

DISTRIBUTION AND INTERACTION OF FUSARIUM CROWN ROT AND
COMMON ROOT ROT PATHOGENS OF WHEAT IN MONTANA AND
DEVELOPMENT OF AN INTEGRATED MANAGEMENT PROGRAM FOR
FUSARIUM CROWN ROT

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Plant Science and Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

June 2010

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June 2010

DEDICATION

This dissertation is dedicated to those angels that have marked my life.

My parents José and Ana,
Who have been supporting and ensuring my future all my life.

My lovely wife Vanessa,
Who has been the angel who has accompanied me on this adventure and whom I hope
will be with me for the rest of my life.

Esta disertación está dedicada a esos ángeles que han marcado mi vida. Mis padres José y Ana, quienes me han apoyado y se han preocupado por mi futuro durante toda mi vida. Mi amada esposa Vanessa, quien me ha acompañado en esta aventura y con quien espero compartir el resto de mi vida.

ACKNOWLEDGEMENTS

I would like to acknowledge and say thanks to my advisor, Dr. Barry J. Jacobsen, who opened the doors of his lab to work with him at the beginning of this process. Who shared his knowledge and wisdom, who had the patience to guide me, and who helped me tirelessly to fulfill this dream.

I would like to thank Dr. Alan Dyer (co-chairman of my committee) for his advice, friendship and support in this process.

I am deeply thankful for the detailed and constructive comments on my research made by my committee members: Dr. Li Huang, Dr. John Sherwood, Dr. Luther Talbert and Dr. Jane Mangold.

During this process several faculty members, staff and students of the Plant Science & Plant Pathology Department and from other departments have collaborated in the success of my doctoral research. Especially thank to Dr. Lisa Rew, Dr. Diane Cooksey, Dr. William Grey, Dr. Perry Miller, David Baumbauer, Ken Kephart, David Wichman, Bob Johnston, Andy Hogg, Jeff Johnston, Jeffrey Holmes, Oliver Neher, Katty Trujillo, Jackie Campbell, Irene Decker, Joanna Dumas, Bobby Bear, and Tamara Parnell.

Finally, to all of the people who contributed in any way to the completion of this project, especially growers and extension agents from the Counties of Judith Basin, Fergus, Phillips, Blaine, Chouteau, Liberty, Hill, Toole, Glacier, and Teton.

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ABSTRACT

This thesis had three objectives: i) Determining distribution of FCR and common root rot (CRR) of wheat in Montana; ii) Determining population dynamics between *F. pseudograminearum* and *Bipolaris sorokiniana* at different wheat development stages, and iii) Development of an integrated disease management program for Fusarium crown rot (FCR) using biological and fungicide seed treatments, cultivar resistance, and induced systemic resistance (SAR). Surveys of 91 fields over two years using qPCR identified FCR in 57% and CRR in 93% of the fields surveyed. *Bipolaris sorokiniana*, *F. culmorum* and *F. pseudograminearum* were isolated from 15, 13 and 8% of tillers respectively. FCR distribution was highly clustered while CRR was uniformly distributed with soil type, elevation and growing degree days influencing distribution. Data from intensively sampled fields estimated yield losses caused by FCR and CRR at 3.2 to 34.9% with losses influenced by pathogen population. This study is the first time qPCR was used to survey the distribution of FCR and CRR and to study the interaction of the respective pathogens. The effect of *F. pseudograminearum* and *B. sorokiniana* inoculum applied singly or in combination at three rates showed high and low rates of *F. pseudograminearum* inoculum reduced *Bipolaris* populations, while *B. sorokiniana* inoculations did not affect *Fusarium* populations in stems. Populations of both pathogens increased from heading until harvest with *Fusarium* colonizing stems earlier than *Bipolaris*. Mixed inoculations increased incidence of infection and co-infection relative to that observed in production fields. Both fungi alone or combined reduced the seedling counts. Grain yield was inversely correlated with *Fusarium* populations. Difenconazole–mefenoxam seed treatment reduced FCR severity between 29.3-50% and fungal and bacterial seed treatments were ineffective. The cv. Volt was identified as partially resistant and had the highest levels of chitinase and β -1, 3-glucanase activity of cultivars evaluated. Induction of SAR by *Bacillus mycooides* isolate BmJ or acibenzolar-S-methyl significantly reduced the severity of FCR compared to water controls. Integration of cultivar resistance plus fungicide seed treatment or SAR induction provided equal control in greenhouse and irrigated trials. In a dryland field trial, integration of all management tools reduced FCR more than individual tools.

CHAPTER 1

GENERAL INTRODUCTION

Wheat crops are commonly affected by the dryland root rot complex (DLRRC) under semiarid conditions. This complex is associated with seedling blight, and rotting of root, crown and stems of the wheat plant. Fusarium crown rot (FCR) and common root rot (CRR) are most commonly associated with DLRRC and have worldwide importance. For Montana, the wheat area of production represents approximately 2.2 millions ha with 96.5% of that area under dryland and semiarid conditions. However, there is inadequate information regarding the distribution, incidence and prevalence of the pathogens involved in the DLRRC in Montana. Moreover, there is not adequate information about the dynamics of infection between the pathogens involved in FCR and CRR diseases during wheat development other than seedling stage. Because current disease management strategies are relative ineffective, new strategies of control are needed to address control of DLRRC. In the first part of this dissertation, based in reported information, were discussed the characteristics of the pathogen species involved in each one of the diseases of the DLRRC, the reported information about interaction between the pathogens involved in the DLRRC, and information regarding strategies of management for FCR and CRR diseases. The third chapter of this dissertation provides results of a regional survey conducted to assess the distribution and severity of FCR and CRR across commercial fields in “the Golden Triangle” of wheat production located in the Northern and Central areas of Montana. Two TaqMan[®] qPCR assays and traditional

methods of pathogen isolation and identification were used to determine FCR and CRR populations and inferred severity within fields. The resulting spatial distributions of FCR and CRR were assessed for their relationships to environmental variables as well as common production practices through a multifactorial analysis. The fourth part of this dissertation examined the pathogen community dynamics between aggressive pathogenic isolates of *Fusarium pseudograminearum* and *Bipolaris sorokiniana*, causal agent of FCR and CRR respectively, within co-inoculated wheat plants at seedling and three later development stages under field conditions using qPCR. These results were then compared with observations made on individual tillers collected from naturally infested commercial spring and winter wheat fields. The final chapter of this dissertation evaluated an integrated pest management program for FCR. In this work, different biological control agents were compared to difenoconazole-mefenoxam seed treatment for control of FCR. Moreover, the level of FCR resistance and the innate activity level of three PR-Proteins on five spring wheat cultivars were assessed by greenhouse experiments as well as the level of induction of SAR with *B. mycoides* BmJ and acibenzolar-*S*-methyl (ASM) was investigated. Finally, results of the integration of host plant resistance, fungicide seed treatment and BCA induced resistance are shown relative to FCR control in both greenhouse and field experiments.

CHAPTER 2

LITERATURE REVIEW

Introduction

Wheat (*Triticum aestivum* L.) is grown on 217 million hectares throughout the world, producing approximately 620 million tons of grain annually from 2004-2006. Wheat provides on average one-fifth of the total caloric input of the world's populations (<http://www.fao.org/>). In Montana, approximately 4.2 million tons of wheat were produced on 2,110,436 ha under dryland conditions and 0.34 million tons of wheat were produced on 76,583 ha under irrigated conditions during 2007 and 2008. During this period 1.26 million hectares were produced under a summer fallow system with the remainder under continuous cropping (<http://www.nass.usda.gov>). The semiarid conditions present in Montana plus the short and nonexistent rotations and widespread use of no-till or conservation tillage system practices favor survival of pathogens causing the dryland root rot complex. The extent of yield loss and distribution of pathogens in this crown and root rot complex in Montana are not well understood.

The dryland root rot complex (DLRRC) is known by a variety of names including dryland foot rot, Fusarium foot rot, crown rot, dryland root rot, and common root rot (Paulitz et al., 2002). The disease complex is dominated by different pathogens in different areas or even by different pathogens during successive growing season in individual fields (Paulitz et al., 2002). In this text, Fusarium crown rot (FCR) is going to be the generic name used to refer to the disease caused by different pathogen species of

the genera *Fusarium*. FCR is primarily caused by *Fusarium culmorum* (W. G. Sm.) Sacc., *F. pseudograminearum* (O'Donnell & T. Aoki; group I) (= *Gibberella coronicola*), and *F. graminearum* Schwabe (group II) (= *G. zaeae* (Schwein.) Petch) (Hogg et al., 2007; Paulitz et al., 2002; Cook, 2010). In other regions, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh., *F. equiseti* (Corda) Sacc., *Microdochium nivale* (Fr.) Samuels & Hallett (= *F. nivale* (Fr.) Sorauer), and several *Fusarium* spp. also have been included and reported in the crown rot disease complex in wheat but are considered less virulent and more restricted environmentally or geographically than the first three species (Cook, 2010). Another important and widespread pathogen causing dryland foot, crown, and root rot in cereal crops is *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. Ex Dastur, syn: *Helminthosporium sativum* P. K. & B.), which is the pathogen involved in common root rot (CRR) disease. Although many species are associated with the dryland root rot complex, *F. pseudograminearum*, *F. culmorum*, and *B. sorokiniana* are considered to be the most pathogenic and economically important pathogens (Burguess et al., 2001; Paulitz et al., 2002; Smiley et al., 2005). FCR and CRR cause similar symptoms, such as grain yield losses, stand reductions, and rotting of seeds, seedlings, roots, crowns, subcrowns, and lower stem tissues. Additionally, some species of *Fusarium* are also able to infect the heads or spikes, causing Fusarium head blight (Cook, 2010). *B. sorokiniana* causes a soilborne disease of seedlings similar to that of *Fusarium* spp., but causes browning and decay of the subcrown internode. However it is not associated with the “white-head” or premature death of wheat tillers caused by FCR pathogens (Paulitz et al., 2002).

