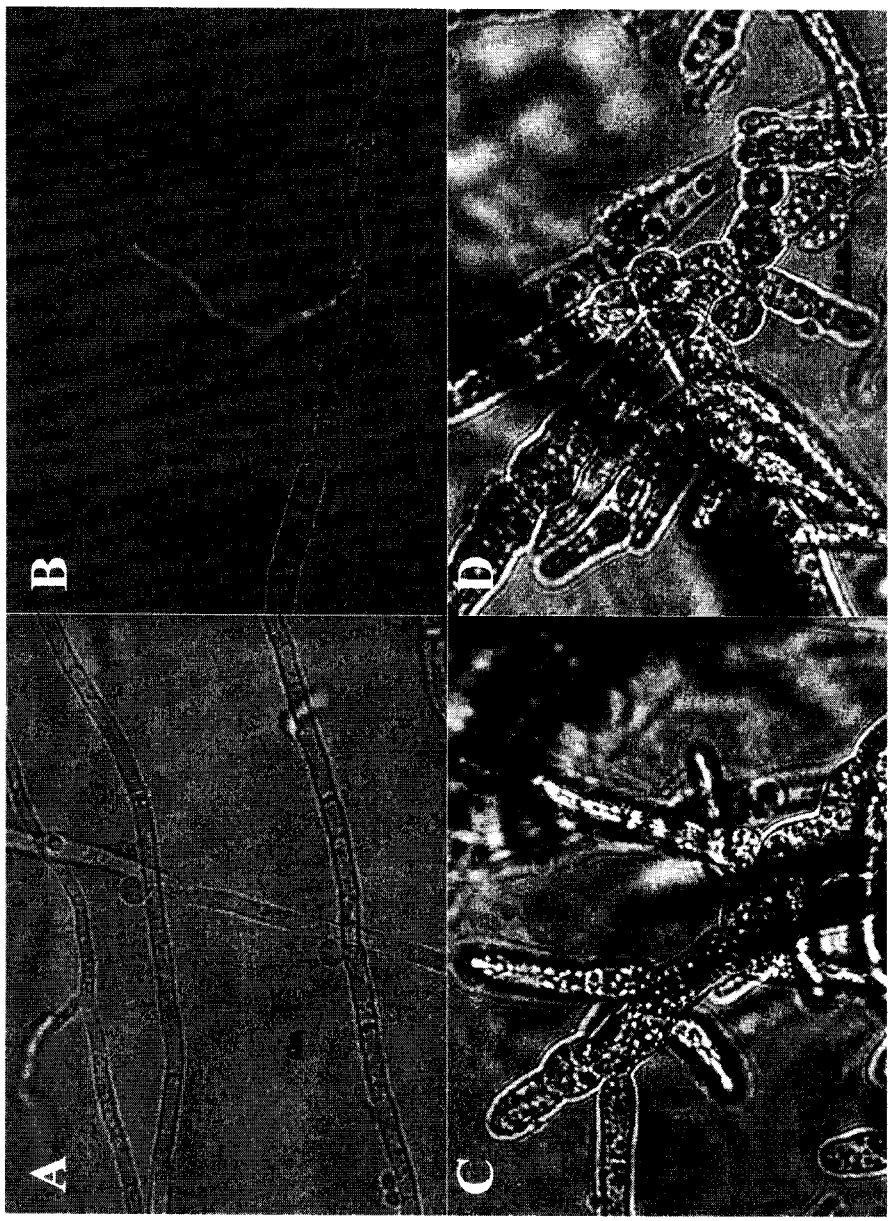


Figure 26

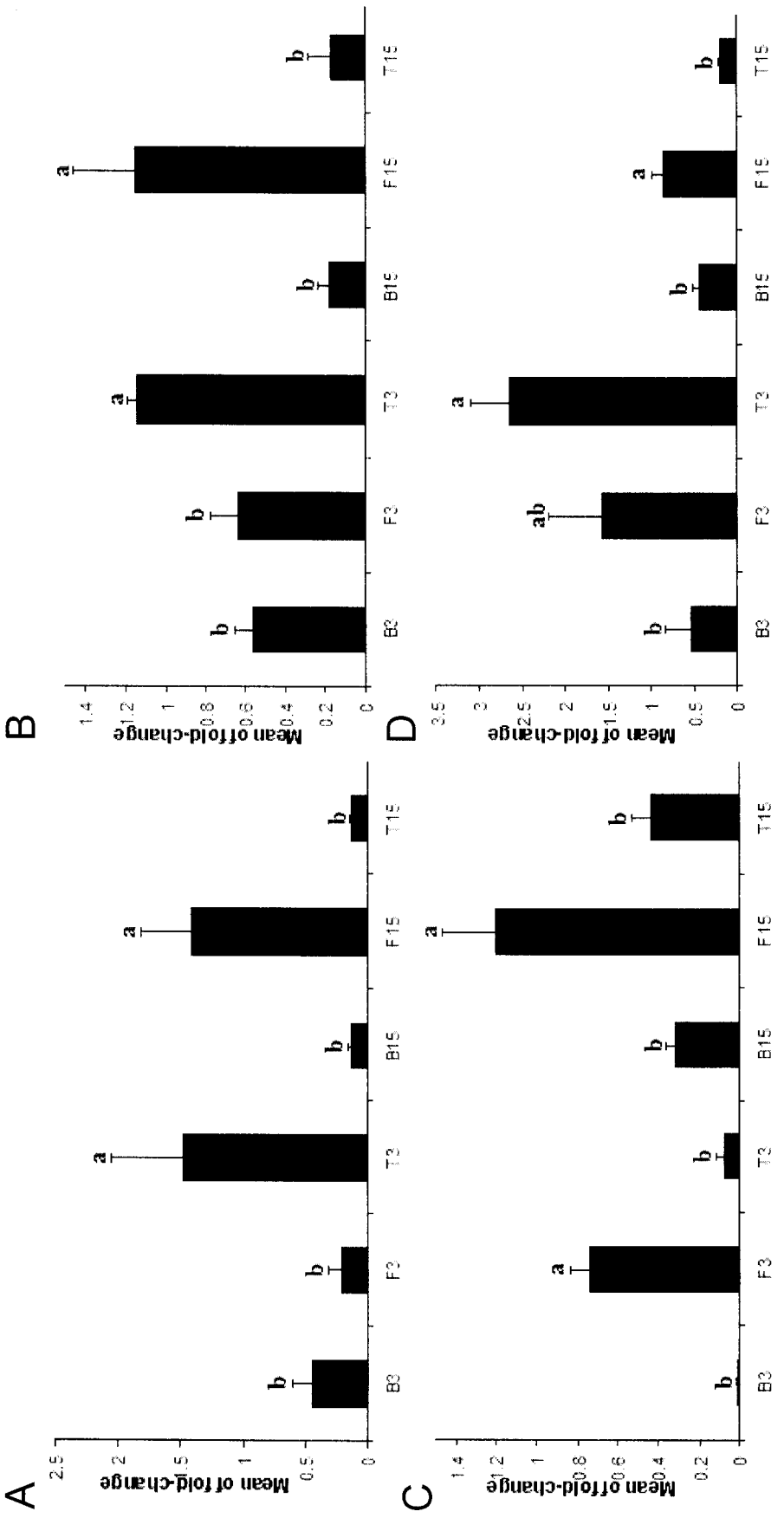


Figure 27

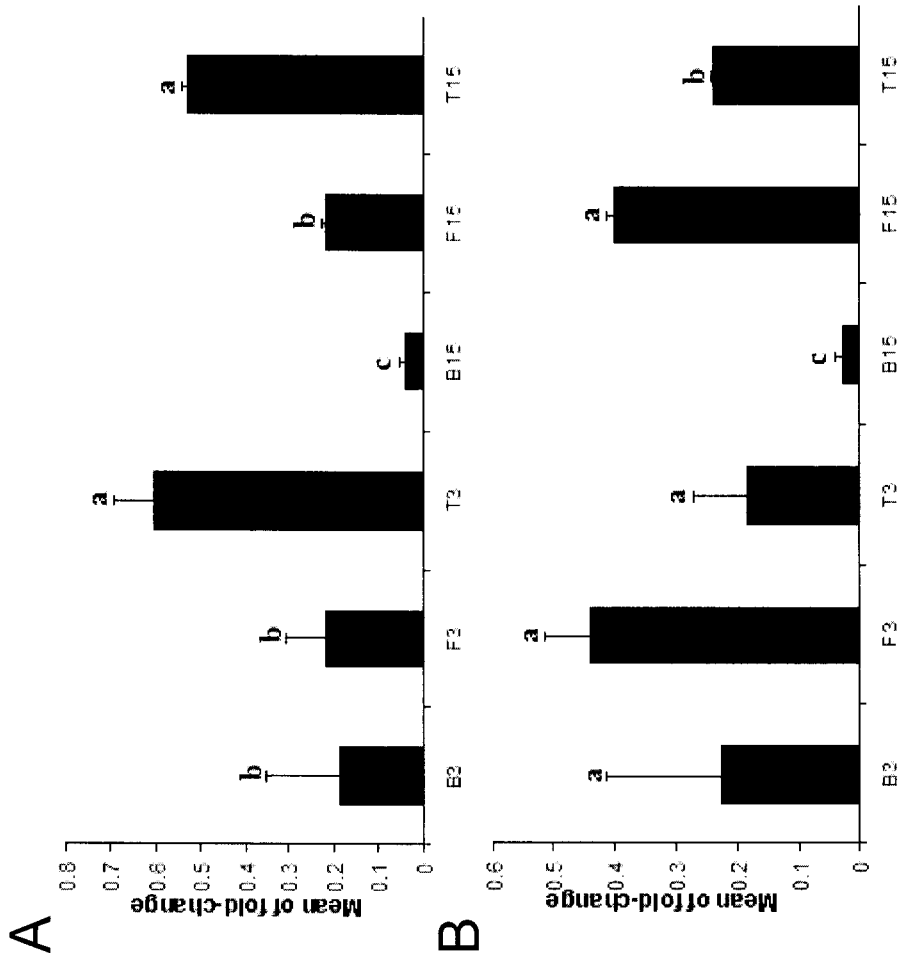

A
B
Mean of fold-change
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
B2
F2
T2
