Quantification of a Novel Biotrophic Mycoparasitic Fungus Using Genus Specific Real-Time PCR for Biocontrol of Phytopathogenic *Fusarium graminearum* in Wheat Root under Controlled Conditions

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Abstract

Fusarium species are well-known causal agents of *Fusarium* root-rot, Fusarium head blight (FHB), and Fusarium damaged kernels (FDK) diseases in Saskatchewan and other provinces of Canada. Our goal is to develop quantitative real-time PCR techniques to determine and evaluate interactions between *Fusarium*-associated biotrophic mycoparasitic fungus SMCD 2220 and 3-acetyldeoxynivalenol (3-ADON) producing *Fusarium graminearum* Schwabe – in and surrounding wheat roots. ITS1F/ITS4 (internal transcribed spacer) sequences from SMCD 2220 biotrophic mycoparasitic fungal isolate and 20 different *Fusarium* strains were aligned, and consensus sequences were verified. Four candidate primer sets from ITS regions were designed based on the non-conserved regions of the consensus sequences. Using the primer set SmyITSF/R, the biotrophic mycoparasite genomic DNAs were amplified from SMCD 2220. This primer set was developed for assessing and quantifying the interactions between SMCD 2220 biotrophic mycoparasite and *F. graminearum*. Well-known necrotrophic *T. harzianum* T-22, was used as the positive control. During *in vitro* studies, only SMCD 2220 was observed to improve wheat seed germination, whereas T-22 induced post-emergence damping-off symptoms. Under controlled phytotron conditions, both SMCD 2220 and *T. harzianum* strains were 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. However, amount of SMCD 2220 DNA detected was no significantly difference between wheat inoculated with *F. graminearum* and without *Fusarium.* In contrary, the amount of *T. harzianum* DNA monitored in the treatment inoculated with *F. graminearum* was observed to reduce significantly, as compared to non-*Fusarium* treatment.

Introduction

Most of the *Fusarium* species are pathogenic to plants and these phytopathogens are responsible for major economically important crops diseases (Bai and Shaner 2004). Fusarium diseases are not only can be found in Canadian wheat plantations, but, pathogenic *Fusarium* spp. can also infect barley, canola, asparagus, and some other crops in Canada (Calman et al. 1986; Fernandez et al. 2007b; Vujanovic,et al. 2006). *Fusarium* species are commonly reported to cause crown and root rot diseases, Fusarium Head Blight (FHB), Fusarium damaged kernels (FDK), and Fusarium wilt in wheat (Fernandez et al. 2007a). Crown and root rot diseases are frequently caused by *F. graminearum, F. culmorum, F. avenaceum, F. oxysporum*, and *F. equiseti* (Fernandez and Jefferson 2004). These pathogenic fungi are observed to show high capability in reducing yields of wheat production, inhibiting seed germination, and affecting the quality of grains (Mavragani 2008). Therefore, it is

important to control the outbreaks of Fusarium diseases or reduce the effects of Fusarium inoculants on crops and increase the plant yields, especially wheat.

Biological control is proposed as promising environmental solution (Pal and McSpadden, 2006) and practical option against Fusarium pathogens (Vujanovic 2008). Hyperparasitism/mycoparasitism is one of biological control mechanisms used to control or suppress plant pathogenic fungi (Howell, 2003; Paulitz and Blanger, 2001). According to Boosalis (1964), mycoparasitism interactions are categorized into two major groups, biotrophic and necrotrophic parasitisms. The classification of the mycoparasitism interactions is based on the fungal parasitic bioactivity and the effects produced on the pathogenic host by the parasitism (Goh et al. 2009). Necrotroph parasitism interactions regularly have broader host ranges as compared to biotrophic parasites because necrotrophs are able to produce non-specific toxic compounds (Barnett, 1963; Boosalis, 1964). The objective of this experiment is to study the mycoparasite-*Fusarium*-wheat root interactions through quantification of a novel biotrophic mycoparasite utilizing genus specific real-time PCR for assessing biocontrol of phytopathogenic *Fusarium graminearum* in wheat root under controlled conditions.

Materials and Methods

Fungal strains and growth conditions

3-acetyldeoxynivalenol (3-ADON)-producing *Fusarium graminearum*, biotrophic mycoparasitic fungus SMCD 2220, and *Trichoderma harzianum* T-22 were retrieved from Saskatchewan Microbial Collection and Database (Saskatoon, SK). All fungal isolates used in this study were grown and maintained on potato dextrose agar (PDA) with antibiotics (Goh et al. 2009).

In-vitro assays

Spring wheat CDC-TEAL 2001 seeds were surface-sterilized prior to using and sowing (Mavragani 2008). Seeds were inoculated together with SMCD 2220 biotroph and T-22 necrotroph on PDA plates.