Fusarium crown rot (FCR) and common root rot (CRR) diseases on wheat are perennial problems in cereal agro-ecosystems and cause significant losses in different regions worldwide (Burgess, et al., 2001; Paulitz, et al., 2002), such as: the Pacific Northwest (Cook 1968; Smiley and Patterson, 1996; Smiley et al., 2005), Canadian Prairies (Bailey et al., 1995a; Hall and Sutton, 1998; Fernandez and Jefferson, 2004; Fernandez et al., 2007a-b; Fernandez et al., 2009), Texas Panhandle (Specht and Rush, 1988), Southeastern Idaho (Strausbaugh, et al., 2004); upper coastal plain area of Mississippi (Gonzalez and Trevathan, 2000), eastern Australia (Backhouse, et al., 2004); South Australia (Fedel-Moen and Harris, 1987), Queensland Australia (Wildermuth, 1986; Wildermuth, et al., 1997), United Kingdom (Pettitt et al., 2003), Turkey (Tunali et al., 2008), north west Iran (Saremi et al., 2007); Brazil (Diehl, 1979), Argentina (De Souza, personal communication) and Chile (Madariaga, personal communication). Semiarid conditions enhance the expression of the diseases because this complex of pathogens reduces the amount of functional root and crown tissue which is critical under moisture-restricted conditions (Papendick and Cook, 1974; Cook, 1981; Bailey et al., 1989; Burgess et al., 2001; Paulitz et al., 2002). The measurable effects on yield are most apparent when the cereals are subjected to water stress later in the growing season and the temperatures are high (Cook 1981; Paulitz, et al., 2002). Moreover, the damage is especially acute when drought occurs during seedling and post-anthesis growth stages (Smiley et al., 2005). However, infections by *F. pseudograminearum* can occur in relatively moist soils (Burgess et al. 1981) and in irrigated systems (Paulitz et al., 2010).

The effect of these DLRRC infections under drought stress can cause yield loss exceeding 50% along with detrimental effects on grain quality such as light test weight (Tunali et al., 2008). In the Pacific Northwest (PNW), Paulitz et al. (2002) determined that 76% of the plants in winter wheat fields can be infested with FCR with estimated losses of 18% in heavily infected fields with a negative impact of US\$76/ha. In a recent survey in the PNW, yield losses associated with the dryland crown and root rot in commercial winter wheat fields were valued at US\$ 219/ha and US\$51/ha considering field losses of 35% and 9.5%, respectively (Smiley et al., 2005). In that survey, the highest damage estimated for field yield losses caused by *F. pseudograminearum* or *B. sorokiniana* were 13 and 16% (US\$48 and US\$58/ha), respectively, while plots inoculated with *F. pseudograminearum* showed grain yield losses of 61%, which were valued in US\$372/ha. In Australia, FCR has been determined as the second most economically important disease in wheat and that the present costs caused by this disease throughout Australia are AUS \$56M per year (Brennan and Murray 1998, cited by Wildermuth et al., 2001). Burges et al. (1981) reported that *F. pseudograminearum* caused up to 26% yield reduction in individual wheat fields in subtropical southern Queensland, Australia.

Recent information about losses associated with CRR in wheat crops is not available. However, Ledingham et al. (1973) had reported losses of 5.7% in a single year in the Canadian prairies, while Wildermuth et al. (1992) had reported losses ranging from 13.9 to 23.9% on a susceptible wheat variety and 6.8 to 13.6% in a partially resistant wheat variety in Australia. In Montana, statewide estimates of the effect of FCR and

CRR losses are not available. Grey estimated FCR-related spring wheat losses at 8% for the counties of Hill, Judith Basin, and Roosevelt in 2002, and those losses were valued at approximately \$4.45 million (Hogg et al., 2007).

These pathogens involved in the DLRRRC may occur singly but they typically co-exist in the same field and even within individual plants. Dominant species in the complex at a specific location can vary from year to year, indicating a high level of adaptation as members of this pathogen complex in response to changes in temperature, seasonal moisture distribution, amount of moisture, and perhaps other edaphic factors (Smiley et al., 2005). In this context it is important to understand the biology involved in the establishment and disease infection of FCR and CRR.

Biology of Common Root Rot of Wheat

The ascomycete fungus *Cochliobolus sativus* (anamorph = *B. sorokiniana*), in addition to common root rot, is involved in other wheat diseases such as leaf spots (spot blotch), seedling blight, head blight, and kernel blight (black point [Smudge]). Among them, seedling blight, CRR, and spot blotch are the most economically important and are highly dependent on environmental conditions (Stein, 2010). For example, in the PNW, among 855 infected tillers only 0.94% winter wheat crowns and subcrown internodes showed infection by CRR pathogen in 1993, while among the 555 infected wheat tillers 5.4% of them were infected with *B. sorokiniana* in 1994 (Smiley and Patterson, 1996). CRR is difficult to diagnose and often goes unnoticed, because above ground symptoms are nondescript (Stein, 2010). However, severe disease results in thinner crop stands, lower number of tillers with heads, reduced head sizes, fewer kernels and low kernel

weight (Tobias et al., 2009; Kumar et al., 2002). The diseased plants generally are randomly distributed in the field, and infected root and associated tissues have dark brown lesions, which expand during the growing season into the crown and lower culm tissues. The dark brown lesions on infected subcrown internodes (Figure 1) have been used to diagnose the disease and to assess severity (Tinline et al., 1975; Wildermuth, 1986; Kokko et al., 2000; Fernandez and Jefferson, 2004; Fernandez et al., 2007a; Fernandez et al., 2009). In general, *B. sorokiniana* has preferences for certain sites which result in a stratified distribution over the roots and crown, and Fedel-Moen and Harris (1987), after taking samples from infected fields, observed that this fungus was concentrated principally in subcrown internodes and culm bases of wheat plants (30 and 28% of incidence, respectively).

Description of Causal Organism. The sexual stage of *B. sorokiniana*, *C. sativus* ((Ito & Kuribayashi) Drechsler ex. Dastur) has been observed in the laboratory on natural media in the presence of opposite mating types. However under natural conditions, the perfect stage has been only found in Zambia, and it has not been reported to occur in any other areas in which the pathogen prevails (Kumar et al., 2002). *B. sorokiniana* is characterized by thick-walled, elliptical to oblong conidia (60–120 μm \times 12–20 μm) with three to ten cells; these conidia are developed terminally on conidiophores, which are single or clustered, simple, erect (6-8 μm \times 110-150 μm) with septations, while the mycelium is composed of hyphae interwoven as a loose cottony mass and appears white or light to dark gray depending on the isolates (Kumar et al., 2002; Stein, 2010).

Recent studies based on large numbers of strains collected on a global basis suggest that *B. sorokiniana* forms a continuum of isolates varying in virulence and aggressiveness, and this variation in virulence is noticeable in wheat and barley (Kumar et al., 2002). Differential reactions of progenies of crosses between isolates that differed in pathogenicity to different grass species indicated complex inheritance involving several genes for pathogenicity (Nelson 1960, 1961). Specificity on the race-cultivar level is indicated by the observation that field populations change to more aggressive races with long-term continuous wheat cultivation (El-Nashaar and Stack, 1989).