B15
F15
T15

Figure 28

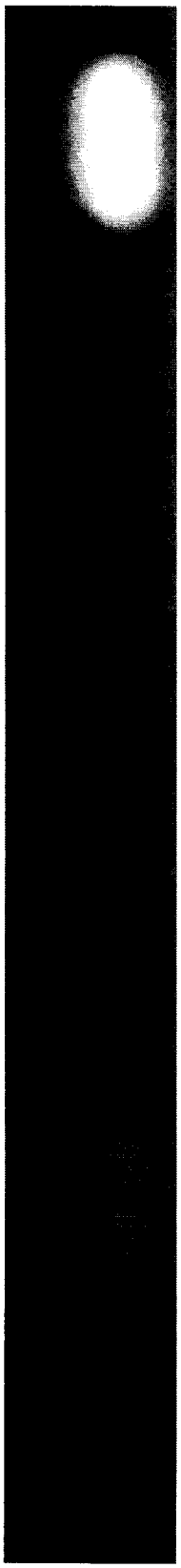


Figure 29

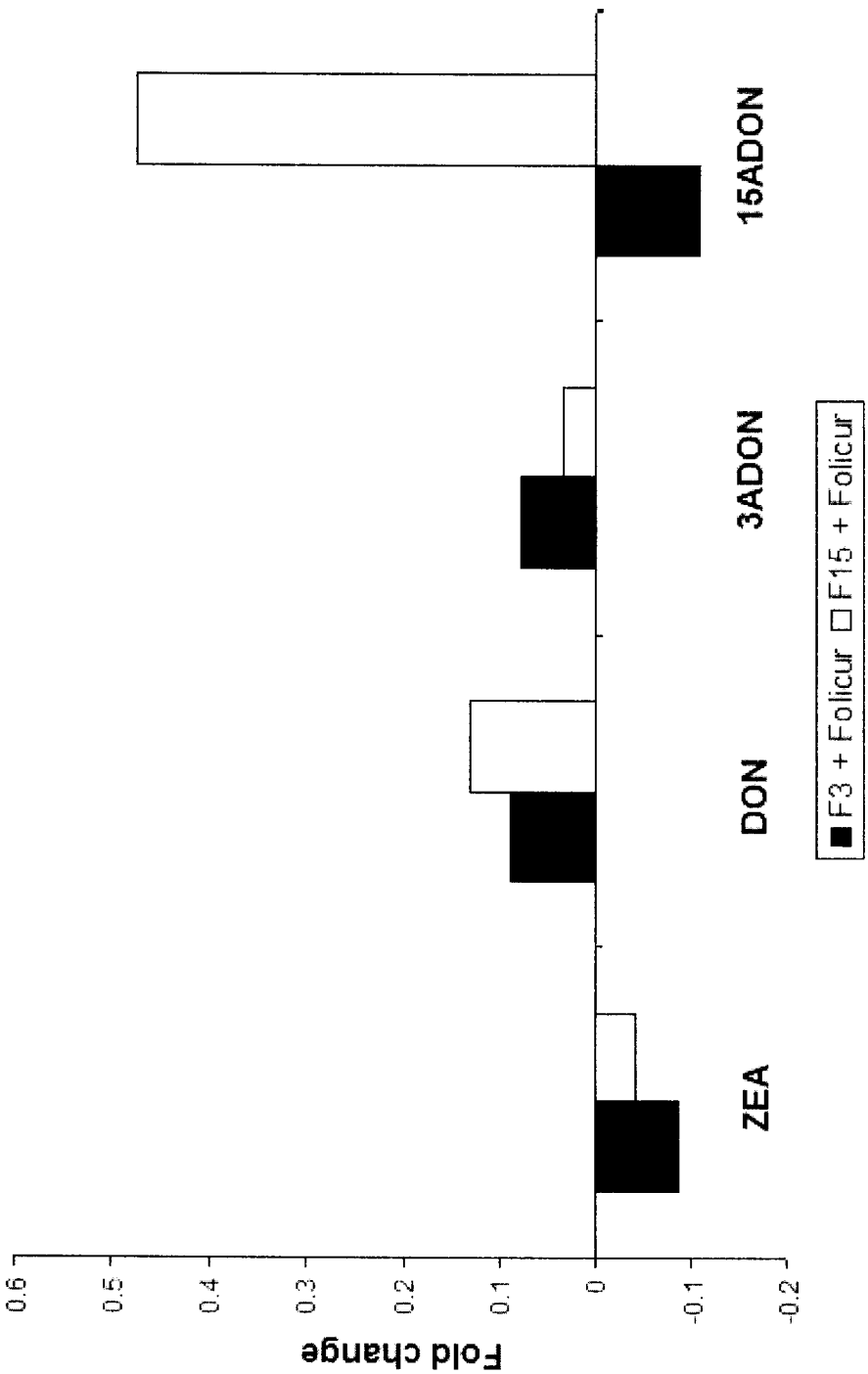

Fold change
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
ZEA
DON
3ADON
15ADON
F3 + Folicur
F15 + Folicur