PCR primers and primers designing

One set of specific PCR primer set (SmyITSF/R) was developed to quantify *Sphaerodes mycoparasitica* in real-time PCR quantification assays. *F. graminearum*- and *T. harzianum*-specific primer sets used in this study were proposed by Nicholson et al. (1998) (Fg16NF/R) and Kim and Knudsen (2008) (TGP4-F/R), respectively. The SmyITS primer set was based upon the non-consensus ITS regions between *Fusarium* species and SMCD 2220. Initial amplifications were performed in a 25 µl reaction mixture (Qiagen Taq Polymerase Core kit) by using Thermal Cycler epgradient S (Eppendorf). The PCR conditions for ITS primer set employed was outlined in Sokolski et al (2004).

Standard

Standard curves for SMCD2220-, *Trichoderma*-, and *F. graminearum*-specific primer sets were generated, based on threshold cycles (Ct), by using a series of 10-fold diluted genomic DNAs from *S. mycoparasitica*, *T. harzianum*, and *F. graminearum*.

Growth conditions and fungal inoculation

Quantification of interactions between mycoparasite-pathogen-wheat roots was conducted on the spring wheat CDC-TEAL 2001. Wheat plants were grown in pots (4 x 4 x 16 cm) with 10 g of soil. Six different treatments were used: 1) control – without fungal inoculants; 2) with

only *F. graminearum* mycelial suspension; 3) with only SMCD2220 mycelial suspension; 4) with only *T. harzianum* mycelial suspension; and 5) with both *F. graminearum* and SMCD2220; and 6) with both *F. graminearum* and *T. harzianum*. Spring wheat plants were grown, watered and fertilized as outlined in Fernandez and Chen (2005).

Real-time PCR quantification

Real-time PCR amplifications of genomic DNAs (for SMCD 2220*, T. harzianum,* and *F. graminearum*) and total DNA extracted from the spring wheat roots harvested at mid-seedling growth (Zadok's growth stage 13) (Zadoks et al. 1974) were carried out in MiniOpticon (Bio-Rad). The reaction mixture for all real-time PCR assays were: 12.5 µl of IQ Supermix (Bio-Rad), 1 µl of each 10 µM forward/reverse primers (Invitrogen), 3.4 µl of BSA (Bovine Serum Albumin) (1.47µg/µl), 6.1 µl of sterilized UltraPure Millipore water, and 1 µl of DNA template.

Statistical analyses

Leaves length (cm), root length (cm), root biomass (g) and seed germination (%); and SMCD2220*, T. harzianum,* and *F. graminearum* genomic DNA quantification from the roots of Spring wheat plants were analyzed by using analysis of variance (ANOVA). Multiple comparisons for more than two samples were analyzed by utilizing Tukey's studentized range test at $P = 0.05$ (SPSS 1990).

Results and Discussions

SmyITSF/R primer set was tested with SMCD2220, seven *Fusarium* species, night different ascomycetous fungal isolates, two zygomycete fungi, and three basidiomycetous fungal strains. This primer set was found only amplifying SMCD2220, not *T. harzianum* and other fungi. Under *In-vitro* assays, wheat seeds inoculated with *T. harzianum* T-22 showed significantly reduction in seed germination as well as leaves and roots length, as compared to uninoculated and with SMCD 2220 (Fig. 1 and 2). Root biomass and root length of *F. graminearum* infected spring wheat were significantly increased with the treatments of biotrophic mycoparasitic SMCD2220 as compared to inoculation with *F. graminearum* alone (Fig. 3).

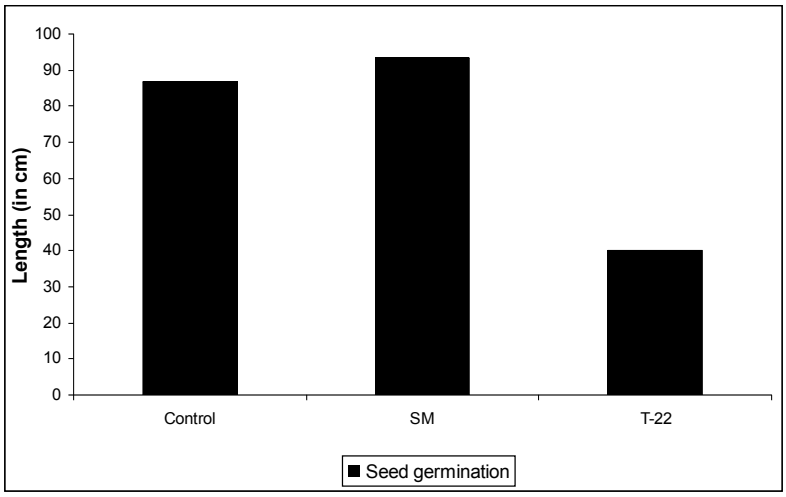

Figure 1. Seed germination for *In-vitro* assays with biotrophic mycoparasite SMCD 2220 and necrotrophic parasitic T-22.