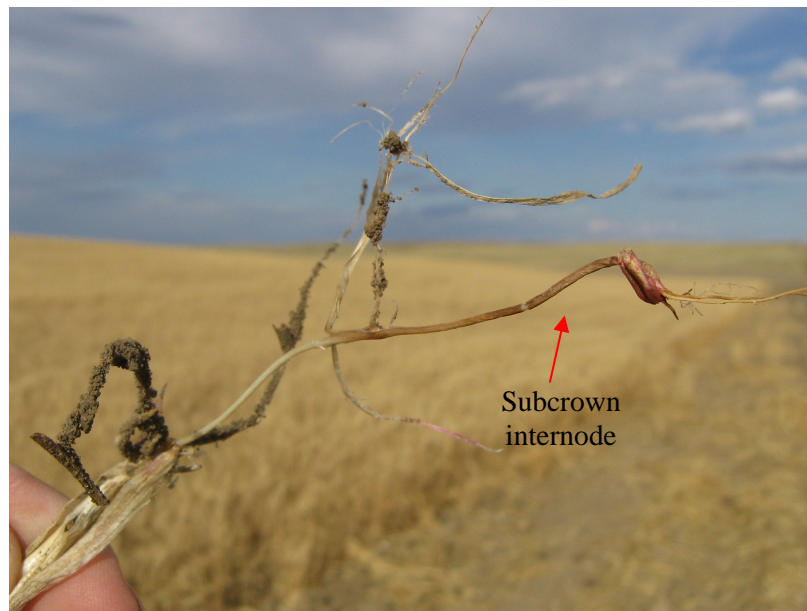


Figure 1. Subcrown internode lesion caused by *Bipolaris sorokiniana* on a wheat plant observed in an infected field in Blaine County in MT.

Disease Cycle of Common Root Rot. *B. sorokiniana* is able to infect both bread (*Triticum aestivum* L.) and durum (*T. durum* L.) wheat and also many related cereal and grass species (Stein, 2010). Barley is considered to be more susceptible to CRR than

wheat (Fedel-Moen and Harris, 1987). This fungus is a poor saprophyte, which requires undecomposed host tissue to survive during its disease cycle (Burgess and Griffin, 1968). *B. sorokiniana* can persist for month as mycelia in host debris or as conidia in the soil (Duczek, 1981). Studies of saprophytic activity of this pathogen showed that percentage of colonization of the wheat straw is proportional to the inoculum potential of the fungus in soil and that *B. sorokiniana* grows vigorously on the rhizoplane and also sporulates abundantly in the wheat rhizosphere (Nair, 1962). A higher number of viable *B. sorokiniana* conidia have been located in the soil profile between 10 to 15 cm deep in commercial fields in the Canadian Prairies (Duczek, 1981). Contaminated wheat seed may also contribute to the survival of the pathogen (Stein, 2010) and to the damping off, root rot and leaf spot phases of this disease (Aggarwal, et al., 2004). *B. sorokiniana* can also be dispersed aurally long distances during combine harvest and conidia have been detected at a rate of 739 spores/m³ downwind from the combined wheat fields and spores were able to germinate 24 h after collection in a mist chamber (Friesen et al., 2001). However, there is no information about the effect of aerial dispersion during harvest on the incidence of CRR.

Under field conditions, *B. sorokiniana* infections occur primary on coleoptiles, subcrown internodes, and primary and secondary roots. Penetration by *B. sorokiniana* of wheat roots is dependent on formation of appressoria-like bodies, which superficially colonize the seedling roots during the first 8 days of infection (Weste, 1975). The natural sesquiterpenoid phytotoxin helminthosporol and its precursor prehelminthosporol isolated from *B. sorokiniana* have been associated with affecting membrane permeability

of mitochondria, chloroplasts, and microsomes of wheat cells by inhibition of proton pumping H^+ -ATPase and 1,3- β -glucan synthase activity; enzymes involved in defense and stress response mechanisms of plant cell (Olbe, et al., 1995; Briquet et al., 1998), and probably these phytotoxins play some role during pathogenesis. Stresses, such as drought, high temperature, freezing, flooding, or mechanical damage, have been shown to predispose the wheat plants to infection by CRR (Stein, 2010).

Most barley and wheat plants are infected by CRR fungi before the inflorescence emerges (Windels and Wiersma, 1992). During the infection progress above the soil surface, secondary conidia are developed and may be dispersed by wind or splashing rain to infect leaves and culms (Stein, 2010). After harvest, conidia and mycelium persist in the soil and undecomposed stubble for 6 or 24 months. Reported soil populations of *B. sorokiniana* in infected fields have varied from 50 to 190 propagules per gram (Wildermuth, 1986) and through the soil profile to a depth of 10 to 25 cm (Duczek, 1981). Also, higher incidence and severity of CRR have been observed in fields where crop stubble has been retained as opposed to where they were removed (Wildermuth, et al., 1997).

Biology of Fusarium Crown Rot of Wheat

Different fungal species of the genus *Fusarium* are associated with this disease complex. Symptoms include seedling blight, root rot, crowns rot, and culm rot, whiteheads, and shriveled grain in wheat and other cereals. *F. culmorum*, *F. pseudograminearum*, and *F. graminearum* are epidemiologically the most important species involved in this complex (Paulitz et al., 2002; Cook, 2010). *F. culmorum* is

associated with cooler semiarid wheat growing regions and *F. pseudograminearum* and *F. graminearum* are dominant in slightly warmer regions (Cook, 1981). Among the other fusaria species, *F. avenaceum*, *F. acuminatum*, *F. oxysporum*, and *F. equiseti* have been considered among the most common widespread fungi isolated from underground tissue of wheat in Canada, but with a low number of tillers infected (Fernandez and Jefferson, 2004). However, *F. avenaceum*, *F. acuminatum*, *F. equiseti*, *F. oxysporum*, and *M. nivale* are considered to be species of lesser importance in the FCR complex (Cook, 2010; Paulitz et al, 2002; Smiley and Patterson, 1996), because they are considered secondary colonizers rather than primary pathogens in semi-arid regions (Burgess et al., 2001). *F. avenaceum*, *F. acuminatum* and *M. nivale* are more pathogenic in areas with wet and cold weathers (Pettitt et al., 2003; Hall and Sutton, 1998) and their infection levels are very dependent of weather conditions (Hall and Sutton, 1998; Pettit and Perry, 2001). However, almost all species of FCR can cause *Fusarium* head blight (FHB), *F. graminearum* is most common cause of head blight and seedling blight of wheat in the USA (Bai and Shaner, 2004; Cook, 2010), while *F. culmorum* is more common in Europe (Wagacha and Muthomi, 2007). FHB infections occur under wet or humid conditions at anthesis or shortly there after (Burrows et al., 2008), while FCR is favored by water stress late in the growth season (Paulitz et al., 2002). All members of the FCR complex produce, under dryland conditions, a chocolate brown discoloration in the first to the third internodes up in the stem, which can be observed when the leaf sheaths are stripped back in the base of the tiller (Fig. 2). When those culm internodes are opened a pink

mycelium is observed inside and its presence can be considered diagnostic FCR (Cook, 1981; Cook, 2010).

Description of Causal Organisms. *Fusarium* species described above are most commonly associated with FCR disease and they are considered “unspecialized” pathogens because they can attack any plant tissue if conditions at the tissue surface are favorable for infection (Paulitz et al., 2002). In general, the anamorph of the different *Fusarium* species may or may not produce macroconidia, microconidia, chlamydospores, and conidia borne on mono or polyphyalides. The typical color of the mycelium on PDA plus the morphological structures mentioned above can be used to identify individual species throughout different synoptic keys (Nelson et al., 1983). Identification to the specie level requires both practice and experience. Recently, specific primers have been developed to identify several of the *Fusarium* species (Scott et al., 2003; Aoki and O’Donnell, 1999; Nicholson et al., 1998), and they can be useful to confirm the pathogen identified with synoptic keys.

The teleomorph *Gibberella* spp. of the complex develop perithecia in clusters on the surface of plant tissues. Perithecia are globose, 125-265 μm in diameter, rough-walled, and varying from bluff to dark blue in color (Cook, 2010). Perithecia produce clavate asci of 4-10 μm wide x 50-80 μm length with six to eight spores. Ascospore are hyaline, ellipsoidal, 3.3-6.5 x 13-17 μm , and one- to three-septate (Cook, 2010). A contrasting biology of the most important member of FCR complex is described in Table 1.

Table 1. Contrasting biology amongst the most important members of the FCR complex¹.

<i>F. graminearum</i> (teleomorph = <i>Giberella zae</i>)	<i>F. culmorum</i> (teleomorph unknown)	<i>F. pseudograminearum</i> (teleomorph = <i>Giberella coronicola</i>)
<ul style="list-style-type: none"> - Lighter macroconidia and less durable chlamydospore. - Produce sexual stage (perithecia and ascospores) that permits longer distance spread. - DON mycotoxin producer 	<ul style="list-style-type: none"> - Heavier macroconidia and more durable chlamydospore. - Produce asexual conidia (macroconidia) and it can have the least efficient dispersal, but a more stable population established. - DON mycotoxin producer 	<ul style="list-style-type: none"> -Lighter macroconidia and less durable chlamydospore. Lighter than <i>F. graminearum</i>. - Produce asexual conidia (macroconidia) and lack of durable survival structure. - DON mycotoxin producer

¹ Dyer, personal communication, 2008

Pathogenic variation among isolates is recognized for species associated with the crown rot complex (Smiley et al., 2005). Comparison of pathogenicity of FCR complex pathogens on hard red spring and durum wheat in Montana showed that *F. culmorum* caused the greatest seedling blight while *F. pseudograminearum* and *F. graminearum* caused greater crown rot (Dyer, et al., 2009).