Figure 30

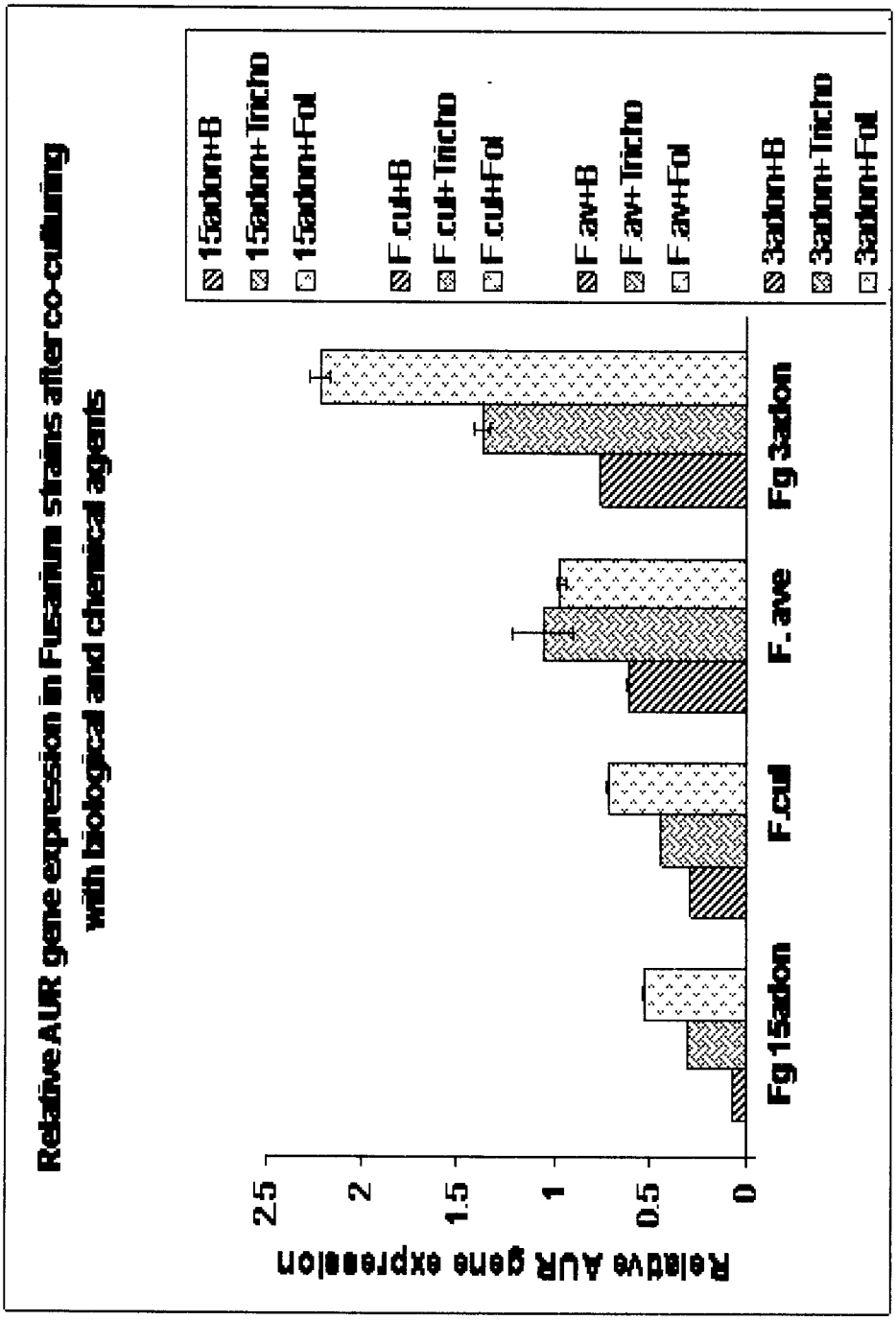

Relative AUR gene expression in Fusarium strains after co-culturing with biological and chemical agents
Relative AUR gene expression
2.5
2
1.5
1
0.5
0
Fg 15adon
F.cul
F. ave
Fg 3adon
15adon+B
15adon+Tricho
15adon+Fol
F.cul+B
F.cul+Tricho
F.cul+Fol
F.av+B
F.av+Tricho
F.av+Fol
3adon+B
3adon+Tricho
3adon+Fol

Figure 31

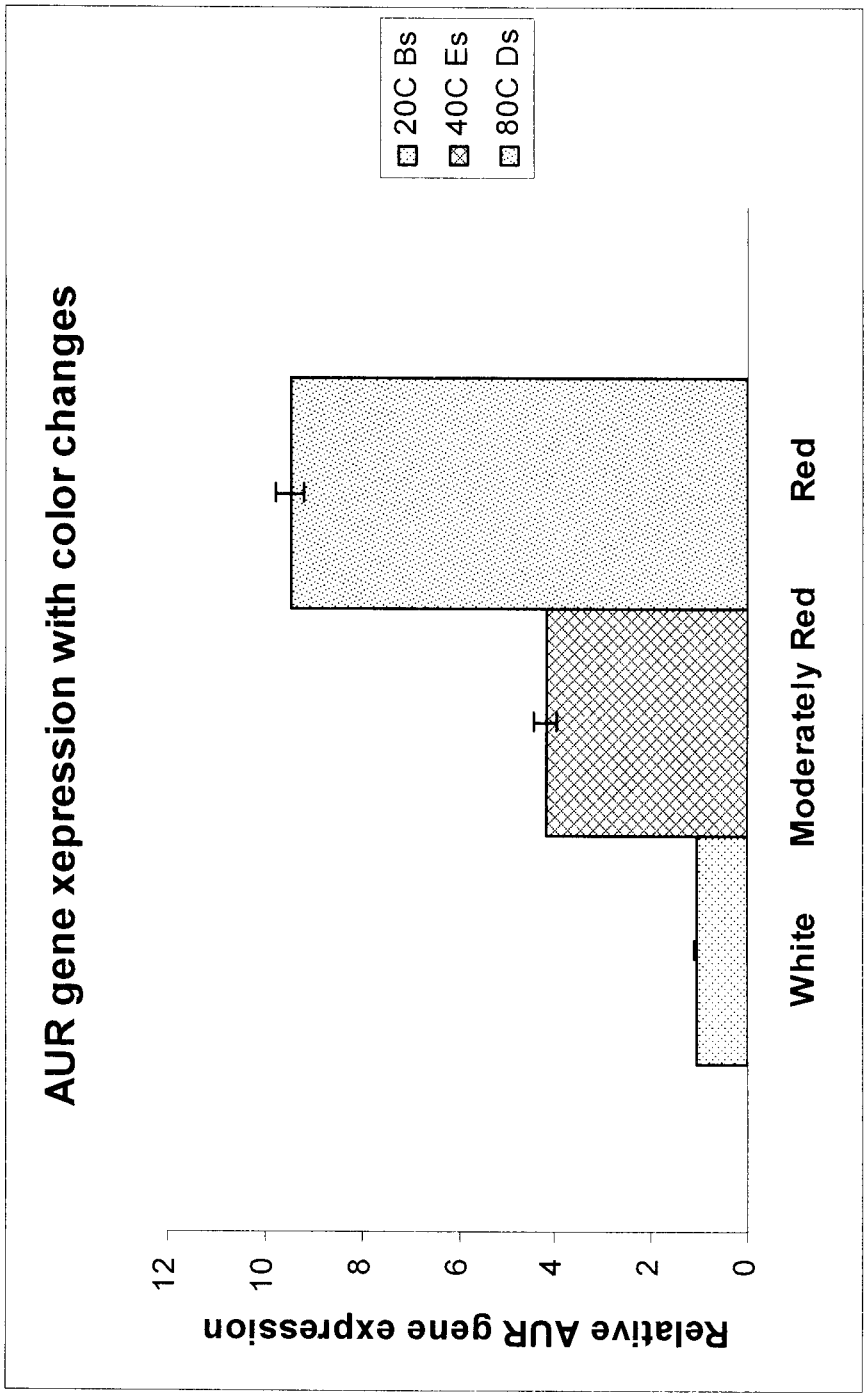
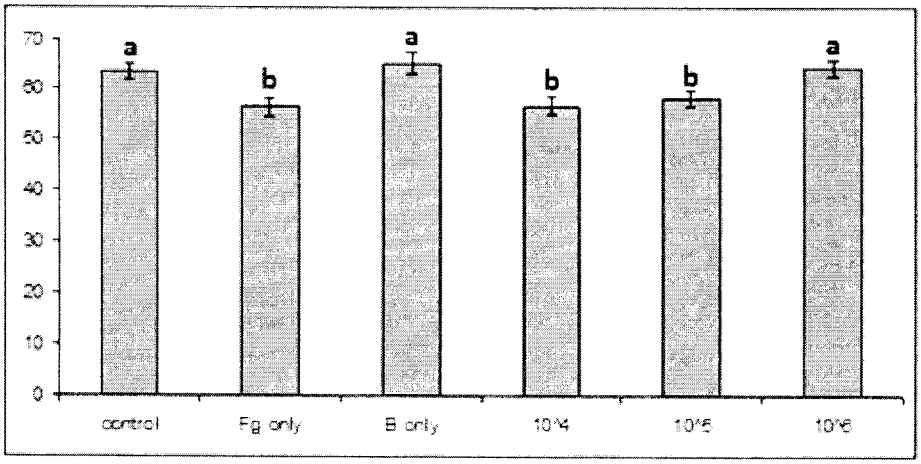
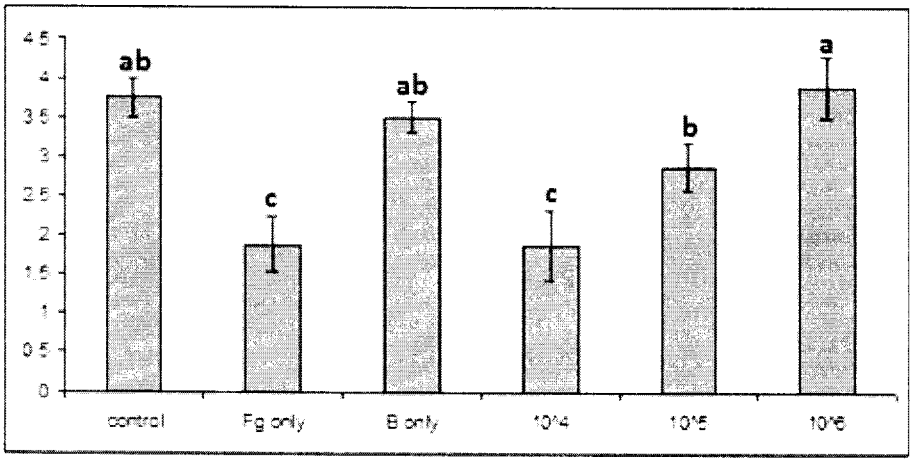