These were further confirmed by using quantification real-time PCR to evaluate quantity of SMCD2220*, F. graminearum,* and *T. harzianum* DNAs in the root of spring wheat challenged with different treatments (Table 1). Amounts of *F. graminearum* DNA detected in the treatments with SMCD2220 and *T. harzianum* were significantly reduced (Table 1). In previous study, treatments with biotrophic mycoparasitic fungal inoculant were observed to show significant suppression of *F. oxysporum* in watermelon plants (Harveson et al. 2002). Amount of SMCD2220 DNA detected was no significantly difference between wheat inoculated with *F. graminearum* and without *Fusarium* (Table 1). In contrary, the amount of *T. harzianum* DNA detected in the treatment inoculated with *F. graminearum* was reduced significantly, as compared to non-*Fusarium* treatment (Table 1). This is concord to the findings proposed by Sivan and Chet (1989). They observed that number of *T. harzianum* counts on root segments decreased as the concentration of *Fusarium* pathogen in soil increased. In addition, they suggested that this is due to the competition between beneficial fungus and pathogen.

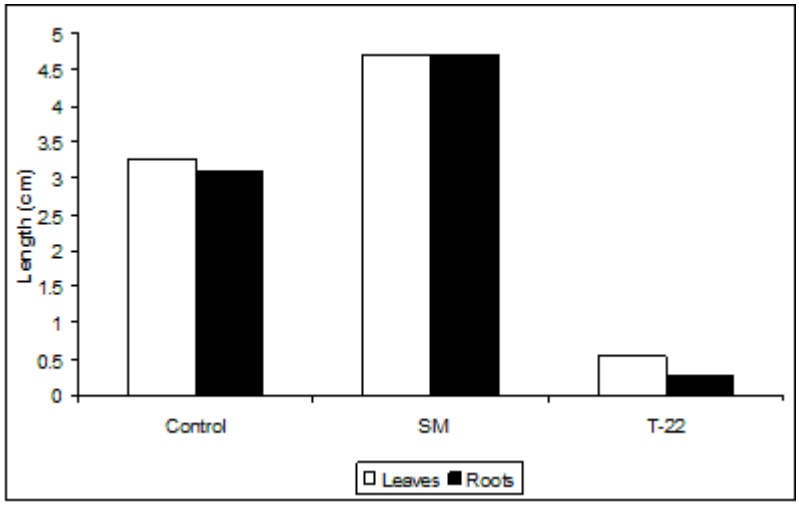

Figure 2. The growth of wheat seedlings under *In-vitro* assays with biotrophic SMCD 2220 and necrotrophic T-22 mycoparasites.

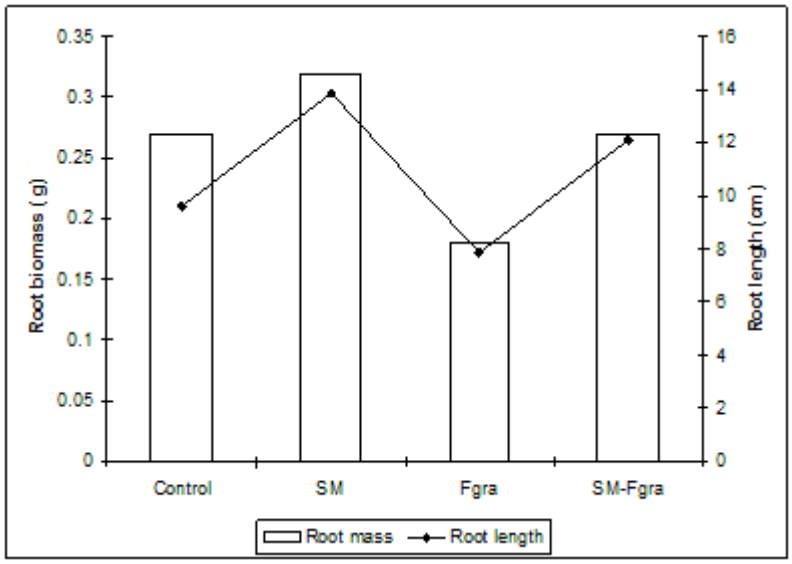

Figure 3. Effects of *S. mycoparasitica* and *F. graminearum* on root biomass (bars) and root length (line) of spring wheat.

Table 1. Amount of DNAs (μg per g of roots) for SMCD 2220, T22, and *F. graminearum* in different treatments by using SMCD 2220, *Trichoderma*-, and *F. graminearum*-specific primer sets with quantitative real-time PCR.

*Treatment	With Fg16NF/R	With SmyITSF/R	With TGP4-F/R
Control	NA	NA	NA
SM	NA	12	NA
T22	NA	NA	180
Fgra	654	NA	NA
SM-Fgra	61	10	NA
T22-Fgra	56	NA	43

* Treatments were: Control = uninoculated control; SM = inoculated with SMCD2220 only; T22 = inoculated with *T. harzianum* only; Fgra = inoculated with *F. graminearum* only; SM-Fgra = inoculated with both SMCD2220 and *F. graminearum;* and T22-Fgra = inoculated with *T. harzianum* and *F. graminearum.*

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