Disease Cycle of Fusarium Crown Rot. Both seed-borne and soil-borne inoculum are important to the epidemiology of FCR (Cook, 1981). Chlamydospores, macroconidia, and mycelium are common survival structures in the soil and in crop residues (Paulitz, 2006; Cook, 1981). *F. culmorum* survives adverse conditions most commonly as viable chlamydospores, while *F. pseudograminearum* and *F. avenaceum* survive most commonly as mycelium inside non-decayed plant residues. This is the

major reason that adoption of conservation tillage practices has been shown to increase FCR caused by *F. pseudograminearum* (Sitton and Cook 1981; Paulitz et al., 2002).



Figure 2. Lower stem internodes with the typical chocolate brown discoloration considered diagnostic of FCR in wheat plants. Plant from an infected field in Choteau County in MT.

Crown infection initially occurs 2-3 cm below the soil surface either through openings around emerging secondary roots or by infection of the newly emerging crown roots (Cook, 1981; Wiese, 1991). Coleoptile infection also occurs through stomata and between epidermal cells (Malalasekera et al., 1973). Infection of the seedling occurs through epidermal cell layers of the coleoptile and then expands into the parenchyma (Pisi and Innocenti, 2001). During pathogenesis FCR pathogens produce an array of enzymes to overcome plant defense responses. Induction of active laccases (Kwon and Anderson, 2002) and enhancement catalase activity (Ponts et al., 2009) by FCR

pathogens has been observed and these enzymes have been associated with reducing or inactivating active oxygen species (AOS) produced by the plant in response to necrotroph infection (Mayer et al., 2001). Moreover, the trichothecene mycotoxin deoxynivalenol (DON) is produced during infection of *F. graminearum* and *F. pseudograminearum* in the wheat stem base (Mudge et al., 2006), and this toxin could have an important role in colonization of the wheat stem considering that DON is an inhibitor of protein synthesis. Thus DON could suppress the production of host defense enzymes and other compounds as has been suggested by Mudge and coworkers (2006). DON also elicits hydrogen peroxide production, programmed cell death and defense responses in wheat (Desmond, 2008b). Additionally, defense responses of the plant during *Fusarium* colonization could be depleted by ammonization and pH modulation of apoplastic fluids by *F. culmorum* infection. Ammonization and pH modulation have shown to modulate the activity of cell-wall-degrading enzymes polygalacturonase and pectin lyase (Aleandri et al., 2007). When FCR pathogens are inside of the plant, colonization of the pith cavity is not restricted by the barrier of lumen at each node (Clement and Parry, 1998) and the lumen appears to provide a pathway for vertical growth while the surrounding parenchyma cells provide a potential nutrient source and humid environment (Mudge, et al., 2006).

Plants infected by FCR rarely show obvious symptoms until after heading (Cook, 1981). However, if wheat plants are under drought conditions, plant defenses weaken and the pathogen infection expands in the vascular tissue and disrupts water movement and prevents recovery of infected plants from the water stress (Cook and Christen, 1976; Hare and Parry, 1996).

Interaction between Fusarium Crown Rot and Common Root Rot

Fernandez and Jefferson (2004) in a survey conducted in the Canadian Prairies showed a negative correlation between both pathogen populations where both FCR and CRR are present. Studies of this interaction dates to the 1930 and 1940 (Hynes, 1938 cited by Ledingham 1942). These studies indicated antagonism between *Fusarium* spp. and *Bipolaris* isolates. Ledingham's work (1942) determined this antagonism effect on seedling emergence of wheat plants inoculated simultaneously with *F. culmorum* and *B. sorokiniana*, whereas *in vitro* tests of conidia germination in film of clear agar on a microscope slide showed inhibition of *B. sorokiniana* germination by *F. culmorum*. Tinline (1977) reported that prepossession of the internode by *B. sorokiniana* infection does not prevent subsequent invasion by *F. culmorum* and *F. acuminatum*, but that prepossession by fusaria pathogens greatly reduces subsequent infection by *B. sorokiniana* in studies of single or combined inoculation of wheat. However, *B. sorokiniana* was a successful secondary invader of internodes infected with *F. sulphureum* (Tinline, 1977). In barley, Scardaci and Webster (1981) determined antagonism between *F. graminearum* and *B. sorokiniana* when barley plants were inoculated simultaneously, resulting in lower levels of seedling blight and root rot. Scardaci and Webster (1981) also determined that when the pathogens were inoculated in sequence, one 21 days before the other, the pathogen that was inoculated first was re-isolated most frequently, indicating the importance of prior colonization and possession of substrate. Fernandez et al. (1985) reported that *F. acuminatum* significantly increased *B. sorokiniana* infection when wheat seedling plants were inoculated with both fungi.

All this work was carried out using autoclaved soil with the objective to avoid masking the interaction of the cereal root rot pathogens by other microflora, and information about the real situation confronted by FCR and CRR pathogens in a cereal field is unknown. Ledingham (1942) reported results of two field interaction experiments, but he only assessed effect on wheat emergence and yield and determined antagonism between *F. culmorum* and *B. sorokiniana*, but he did not determine effect on populations of those fungi in crown or stem tissues during the period from heading to maturity.

Management of Fusarium Crown Rot and Common Root Rot

Despite the antagonistic interactions reported between FCR and CRR pathogens, both FCR and CRR pathogens are commonly isolated from the same fields in the Canadian Prairies (Hall and Sutton, 1998; Fernandez and Jefferson, 2004; Fernandez et al., 2007b; Fernandez et al., 2009) and the PNW (Cook 1968; Smiley and Patterson, 1996; Smiley et al., 2005). The management strategies for both diseases are similar. Cook (2010), Stein (2010), Kumar et al. (2002), and Wiese (1991) have recommended control practices, such as: use of clean and chemically disinfected seed, management of date of seeding, proper fertilization, use of tillage, crop rotations avoiding other cereals, use of cultivars with resistance or tolerance, and/or with resistance to water stress. However, no single management strategy has been shown eliminate root and crown rots, but combined practices mentioned have proven useful but do not provide high levels of control.

Chemical Disinfection of Seed. Fungicide seed treatments are recommended for the management of FCR and CRR combined with healthy seeds, because this reduces seedling blight, as has been widely observed (Stein 2010; Cook, 2010). For example emergence of winter wheat in fields tended to be superior for seed treated with difenoconazole (Dividend[®]), alone or mixed with Apron (Smiley et al., 1995). Extension plant pathologists at Montana State University recommend using seed treatment fungicides such as Vitavax Extra (carboxin + imazalil + thiabendazole), Dividend XL or RT (difenoconazole + mefenoxam), Raxil XT or MD (tebuconazole + metalaxyl), Raxil MD Extra (tebuconazole + metalaxyl + imazalil), Baytan (tridimenol), and RR, Flo-pro, NuZone (imazalil) to promote healthy seedling growth (Dyer et al., 2007). However, chemical control is limited to early stages of the wheat growth, because the fungicides do not maintain their efficiency beyond 2-4 weeks (Balmas, et al., 2006), and later infections can be observed, especially in winter wheat. However, soils infected by CRR generally have shown that use of seed treatment results in increased yield in both winter wheat (Stein, 2010) and spring wheat (Stack, 1992).

Management of Date of Planting. Use of cultivars with high yield potential and following the recommended date of planting in a geographic area can be simple actions that reduce the incidence of both diseases. In general, early planting promotes disease in winter wheat (Cook, 2010). In the PNW, assessment of date of planting showed that FCR were more prevalent in early-planted winter wheat and was generally reduced or absent in planting made later in fall (Smiley et al., 2009). Late-autumn seeding of winter wheat also reduces seedling exposure to warm soil and limits the amount of vegetative

growth that can lead to premature reduction of soil water and water stress on the plants that promotes damage by both pathogens (Cook, 2010) Despite the effectiveness of this practice, management of date of planting depends on the amount of acres managed by each farmer and weather conditions associated with each area of production.