AUR gene xepression with color changes
20C Bs
40C Es
80C Ds
Relative AUR gene expression
0
2
4
6
8
10
12
White
Moderately Red
Red

Figure 32

A: Height of plants (cm)



B: Spikes per plant (average number)



C: Weight of 5 spikes (g)

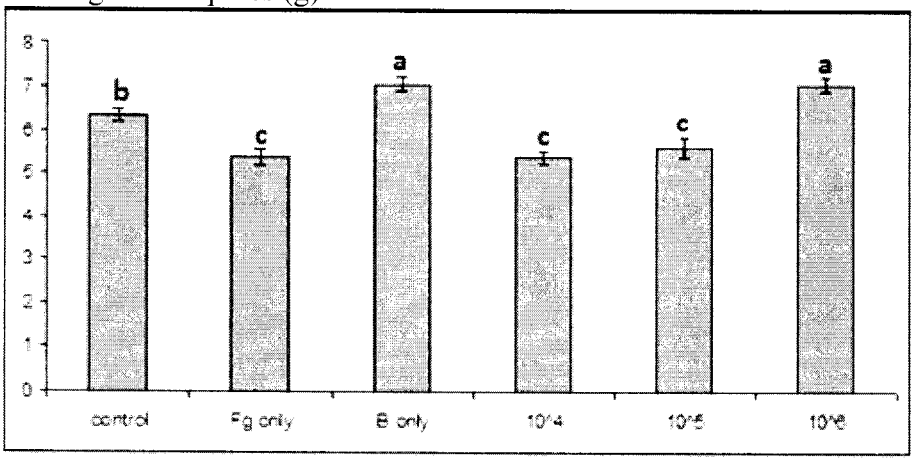
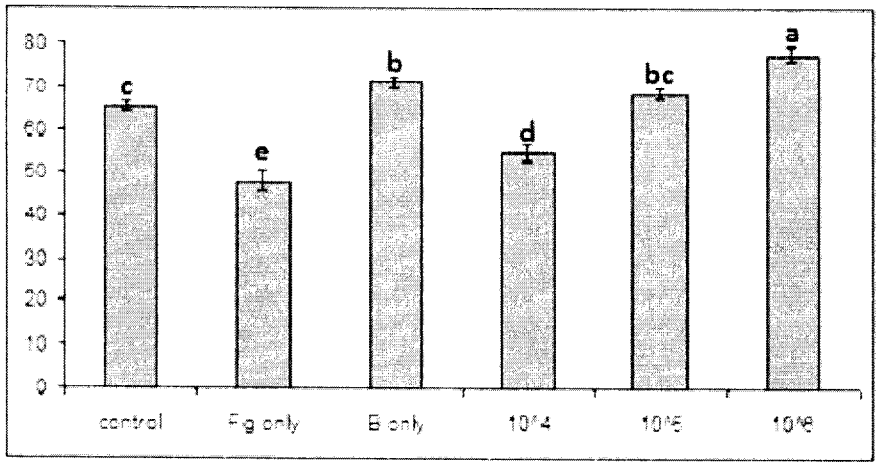
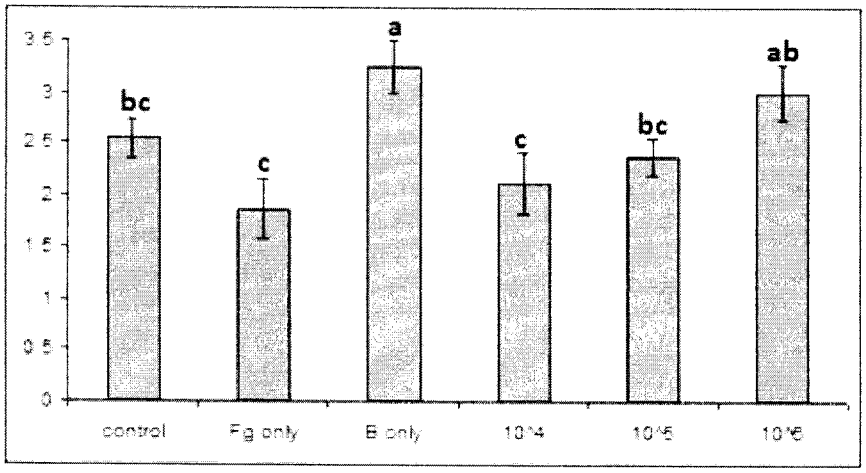


Figure 33

A: Height of plants (cm)



B: Spikes per plant (average number)



C: Weight of 5 spikes (g)

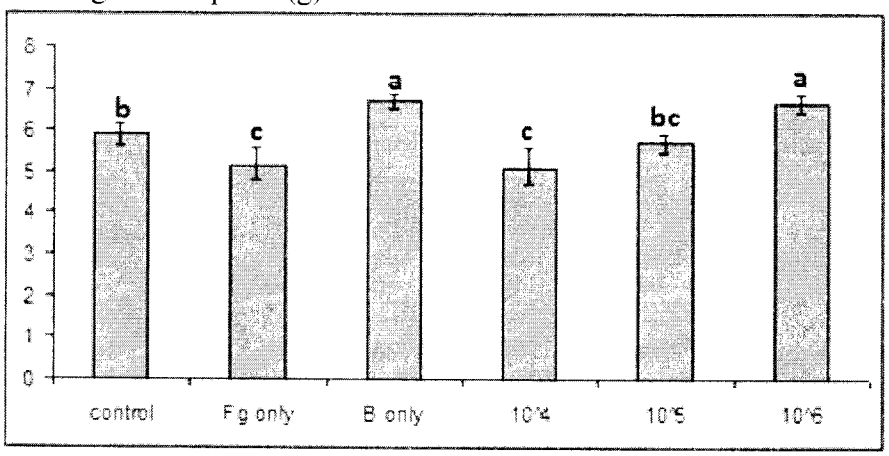


Figure 34

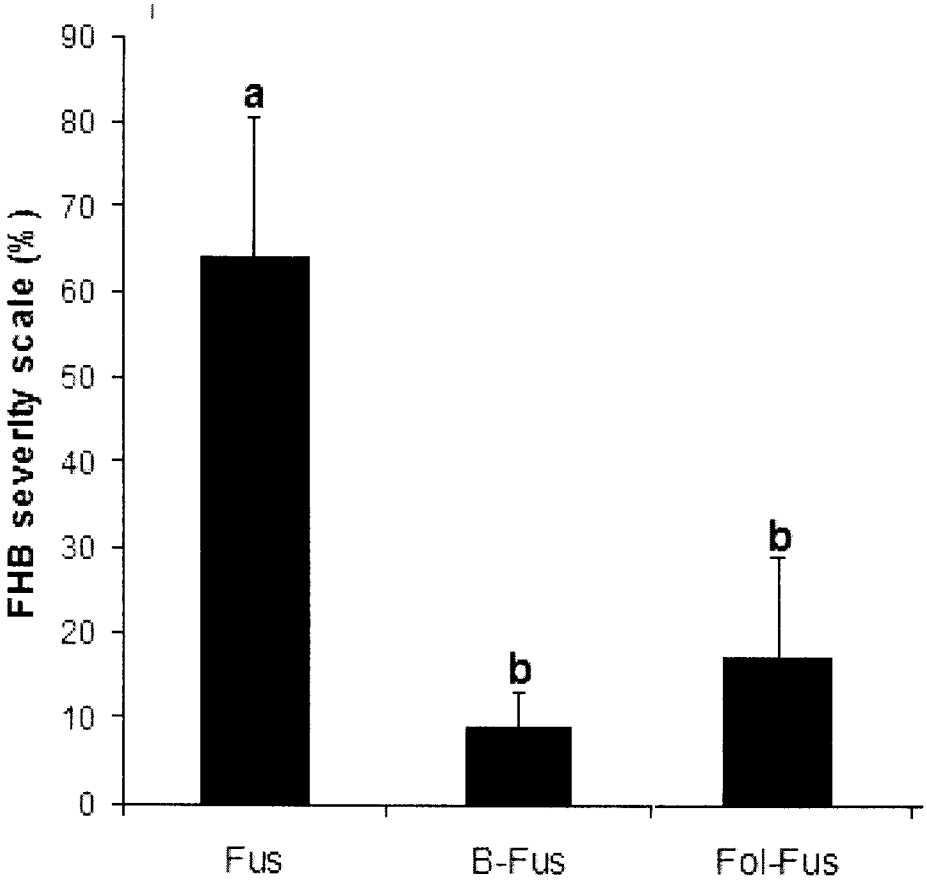

FHB severity scale (%)
90
80
70
60
50
40
30
20
10
0
a
b
b
Fus
B-Fus
Fol-Fus

Figure 35

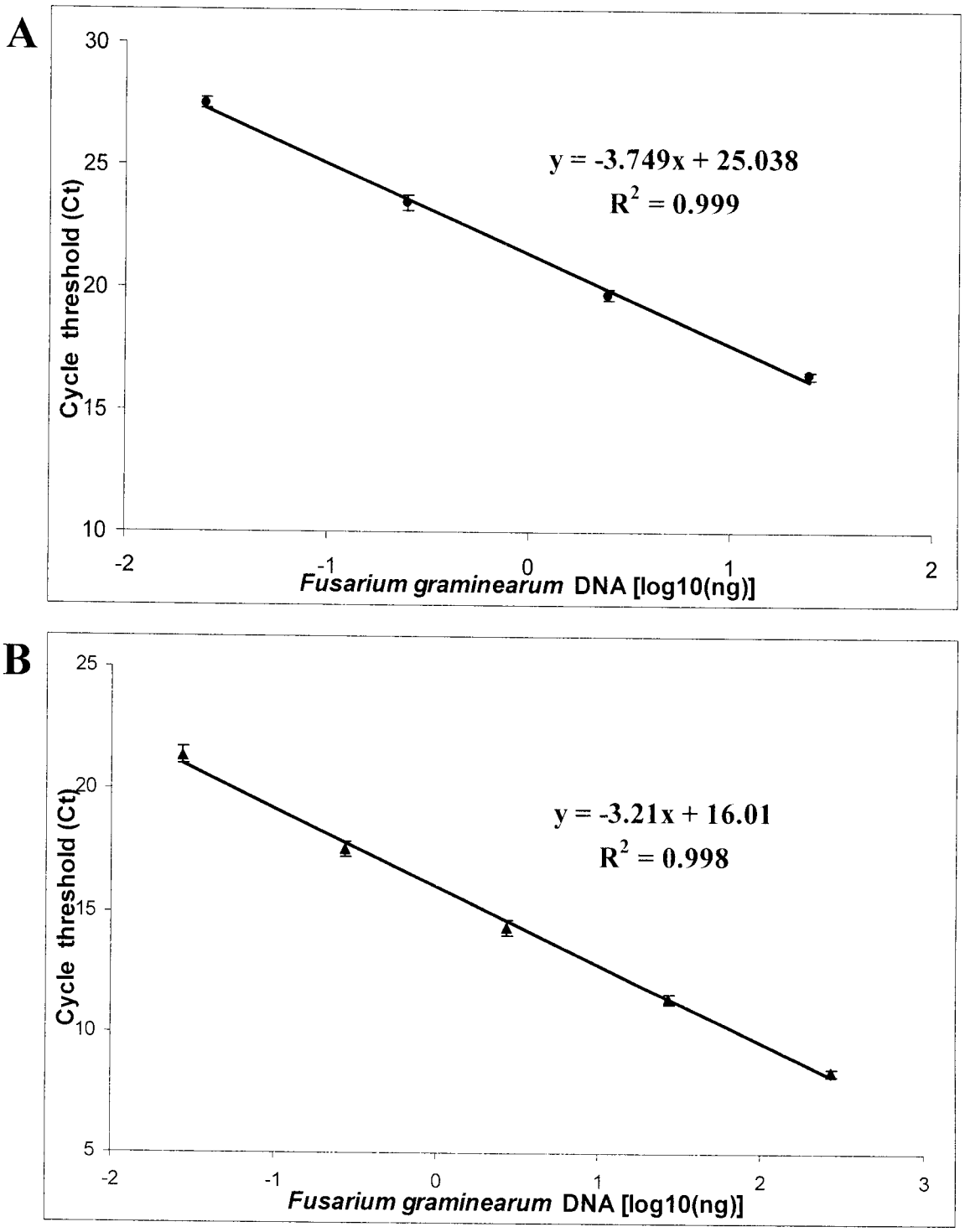

A
30
25
20
15
10
Cycle threshold (Ct)
y = -3.749x + 25.038
R2 = 0.999
-2
-1
0
1
2
Fusarium graminearum DNA [log10(ng)]
B
25
20
15
10
5
Cycle threshold (Ct)
y = -3.21x + 16.01
R2 = 0.998
-2
-1
0
1
2
3
Fusarium graminearum DNA [log10(ng)]