Crop rotation with Non Cereals. Crop rotation is the single most effective method for control of both diseases, but it is most effective in reducing seedling and root rot symptoms caused by *B. sorokiniana* (Stein, 2010). In Canada, where CRR prevails, rotation, including two or more years of flax (*Linum usitatissimum*) reduced the amount of viable inoculum of *B. sorokiniana* in the soil (Conner et al., 1996). Crop rotation also allows the growers to limit alternative hosts and to control the more ephemeral *Fusarium* species (Wiese, 1991) and significantly reduces the pathogenic fitness level of *F. graminearum* and *F. pseudograminearum* on wheat (Akinsanmi et al., 2007). However, about half of the inoculum of *Fusarium* spp. and *B. sorokiniana* present after harvest is functional a year later, and about 10% can survive for nearly two years (Wiese, 1991). Rotation with a broadleaf crop such as peas or soybean has proven beneficial to limit damage from both FHB and FCR caused by *F. graminearum* with a break of at least two years without cereal being most effective in reducing damage from FCR caused by *F. pseudograminearum* (Burgess et al., 2001; Cook, 2010). The longevity of chlamydospore inoculum of *F. culmorum* makes use of rotation more challenging, as evidenced by experiments that showed a two year break did not provide effective control of this species (Cook, 1981; Cook 2010). Fernandez et al. (2007b) reported that summer-fallow was associated with increased infection by *B. sorokiniana*, whereas the relative

levels of *Fusarium* spp., except for *F. acuminatum* and *F. equiseti*, appeared to consistently decrease when there was summer-fallow in the previous year, or in one of the previous 2 years. Both *B. sorokiniana* and *Fusarium* species have large host ranges, which includes numerous grass species, therefore crop options for the rotations are limited. Additionally, crop rotation options are limited in some areas because low rainfall limits commercial economic alternatives (Strausbaugh et al. 2005).

Cultivar Resistance or Tolerance. Use of resistant cultivars would be the most effective and efficient measure to reduce the impact of both diseases, however resistance in commercial cultivars to either or both diseases is only partial (Tobias et al, 2009; Stein, 2010; Cook, 2010) and disease outbreaks are common and can be severe when climatic conditions are favorable for the pathogens on these partially resistant cultivars (Burgess et al., 2001; Strausbaugh et al. 2005). Resistance to CRR has been developed and evaluated recently in hard red spring wheat cultivars in North Dakota (Tobias et al., 2009). Tobias et al. (2009) reported variation in disease severity in the subcrown internode ranging from 20 to 60%, and resistant responses were strongly affected by environmental conditions. Variation among cultivars has also been reported in earlier studies with CRR on wheat (Harding, 1972; Bailey, et al., 1988). Commercial genotypes ND 722, AC Candillac, HJ 98, Argent and Scholar have shown better or similar level of disease severity than Amidon, a partially resistant cultivar to CRR used as control (Tobias et al., 2009). Also, resistance to CRR has also been obtained after crossing *Aegilops ovata* with *Triticum aestivum* using Chinese Spring *ph1b* genetic stock and the cultivar 'Leader'. This germplasm represents a different source of genes for resistance to

CRR than generally available in spring wheat cultivars (Bailey et al., 1995b). Also, resistance to CRR was transferred into wheat via crossing with Agrotana, a resistant wheat relative *Thinopyrum ponticum* (Podp.) partial amphiploid line. Evaluation of CRR reactions showed that selected advanced lines with blue kernel color derived from a wheat × Agrotana cross expressed more resistance than the susceptible *T. aestivum* ‘Chinese Spring’ parent and other susceptible wheat check cultivars (Li et al., 2004).

Resistance to *Fusarium* pathogens is associated with FHB resistance (Bai and Shaner, 2004) or directly to crown root rot disease (Smiley et al., 2003). Two most important types of resistance to FHB in wheat are described: resistance to initial infection (referred as type I) and resistance to spread of FHB symptoms within a spike (referred to as type II). Type II resistance has been found in a number of wheat cultivars and appears to be more stable and less affected by nongenetic factors than type I resistance (Bai and Shaner, 2004). However, while high resistance to FHB has been described (Bai and Shaner, 2004), work done by Xie et al. (2006) and Li, et al. (2010) suggested that FHB resistant germplasm did not offer any resistance to FCR. The idea of resistance inversion has been proposed for the observed phenomenon of differential resistance to FCR and FHB in wheat, where one plant genotype displays a resistance phenotype at one development stage but a susceptible reaction to the same pathogen at another stage (Li, et al., 2010). However, seedling and adult-plant tolerance (partial resistance) to some members of the FCR complex such as *F. pseudograminearum* have been reported (Collard et al., 2005; Bovill et al., 2006; Li, et al., 2010; Cook 2010), and is associated with reduced damage to stem base tissue and to increased wheat yield (Wildermuth et al.,

2001). Seedling resistance, but not adult-plant resistance, has been associated with the phenotypic expression of a genetically determined depth at which crown tissue is formed for each wheat cultivar or breeding line (Wildermuth et al., 2001). Collar et al. (2005), working with molecular markers associated with partial seedling resistance to this disease, and using a population of doubled haploid lines constructed from crosses between '2-49' (partially resistant) and 'Janz' (susceptible) parents demonstrated that the trait is quantitatively inherited and that none of the QTLs identified with resistance to FCR were located in the same region as resistance QTLs identified in other populations segregating for FHB, caused by *F. graminearum*. Seedling resistance has been linked by QTLs located on chromosomes 2B, 2D and 5D in progenies obtained from the cross between 'W21MMT70' (partial resistance) x 'Mendos' (susceptible). The 2D and 5D QTL were inherited from the line 'W21MMT70', whereas the 2B QTL was inherited from 'Mendos' (Bovill et al., 2006). These loci are different from those associated with crown rot resistance in other wheat populations that were examined by Collar et al. (2005), who determined that resistant QTL's were located on chromosomes 1D and 1A. Bovill et al. (2006) suggested that those loci may represent an opportunity for pyramiding QTL to provide more durable resistance to FCR.

According Smiley et al. (2003), genetic tolerance to FCR is important during years when the disease pressure is moderate and it is ineffective when disease pressure is high. In the PNW, Smiley and Yan (2009), working with winter wheat cultivars, have screened for tolerance to FCR in naturally infested and inoculated soils, and the result for phenotypic tolerance response in individual cultivars has been highly variable over years

and test sites. However, these authors in a cooperative effort between Australian and USA researchers have identified significant differences among spring wheat entries in the PNW (Smiley and Yan, 2009).

The losses caused by FCR and CRR are significant, and active wheat screening and breeding programs for dryland root rot resistance and tolerance have been actively initiated in different locations worldwide with the support of CIMMYT (Nicol et al., 2004, Smiley et al., 2003, Bruckner, personal communication).

Effect of Crop Nutrition. Soil fertility must be adequate to support vegetative growth but it needs to be based on water supplies to avoid FCR and CRR. Excessive nitrogen under low-rainfall conditions promotes vegetative growth and especially tiller formation beyond what can be sustained with the water stored in the soil. These conditions favor water stress on the plants during heading and grain fill, which predisposes the crop to severe foot and crown rot caused by FCR and CRR (Papendick and Cook, 1974; Cook 1980; Burgess et al., 2001; Cook, 2010; Stein, 2010). Cook (1980) recommended the rate of nitrogen application needs to be based on a soil test for residual nitrogen. Nitrogen fertility should not exceed 60-75 kg/ha in areas with <240 mm average annual precipitation. Moreover, zinc-deficiency has been linked with higher levels of infection caused by *F. pseudograminearum* on wheat in glasshouse trials (Sparrow and Graham, 1988). Also, wheat genotypes with more efficient capacity to extract zinc from soils poor with zinc availability have been associated with reduction of FCR severity as well as increasing plant vigor (Grewal et al., 1996).

Effect of Tillage Practices and Stubble Management. Preceding cropping sequences and agronomic practices can affect the level of inoculum of both pathogens, its distribution in the field, and define the incidence of infected plants by FCR and CRR (Burgess et al., 2001). Both diseases have shown higher incidence and severity where stubble is retained than where it is removed (Wildermuth et al., 1997; Paulitz et al., 2002; Cook 2010). Management of infected stubble through postharvest burning, fire plus harrowing in fall season, stubble incorporation by disc cultivators that invert the soil and surface residue, or stubble retirement from the field will greatly reduce the sources of inoculum for FCR and CRR diseases (Burgess et al., 2001). *B. sorokiniana* is favored by conventional-tillage in wheat (Diehl et al. 1982; Salas and Stack, 1988; Mathieson, et al. 1990; Tinline and Spurr 1991; Fernandez et al., 2007b) and in barley production (Fernandez, et al., 2007a). This increase of *B. sorokiniana* by conventional-tillage could be associated with greater spread of conidia in the soil profile because disc plowing promotes the fragmentation of stubble. Soilborne conidia are the most important source of infection (Duczek et al., 1985) and they are able to survive for months in the soil (Duczek, 1981). On the other hand, plowing that promotes the fragmentation and decomposition of stubble reduces infection by FCR pathogens, such as *F. pseudograminearum* or *F. graminearum* (Burgess et al., 2001; Steinkellner and Langer, 2004), with lesser effects on persistent chlamydospores of *F. culmorum* (Windels and Wiersma, 1992). In dryland agriculture the use of summer fallow to conserve soil moisture and release organic nitrogen has caused widespread adoption of moisture-conserving minimum tillage systems (Padbury et al., 2002). Use of no-till and

conservation tillage system practices in a wheat-fallow production system has been associated with higher levels of *Fusarium* infections (Smiley et al., 1996; Bailey et al., 2001) and a population change from *F. culmorum* to *F. pseudograminearum* (Sitton and Cook 1981; Paulitz et al., 2002). It has also been suggested that *F. pseudograminearum* is more aggressive than *F. culmorum*, which may explain the increase in FCR severity under conservation tillage systems (Paulitz et al., 2002; Smiley et al. 2005a). *F. pseudograminearum* is a strictly residue born pathogen that depends on infesting late season tillers for survival between cropping periods (Sitton and Cook 1981; Pereyra and Dill-Macky, 2004). This fact could increase the selection pressure on the pathogen to capture residues in order to survive a prolonged non-cropping period (Dyer et al., 2009). According to Bailey (1996), prior to the adoption of conservation tillage, the impact of these factors on FCR was largely unknown and could not have been properly considered in making predictions on the impact of conservation tillage practices on this disease.

Effect of Other Cropping Practices. Cook (1980) has recommended increasing the distance between rows to reduce crown and root rot infection in semiarid areas. The wide-row spacing results in a reduced seeding density and hence a slower rate of soil water use per unit of area of the field (Papendick and Cook, 1974; Cook, 1980). Cultural practices to reduce moisture loss from the soil would logically be associated with reduction of crown and root rot diseases (Papendick and Cook, 1974). Improved infiltration and reduced water runoff during precipitation or snow melt by working the field with chisel plow is thought to reduce crown and root rot diseases by making more water available (Cook, 1981). Controlling weeds in summer fallow land and during crop

development should also reduce the infection by these diseases, because weeds deplete soil moisture that predisposes plant roots to infection in fall. However, studies conducted in the Northern Great Plain of Canada have determined that previous glyphosate applications in summer-fallow were associated with lower *B. sorokiniana* and higher *Fusarium* spp. levels in barley and wheat grown under minimum-till management (Fernandez et al, 2005; Fernandez et al 2007a-b, Fernandez et al, 2008; Fernandez et al, 2009). The effect of glyphosate application also has been associated with higher levels of *Fusarium* head blight on spring wheat in the Canadian prairies (Fernandez et al, 2005; Fernandez et al 2007a-b; Fernandez et al, 2008; Fernandez et al, 2009).

New Strategies of Control for Crown and Root Rot Diseases

The management cropping practices discussed previously make controlling the crown and root rot complex challenging and emphasize the need for other control alternatives. Wildermuth et al. (1997) have suggested that some form of biological suppression may be operating to limit maximum incidence of crown and root rot infections in Australia. Biological control agents (BCAs) have shown promise for the control of CRR disease (Shivanna et al., 1996; Knudsen et al., 1995; Kumar et al., 2002; Dal Bello et al., 2003) and FCR disease (Huang and Wong, 1998; Dal Bello et al., 2002; Johansson et al., 2003; Luongo et al., 2005; Khan et al., 2006; Singh et al., 2009). Plant growth-promoting fungal isolates of *Phoma* spp. from zoysiagrass (*Zoysia* sp.) rhizosphere have been reported to suppress *B. sorokiniana* due to competitive root colonization (Shivanna et al., 1996). Successful antagonists against seed-borne *B. sorokiniana* were *Chaetomium* sp., *Idriella bolleyi*, and *Gliocladium roseum* (Knudsen et

al., 1995). *Piriformospora indica*, a plant growth-promoting root endophytic basidiomycete, has resulted in a considerable increase in growth and yield relative to non-infested controls for CRR (Kumar et al., 2002). Under greenhouse conditions, *Bacillus subtilis* 3 and *Gliocladium roseum* reduced the level of infection of seedling blight caused by *B. sorokiniana* on two wheat cultivars in Argentina, but under field conditions, biocontrol of the disease was not achieved (Del Bello et al., 2003).

Two approaches have been considered in the control of FCR by using BCA's: 1) Manipulation of microbial antagonists to increase the rate of mortality of *Fusarium* spp. in cereal residues (Wong et al., 2002; Luongo et al., 2005; Singh et al., 2009), and 2) seed treatment with BCAs (Dal Bello et al., 2002; Khan et al., 2006). Assessment of saprophytic fungi obtained from cereal tissues or necrotic tissues of other crops have shown that isolates of *Clonostachys rosea* consistently suppressed sporulation of *F. culmorum* and *F. graminearum* on wheat straw (Luongo et al., 2005), while *Trichoderma harzianum*, *F. equiseti*, and *F. nygamai* showed strong antagonism in dual culture interaction with *F. pseudogramineraum* (Singh et al., 2009). These have been validated in bio-assays conducted under controlled conditions but results have been variable for different *Fusarium* spp (Luongo et al., 2005). In addition, BCA performance was strongly affected by temperature and water potential (Singh et al., 2009). Seed bio-based treatment has proven a promising method for enhancing biological control of plant diseases. Huang and Wong (1998) working with a rifampicin-resistant isolate of *Burkholderia (Pseudomonas) cepacia* (A3R) significantly reduced crown rot symptoms caused by *F. pseudograminearum* on wheat in glasshouse and field experiments and

increased grain yield significantly in one of two field experiments. Johansson et al. (2003) tested the action of 164 bacterial isolates against both *F. culmorum* and *M. nivale* as causal agents of seedling blight of wheat in field experiments during five consecutive growing seasons. Their research determined that three fluorescent pseudomonads and *Pantoea* sp. isolate MF 626 were able to increase the number of established wheat plants under field conditions, when wheat seeds were coinoculated with *F. culmorum*. Del Bello et al. (2002), assessed fifty-two bacterial strains and six *Trichoderma* spp. isolated from the wheat rhizosphere for biocontrol of seedling blight of wheat caused by *F. graminearum*. Among isolates tested *Stenotrophomonas maltophilia*, three strains of *Bacillus cereus* and one isolate of *T. harzianum* increased the plant stand, height and dry weight in different wheat cultivars, but they did not cause a significant decrease in the percentage of diseased plants. Khan et al. (2006) working with pseudomonads and chitosan against *F. culmorum* reported the induction of a wheat class III plant peroxidase gene, which suggested that part of the biocontrol activity of these bacteria and chitosan might be due to the induction of systemic acquired resistance (SAR) in host plants.

One promising strategy to control diseases is induced resistance. In the broadest sense, induced resistance means the control of parasite or pest by activation of genetically programmed plant defense pathways before infection or infestation (Kumar et al., 2002; Kogel and Langen, 2005). Resistance and defense gene expression can be induced locally at the site of pathogen infection or in non-inoculated tissues in a process known as systemic acquired resistance (SAR) (Agrios, 2005). Induced resistance to microbial pathogens, resembling the SAR response, can be obtained by applying defense-signaling

compounds that activate the defense signaling pathways (Kogel and Langen, 2005; Mayer et al., 2001; Kessmann et al., 1994). In cereals, pathways of SAR induction are regulated by salicylic acid (SA) and jasmonic acid (JA) and their cellular targets (Kogel and Langen, 2005). Downstream signaling components, such as nonexpressor of pathogenesis-related gene 1 (NPR1) are deployed during SA-dependent defense and orchestrate cross-talk between SA and JA pathways (Spoel et al., 2003). The antagonistic SA and JA pathways elicit the accumulation of distinct subsets of defense-related proteins. SA-dependent pathways are associated with Pathogenesis-Related proteins (PR-proteins) such as peroxidase, chitinase, β -glucanase and *PR-1* (Durrant and Dong, 2004). The JA pathway is associated with production of thionins, defensins and proteinase inhibitors (Xu et al., 2001). Almost all classes of PR-proteins induced in plants in response to attack by microbial or insect pests have been identified in wheat, and some PR-like proteins are regulated developmentally, whereas other members of the same group are inducible by some stress (Muthukrishnan et al., 2001). PR-proteins are defined as proteins coded by the host plant but induced only in pathological or related situations (Antoniw et al., 1980), while PR-like proteins are related to PR-proteins accumulated in normal (uninfected) plants in certain tissues or developmental stages (Van Loon and Van Strien, 1999).