Figure 36

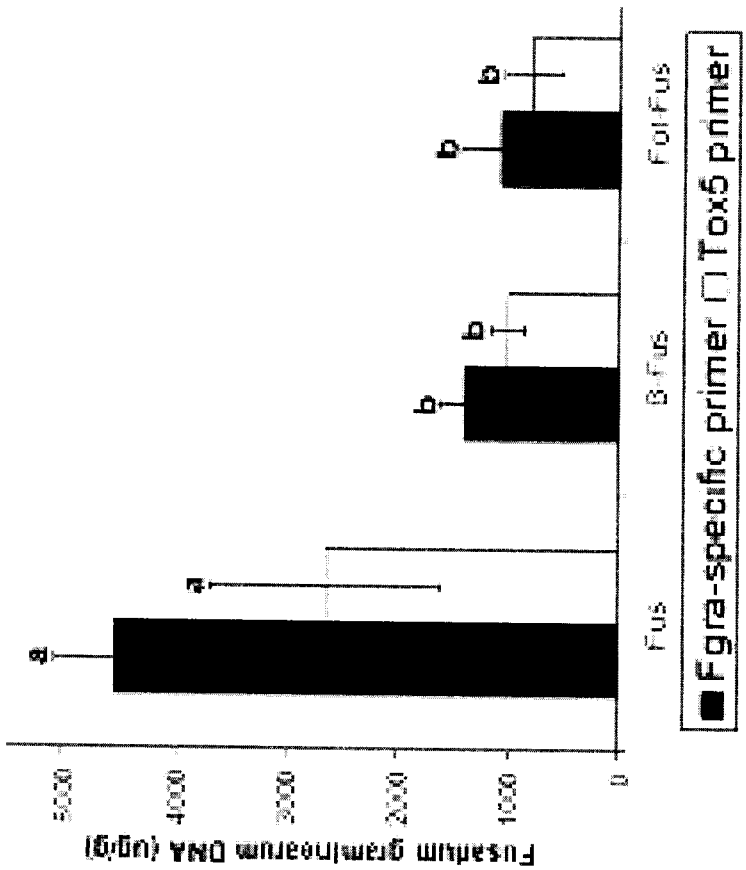

Fusarium graminearum DNA (ug/g)
5000
4000
3000
2000
1000
0
a
a
b
b
b
b
Fus
B-Fus
Fol-Fus
Fgra-specific primer
Tox5 primer

Figure 37

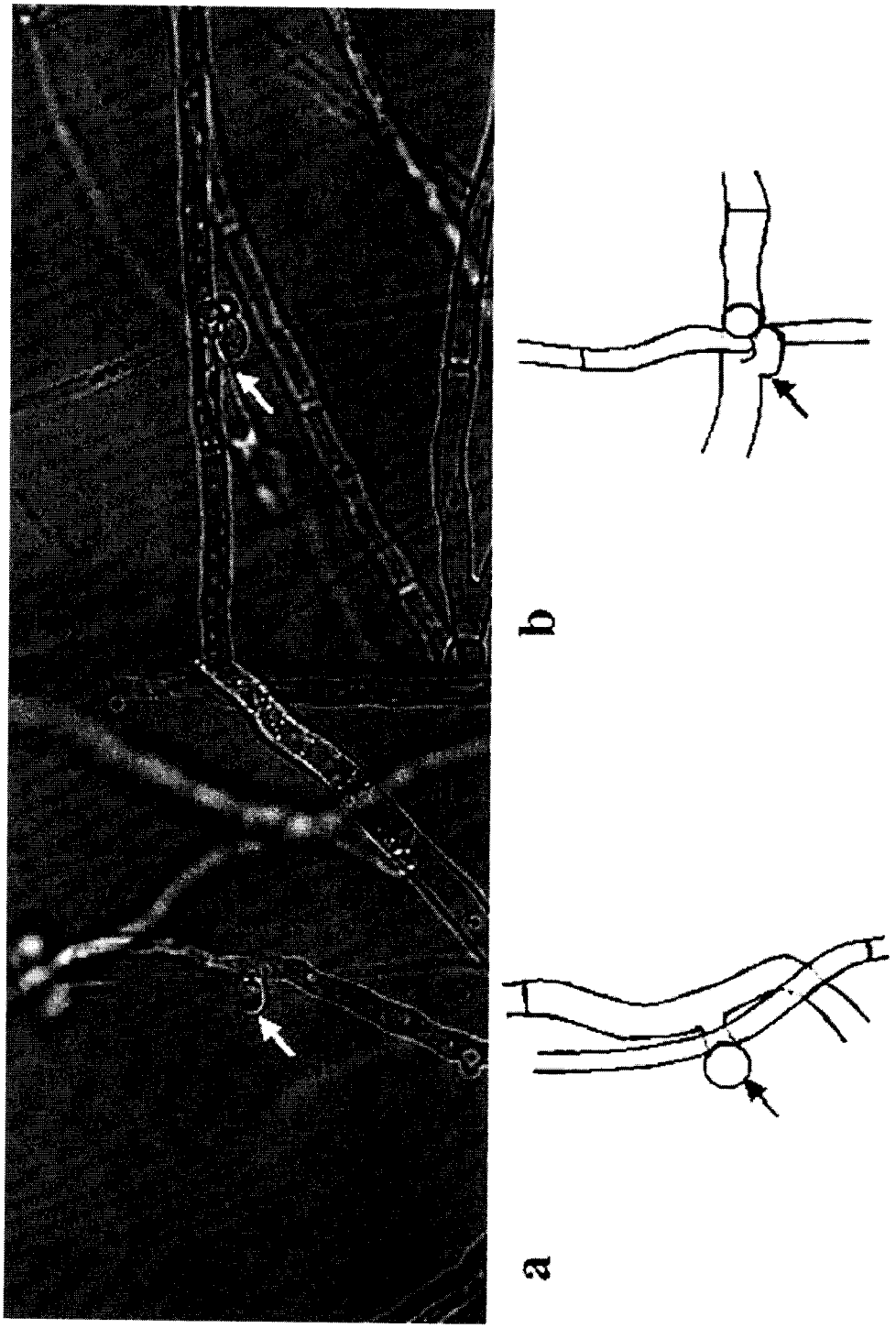


Figure 38

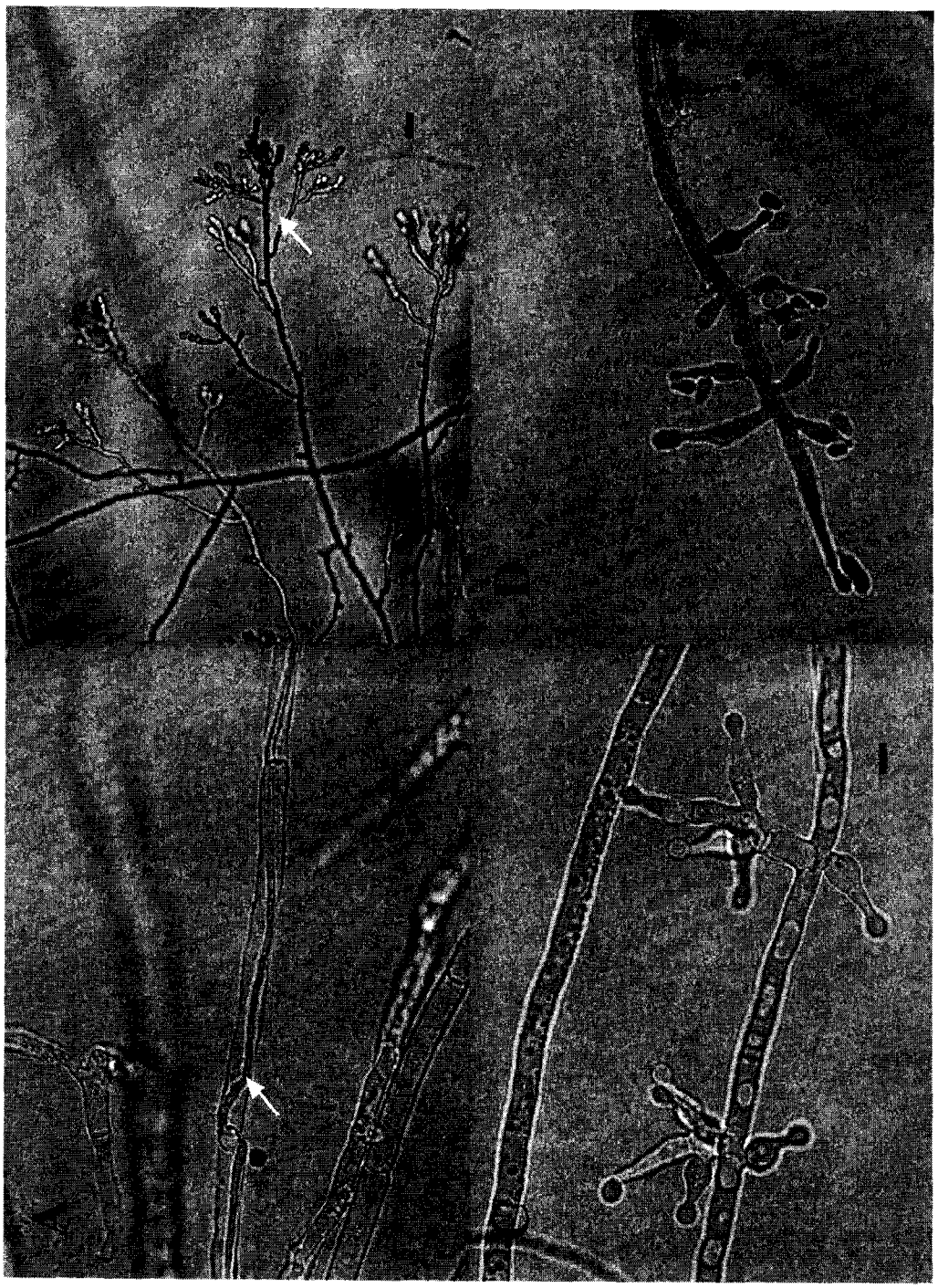


Figure 39

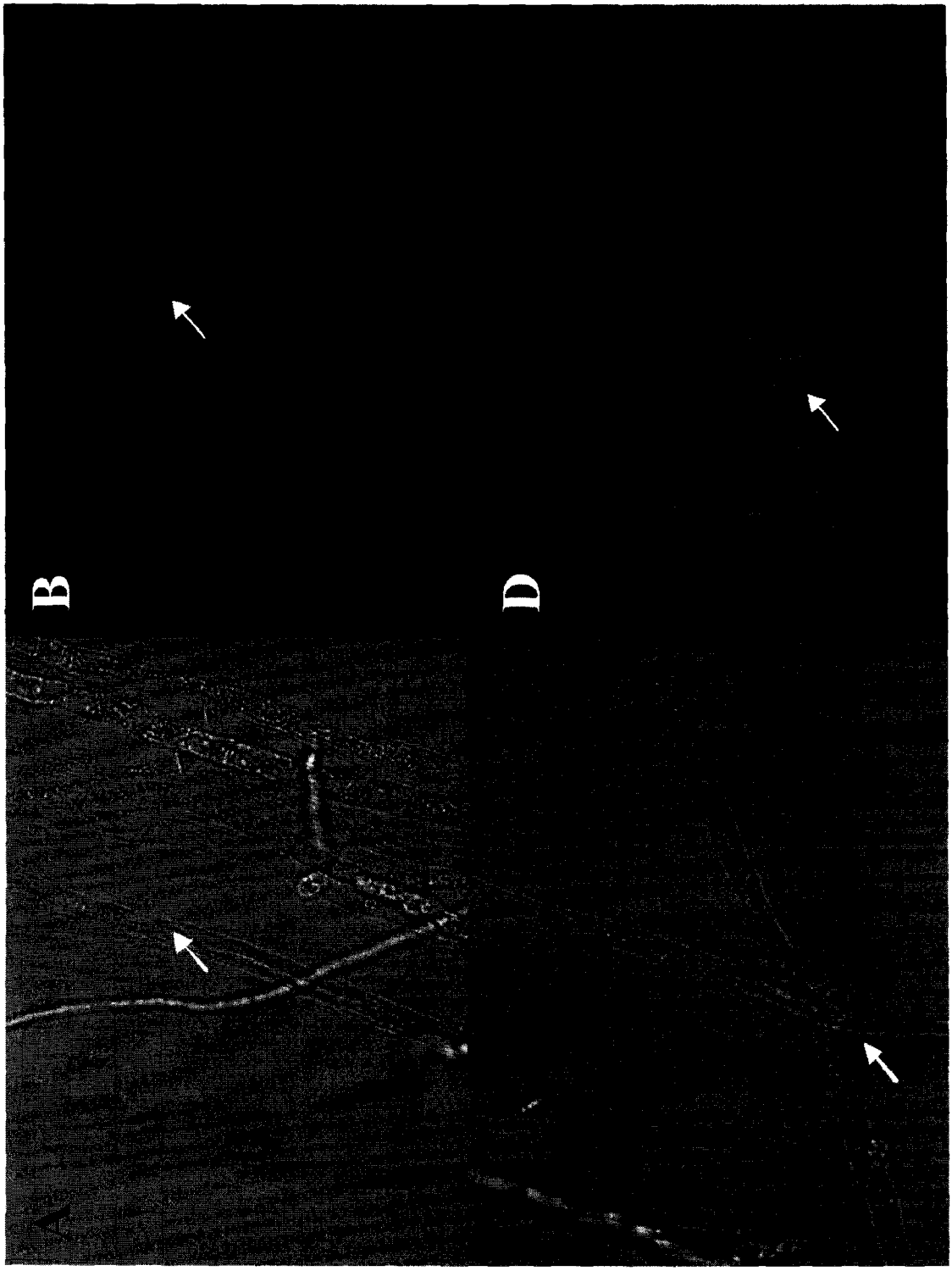


Figure 40

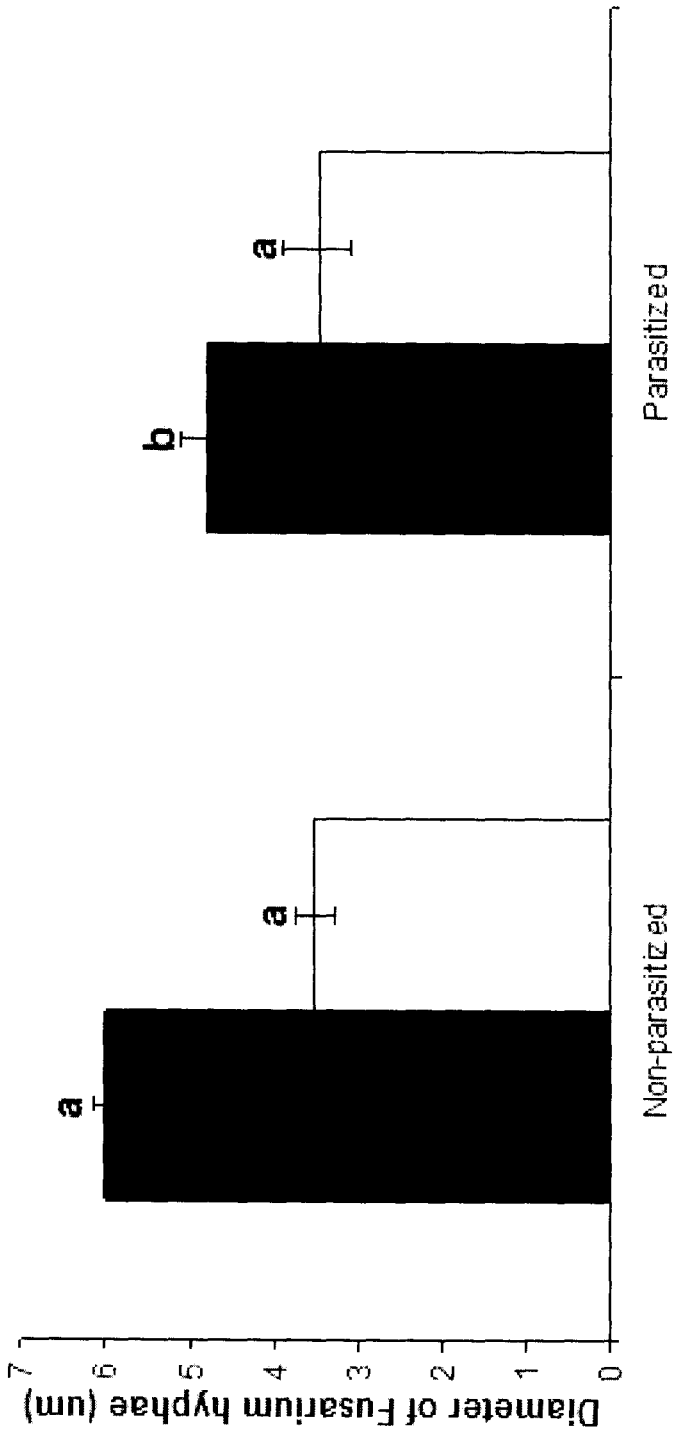

Diameter of Fusarium hyphae (um)
0
1
2
3
4
5
6
7
a
a
b
a
Non-parasitized
Parasitized