Few studies have been conducted to explore response to infection with crown and root rot pathogens in wheat. Liljeroth et al. (2001) studied the accumulation of PR-proteins in the roots of barley and wheat in response to root infection with *B. sorokiniana*. In this work, several PR proteins, particularly *PR-1* and thaumatin-like

protein (*PR-5*) accumulated in response to infection. Root-inoculated plants induced isoforms of chitinase (*PR-3*) and thaumatin-like proteins differently expressed in leaves and roots, which indicated tissue-specific expression and systemic induction. Liljeroth et al. (2001) also observed that tissue differences occur in roots, with chitinase proteins constitutively being expressed in the root cortex but not in the stele. Also, these authors observed that infection of roots close to the root tip caused a stronger accumulation of PR proteins compared with infection of older segments of seminal roots. On the other hand, Desmond et al. (2006) studying the molecular host-interaction between *F. pseudograminearum* and wheat showed the induction and expression of eight defense genes in a susceptible and a partially resistant cultivar of wheat where plants were infected by the pathogen. Additionally, they were able to show that the induction of those genes by using methyl jasmonate and benzo (1,2,3)thiadiazole-7-carbothionic acid-S-methyl ester (BTH, Bion) treatments delayed disease development caused by infection of *F. pseudograminearum* on the cultivars. In two gene expression research projects, induction of chitinase gene expression on wheat seedling had been observed in response against *F. pseudograminearum* (Desmond, et al. 2006) and *F. asiaticum* (Li, et al., 2009) infection on wheat seedling plants. According to Muthukrishnan et al. (2001), the hydrolytic enzyme chitinase and β -1, 3-glucanase activity in plants exposed to fungal pathogens have two consequences: one is the release of cell wall fragments from the pathogen containing oligosaccharides, which have been shown to elicit defense responses (Woo et al., 2006), and second, is the interference of assembly of the fungal cell wall when this is being assembled by elongation of preexisting primers of oligo-N-

acetylglucosamines and oligo- β -1,3-glucans by chitin synthase and β -1,3- glucan synthase, respectively (Mauch et al., 1988). In addition, high concentrations of lytic enzymes may result in dissolution of the fungal cell wall when its substrates are exposed (instead of being buried under layers of other polymers) (Woo et al., 2006).

Over-expression of the chitinase gene and chitin-binding (*PR-4*) gene in wheat seedlings has been associated with seedling resistance to *F. asiaticum* infection, which also has had a correlation with high expression of a plant cytochrome P450 gene *CYP709C1*, which is involved in detoxification of exogenous compounds (Li, et al., 2010). Pathogenesis-related proteins of the *PR-4* family have been shown to have distinct antifungal activities in coleoptiles and roots against *F. culmorum* (Caruso et al. 2001; Bertini et al., 1994). This *PR 4* has chitin-binding activity and has been demonstrated to possess an RNase activity that may be part of a mechanism for inhibiting invading pathogens (Caruso et al., 2001). Moreover, Desmond et al. (2006) also showed that in response to FCR, thaumatin-like proteins (*PR-5*) were highly expressed after inoculation with *F. pseudograminearum*.

By using a GeneChip[®] Wheat array, Desmond et al. (2008a) showed that after one day of inoculation with *F. pseudograminearum*, 1248 unique genes were induced in response to the inoculation in the stem base of 2-week-old wheat seedlings when compared to mock-controls. Among these genes, the largest classes of induced genes were associated to anti-microbial proteins, such as chitinase, β -1, 3-glucanase, *PR-1*, *PR-10*, *PR-5*, peroxidases, germin-like proteins, detoxifying proteins such as glucosyltransferase or cytochrome *P450*. This array of genes involved in the response of

the plant to crown rot pathogens raise the complexity of the process of resistance to this necrotrophic disease, and the necessity of going in depth in the study about genes which correspond to different sources of defense during induction in wheat plants.

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CHAPTER 3

**Distribution and severity of Fusarium crown rot and common root rot of wheat in
Montana using real-time qPCR and conventional isolation**

Contribution of Authors and Co-Authors

Manuscript in Chapters 3

Chapter 3:

Co-Author: Alan T. Dyer, Andy C. Hogg, Lisa J. Rew, and Barry J. Jacobsen

Contributions of Dr. Alan T. Dyer provided economical support to this survey, real-time processing of samples and aided in the statistical analysis. M.Sc. Andy C. Hogg collaborated with his expertise in developing a probe to quantify *Bipolaris sorokiniana* infection in first internode of wheat plants and he contributed during the process of pathogen identification. Dr. Lisa J. Rew contributed in the process to extract environmental variables data from a GIS database for the area of sampling and assistance in mapping used in the manuscript. Dr. Barry J. Jacobsen provided financial assistance, guidance and laboratory facilities. Also, Dr. Dyer and Dr. Jacobsen then worked on all parts of the manuscript through its successive revisions, reading and approving the final manuscript.

Manuscript Information Page

- Authors: Ernesto A. Moya, Alan T. Dyer, Andy C. Hogg, Lisa J. Rew, and Barry J. Jacobsen.
- Journal name: Plant Disease
- Status of the manuscript
 - Prepared for submission to peer-reviewed journal
 - Officially submitted to a peer-reviewed journal
 - Accepted by a peer-reviewed journal
 - Published in a peer-reviewed journal
- Publisher: American Phytopathological Society
- Data of submission (for submitted manuscript)
- Date manuscript will appear (for accepted work)
- Issue in which manuscript appears in (for published works)

Distribution and severity of Fusarium crown rot and common root rot of wheat in Montana using real-time qPCR and conventional isolation

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ABSTRACT

This study was conducted to determine the distribution of *Fusarium* crown rot (FCR) and common root rot (CRR) associated with wheat (*Triticum aestivum* L.) in 91 fields in Montana by using real-time quantitative Polymerase-Chain-Reaction and conventional isolation methods during the 2008 and 2009 crop seasons. Significant correlations were found between detection methods for both diseases ($P < 0.001$). FCR was observed in 57% of the fields and CRR was the most widespread disease being detected in 93% of the fields surveyed. Percent incidence based on isolation from individual tillers as determined by traditional isolation methods, was *Bipolaris sorokiniana* (15%), *F. culmorum* (13%) and *F. pseudograminearum* (8%). Eyespot pathogens, *Oculimacula yallundae* and *O. aciformis*, were isolated from 49% of the fields and several *Fusarium* species considered to be less pathogenic on wheat were also found at variable

frequencies. FCR populations were highly variable across the regions as indicated by both isolation and qPCR methods. FCR was not detected in any fields from the Gb5 soil types of Judith Basin and Fergus Counties. Elevation, soil type, and temperature affected spatial distributions of both diseases and pathogens. High FCR populations as indicated by qPCR were associated with spring wheat crops rather than winter wheat ($P < 0.001$). Data from eight intensively sampled fields showed that FCR and CRR could produce yield losses in a range of 3.2 to 34.9%. This study is the first time qPCR was used to survey the FCR and CRR diseases of wheat and the merits and weakness of qPCR relative to traditional isolation methods are discussed.

INTRODUCTION

Fusarium crown rot (FCR) and common root rot (CRR) are important diseases of wheat and barley, causing yield losses, stand reductions and rotting of root, crown, subcrown, and lower stem tissues (18,19,20,24,43). Fusarium crown rot is generally accepted as a disease complex dominated by different pathogens in different areas or even by different pathogens during successive growing seasons in individual fields (43). The FCR complex most commonly involves *Fusarium culmorum* (W. G. Sm.) Sacc., *F. pseudograminearum* (O'Donnell & T. Aoki; group I) (= *Gibberella coronicola*), and *F. graminearum* Schwabe (group II) (= *G. zaeae* (Schwein.) Petch) (43,55). In some regions, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh., *F. equiseti* (Corda) Sacc., and *Microdochium nivale* (Fr.) Samuels & Hallett (= *F. nivale* (Fr.) Sorauer) have also been associated with FCR (43). In contrast to FCR, CRR is caused solely by *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. Ex

Dastur). Because FCR and CRR are perennial and widespread problems in wheat and barley production, several surveys have been conducted to determine the pathogens involved, estimate yield losses, and to examine environmental factors associated with these diseases. Surveys have been carried out in the Pacific Northwest (12,55), Canadian Prairies (18, 19, 20,24,26), Texas Panhandle (57), Southeastern Idaho (58); upper coastal plain area of Mississippi (25), Colorado and Wyoming (28), eastern Australia (4); South Australia (17), Queensland Australia (65,66), United Kingdom (44), Turkey (63), north west Iran (46); and Brazil (14). Unfortunately none of these surveys have included the major production areas of Montana where heavy root and crown rot losses have recently been reported (29).

While wheat production in Montana is widely dispersed, the north central and central regions of the state are known as “the Golden Triangle” and this area represents approximately 43.5% of the state’s 2.19 million hectares of annual wheat production (<http://www.nass.usda.gov>). Within this region, adoption of no-till farming practices has become popular because they conserve soil moisture, fuel and labor inputs plus reducing soil erosion (15,30). The adoption of no-till wheat cropping systems has also been linked to increase severity of FCR (9,42,52) and CRR (66). In Montana’s wheat agroecosystem, incorporation of no-till and conservation tillage system practices may also be associated with a possible change in FCR pathogen species from *F. culmorum* to *F. pseudograminearum* (15). Other production practices beside tillage that have been linked to increased FCR and CRR severity include short or no crop rotations and glyphosate herbicide treatments used in chemical fallow or preplant weed control (19,20,21,22,24).

Information regarding disease and pathogen incidence, yield losses, and the associated production practices is needed in order to determine better target control practices in “the Golden Triangle”. Also documentation of cropping practices is important considering the impact of cropping practices on dryland root rot diseases and the widespread perception that these diseases are becoming more important.