Figure 41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001253

A CLASSIFICATK)N ( )F SIJB.IECT MATTER

IPC: ***C12N 15/31*** **(2006.01).** ***AOlN 63/0-1*** *(2006.01).* ***AOlP 3/00*** (2006.01). ***CO7K 1-1/37*** **(2006.01).** ***C12N 1/1-1*** (2006.01). ***Cl 2N 15/63*** (2006.01). ***C12N 15/80*** (2006.01). ***Cl 2Q 1/68*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation seaiched (classification s} stem followed h\ classification symbols)

C12N 15/31 (2006.01). ***AOlN 63/0-1*** *(2006.01).* ***AOlP 3/00*** (2006.01). ***C07K 1-1/37*** (2006.01).
C12N 1/14 **(2006.01).** ***C12N 15/63*** (2006.01). *C12N* ***15/80*** **(2006.01).** ***C12CJ 1/68*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
STN (Biosis, CaPlus, Dissabs), Total Patent, EPOQlJE, Scopus, NCBI, Canadian Patent Database
Keywords lmcoparasit*, Sphaerodes, Fusanum, biocontrol, plant pathogen, biotrophic

C DOCUMENTS CONSE)ERED TO BE RELEVANT

Categoπ *	Citation of document, with indication, where appropriate, of the relevant passages	Rele\ ant to claim No
P, X	CK)H, Y K and VU.TANOVIC, V Biotrophic m\ coparasitic interactions between *Sphaerodes mγcoparasttica* and phUopathogenic *Fusanum* species Biocontrol Science and Technolog\ 30 Apπl2010, VoI 20,No 9, pages 89 1-902 eISSN 1360-0478	1, 2, 6, 8, 9 and 11-16
P, X	VU.TANOVIC, V and GOH, Y K *Sphaerodes mγcoparawtwa* sp no\ , a new biotrophic m\coparasite on *Fnsarium aveiiaceum, F. graniinearuni* and *F. oxwsponim* M\ cological Research 1 September 2009, VoI 113, pages 1172-1 180 e ISSN 1469-8102	1, 2, 6, 8, 9 and 11-16
A	HAVERSON, R M et al No\el use of a P\ienom\cetous M}coparasite for Management of Fusanum Wilt of Watermelon Plant Disease September 2002, VoI 86, No 9, pages 1025-1030 ISSN 0191-2917	1, 2, 6, 8, 9 and 11-16

[ ] Fuithei documents aie listed in the continuation of Bo\ C

[ ] See patent famih annex

* Special categories ol cited documents

Λ document defining the general state oi the art which is not considered to be o± particular rele\ ance

E earlier application or patent but published on or alter the international filing date

L document \Inch ma\ thro\ doubts on prio πt\ clami(s) or whichis cited to establish the publication date o± another citation or other special reason (as specified)

I) document referring to an oral disclosure use exhibition or other means

P document published prior to the international filing date but later than the pπorit Λ date claimed

T later document published alter the international films, date or prio πt\ date and not m conflict \ ith the application but cited to understand the principle or theoi Λ underhmg the m\ention

X document ol particular rele\ ance the claimed m\ ention cannot be considered no\ el or cannot be considered to m\ oh e an m\ enti\ e step \ hen the document is taken alone

Y document ol particular rele\ ance the claimed m\ ention cannot be considered to m\ oh e an m\ entrv e step \ hen the document is combined \ Ith one or more other such documents such combination being ob\ ions to a person skilled m the art

& document member ot the same patent tamih

Date of the actual completion of the international search	Date of mailing of the international search report
10 No\ embei 2010 (10-1 1-2010)	18 No\ ember 2010 (18-1 1-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Properh Office Place du Poitage I, Cl 14 - 1st Flooi, Box PCT 50 Victoria Stieet Gatineau, Quebec KlA 0C9 Facsimile No 001-819-953-2476	Christine Teixeira (819) 994-0244

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001253

Box No. **II** **Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [ ] Claim Nos

because the\ relate to subject matter not required to be searched b\ this Authority, nameh

2 [ ] Claim Nos

because the\ relate to parts of the international application that do not comph with the piescribed requirements to such an extent that no meaningful international seaich can be carried out, specificalh

3 [ ] Claim Nos

because the\ aie dependent claims and aie not diafted in accoidance with the second and thud sentences of Rule 6 4(a)

Box No. **Ill** **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple in\ entions in this international application, as follow s

see Extra Sheet

1 [ ] As all required additional seaich lees were timeh paid b\ the applicant, this international search report co\ers all seaichable claims

2 [ ] As all seaichable claims could be seaiched without effort |ustif\ing additional fees, this Authority did not imite payment of additional fees

3 [ ] As onh some of the requiied additional search fees were timeh paid b\ the applicant, this international search report co\eis onh those claims foi which fees weie paid, specificalh claim Nos

4 [X] No required additional search fees were timeh paid b\ the applicant Consequenth , this international seaich report is restricted to the invention first mentioned in the claims, it is cσvered b\ claim Nos

1. 2. 6 and 9 (wholh) and 8 and 11-16 (partialh)

Remark on Protest

[ ] The additional search fees were accompanied b\ the applicant `s protest and, wheie applicable, the pa\ment of a protest fee

[ ] The additional search fees were accompanied b\ the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation

[ ] No protest accompanied the pa\ment of additional search fees

Indention 1 Claims 1. 2. 6 and 9 (wholh) and claims 8 and 11-16 (partialh)

are directed towards a biologicalh pine culture of *Sphaerodes my coparasitta* strain IDAC 30 1008-00 1/SMCD2220-01 compositions compiising said stiain and use theieof foi contiolling disease symptoms caused by *Fiisarium spp* in plants

Indention 2 Claims 3-5. 7. 8 and 10-16 (all paitialh)

are directed towards a nucleic acid molecule fiom a culture of *Sphaerodes my coparasitia* comprising a nucleotide sequence set foitli in SEQ ID NO 1. a nucleotide construct comprising said nucleic acid molecule, a microbial cell comprising said nucleic acid molecule and use of said microbial cell for controlling disease symptoms caused by *Fusarmm spp* in plants

Imentions 3 and 4 Claims 3-5. 7. 8 and 10-16 (all paitialh)

are directed towards the same subject mattei as Im ention 2 but ielating to SEQ ID NOs 2 and 3 iespectn eh

Imention 5 Claim 17 (wholh) and claims 19 and 20 (partialh)

are directed towards an exocellular protein from a culture of *Sphaerodes my coparasiTia* stiain IDAC 30 1008-00 lliaung a moleculai weight of 13 LDa. compositions comprising said protein and use thereof for controlling disease sunptoms caused by *Fusarmm spp* m plants

Imention 6 Claim 18 (wholh) and claims 19 and 20 (partialh)

are directed towards the same subject mattei as Imention 5 but ielating to a piotein hauiig a moleculai weight of 50 LDa

Im ention 7 Claims 21-23 (wholh)

are directed towards a method foi testing a sample of plant seeds for the presence of aurofusa πn and primers for the detecting the expression of aurofusarin

The claims are diiected to a pluialih of alleged imentions as indicated abo-\e The mere fact that the nucleic acids and exocellulai proteins are der ıv ed from *Sphaerodes my coparasitia* is not sufficient to establish unit} of in\ention as the disclosed nucleic acid molecules comprising the nucleotide sequences set foitli in SEQ ID NOs 1-3 are stracturalh distinct and the structures of the 13 IdDa and 50 LDa exocellulai proteins are not e\en disclosed Moreo\er. the instant application fails to pirn ide substant π e support demonstrating a significant structural element that is responsible foi the function oi biological actn ih of said nucleic acids and exocellulai proteins, therefoie. there is no special technical featuie that is common to the claims of Imentions 1-6 Furthei. the claims of Imention 7 are directed to primers der π ed from the aurofusaiin gene of *Fusarmm spp* . consequently . there is no common special technical feature linking the groups of in\entions