To date, FCR and CRR surveys have been primarily conducted through the isolation and culturing of fungal pathogens from infected tissues and/or through the assessment of visual symptoms characteristic for the respective diseases. Unfortunately, the isolation methods typically used for these surveys are labor-intensive and are often confounded by factors such as competition and antagonism from other organisms within the tissues, the expertise of the individuals isolating and identifying species, and the selectivity, and sensitivity of the media assays (30). On the other hand, visual assessment of the diseases is labor intensive, less pathogen specific and subject to the vagaries of the individuals involved (29,56). With the introduction of quantitative real-time polymerase chain reaction assays (qPCR), these problems may be solved as qPCR has the potential to make disease assessments quickly and accurately (48). The value of qPCR for conducting disease surveys has already been demonstrated for a number of plant disease systems (31,32,35,64).

Because of the need to understand the disease dynamics in Montana and the advent of modern molecular methods, this study was conducted to assess the distribution and severity of FCR and CRR across commercial wheat fields in “the Golden Triangle” of Montana. To accomplish this, two TaqMan[®] qPCR assays were used to determine FCR

and CRR severity within fields. Because of their recent development, qPCR results were compared with the traditional methods of pathogen isolation and identification to examine their relative performance in this pathosystem. The resulting spatial distributions of FCR and CRR were assessed for their relationships to environmental variables as well as common production practices through a multifactorial analysis.

MATERIAL AND METHODS

Description of survey counties and sampling. During the summer of 2008 and 2009, a survey was conducted involving 40 and 51 commercial wheat fields respectively from ten counties throughout the north central and central regions of Montana (Figure 1). The counties sampled were Blaine, Choteau, Fergus, Glacier, Hill, Judith Basin, Liberty, Phillips, Teton, and Toole. For each county between two and nine fields were sampled each year. Sampled fields were randomly chosen with the help of the county extension agents of the respective counties. Fields selected were over 60 acres in size and were separated by a minimum of 2 kilometers in distance. For each field, plant samples were collected within 14 days of harvest (either before or after). During 2008, samples were collected between the August 11th and 16th, while during 2009, samples were taken between August 10th and 25th. For each field, samples were collected at 20 sites along a 600 m diagonal transect, with each site being approximately 30 m apart. Transect direction was variable and always started in 30 m inside of the field, and the transect always intended to cover a representative area of the field. At each sampling site, 3 randomly selected tillers were collected for a total of 60 tillers per field. The selected

tillers were taken back to the laboratory where they were cleaned, dried, and stored at 4°C until they could be processed using qPCR or media-based isolation.

Coordinated with the field sampling, growers were surveyed about their cropping systems using an electronic or a paper survey during the two year study. Survey questions detailed wheat type (spring vs winter), cropping intensity, crop rotation, glyphosate usage, fungicide seed treatments and nitrogen fertilizer applications. This information was used to categorize the crops/fields according to crop production factors. This data was then used to determine association of these factors with disease severity as measured by qPCR quantification of targeted DNA sequences.

Quantification of crown and root rot infection on field samples. For DNA extractions and qPCR quantification, 3mm stem sections were cut from the first internode of 30 randomly selected tillers per field sample. Subsequent, handling, processing and DNA extraction of the stem sections followed previously established protocols (30). Real-time quantification of pathogen populations was conducted using probes dual-labeled with the 6- carboxyfluorescein (6-FAM) fluorescent reporter dye and the 6-carboxyltetramethylrhodamine (TAMRA) fluorescence quencher. The probe and primers used for quantification of *Fusarium* populations were previously described by Strausbaugh et al., (59) and modified by Hogg et al., (29). This assay was specific for the three main species in the FCR complex, *F. culmorum*, *F. pseudograminearum* and *F. graminearum*. The probe and primers used for *Bipolaris sorokiniana* were designed using the partial DNA sequence of the glyceraldehyde-3-phosphate dehydrogenase-like (*gpd*) gene from *B. sorokiniana* isolate ND93-1 (Gene Bank accession no. EF513209.1).

The probe sequence was 5'-TCCATGGAGCGAGACTGGGCGC-3', which is located between the 377bp and 398bp of the *gpd* sequence. The forward and reverse primer sequences were 5'-GAAGGACCCCGCCAACA-3' and 5'-CCGCTACACTCGACGACGTAGT-3', respectively. All amplifications were carried out in a Rotor Gene Q (QBiogene Inc.) using a two-step protocol with the following thermocycling parameters: 2 min at 55°C, 10 min at 94°C, and 40 cycles of 15 s at 94°C and 60 s at 60°C. QPCR reactions (25 µl) consisted of 12.5 µl of Universal TaqMan Master Mix (PE Applied Biosystems), 2.5 µl of 2 µM TaqMan probe, 2.25 µl of a 1:1 mixture of 20 µM forward and reverse primer solutions (Integrated DNA Technologies, Inc. Coralville, IA), 2.75 µl of molecular grade water, and 5 µl of DNA sample. Each real-time run included five, 10-fold-dilution standards, ranging from 660 copies to 6.6 million copies. Standard curves produced by plotting the log of the DNA standards concentrations versus the C_t values showed strong relationships (R values always exceeded 0.99). Both the DNA standards and no-template control (NTC) samples were run in triplicate for each real-time run. DNA field samples were run three times and the average of the three runs was considered the level of crown and root rot infection present in the field. To avoid artifacts from primer dimers, a cut-off value of 36 cycles was established for C_t , the cycle number at which the fluorescence generated within the reaction crosses the threshold for being significantly different from the baseline or background signal.

For *Fusarium*, the DNA standard used for absolute quantification during qPCR was a cloned fragment of the *tri5* gene from *F. culmorum*, isolate 2223 (29). For *Bipolaris*, a

507 bp fragment of the *gpd* gene from isolate 2243 (collected in Chester, MT) was cloned using Invitrogens TOPO TA cloning kit as per the manufacture instructions (Carlsbad, CA). A *gpd* gene fragment was amplified using a forward primer 5'-TAAAGCTGACCCTGTGTCTCAGCA-3' and a reverse primer 5'-AGAGAACCTCAATGTCGGGCTTGT-3'. Colony selections were confirmed by PCR amplification. Because the *Bipolaris sorokiniana* assay was new, its specificity and sensitivity was tested across the following plant pathogens: *F. culmorum*, *F. pseudograminearum*, *F. graminearum*, *F. sambucinum*, *F. avenaceum*, *F. acuminatum*, *F. solani*, *F. oxysporum*, *F. moniliforme*, *F. equiseti*, *F. poae*, *F. sporotrichoides*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Cephalosporium gramineum*., *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, *Phialophora* spp., *Sclerotinia sclerotium*, *Aphanomyces cochlioides*, *Penicillium claviforme*, *Oculimacula yallundae* (syn *Tapesia yallundae*), *Rhizoctonia solani*, and *Bipolaris maydis* (Nisikado) Shoemaker (teleomorph = *Cochliobolus heterostrophus* (Drechs.) Drechs.; synonym = *Helminthosporium maydis* Nisikado). For each species, 10 ng of DNA was tested via qPCR to check for nonspecific reactions and experiments were repeated once in their entirety. The only fungi amplified by this assay were *Bipolaris maydis* and *B. sorokiniana*. Both species had similar amplification curves.

Isolation and identification of crown and root rot pathogens. To determine the root and crown rot pathogens involved in the infection of the stems and to corroborate qPCR results, additional 3 mm segments were excised from the first internodes of the 30 wheat tillers used for qPCR quantification. These new segments were used to assess presence

of *Fusarium* spp., *B. sorokiniana*, and other pathogen species in these tissues through culturing on a general and selective media. One half of the segments were plated onto 50% potato dextrose agar (PDA) amended with antibiotics (100 µg of streptomycin sulfate, 100 µg tetracycline sulfate, and 50 µg of neomycin sulfate). The other half of the stem segments were plated onto a media selective for *Bipolaris* (57), since the slow growing of *B. sorokiniana* was easily overgrown by *Fusarium* mycelia on PDA. Prior to plating, stem segments were disinfected with 0.54% sodium hypochlorite solution for thirty seconds, rinsed with sterile distilled water, and dried on a sterile paper towel. Five disinfected segments were placed on each media plate. Plates were incubated at $23\pm 1^{\circ}\text{C}$ and monitored daily for fungal growth. Any resulting fungal colonies were isolated by hyphal tipping onto fresh PDA plates. All resulting isolates were identified to genus by morphology and culture characteristics. All resulting *Fusarium* cultures were transferred to carnation leaf amended media (CLA media) for identification to species using traditional species identification characteristics (38,62). Isolates of *B. sorokiniana* and other fungi were identified under microscope according the characteristics of their conidia and mycelia (6). The number and identity of fungi were recorded for each sample. These data were used to correlate relationships between isolation frequencies and the population sizes as determined by the qPCR assays. In consideration that *F. culmorum*, *F. pseudograminearum*, and *F. graminearum* are all detected by the qPCR *Fusarium* assay, these pathogens were considered as the members of the FCR complex when used to compare isolation and qPCR assay results.

Assessment of relationship between diseases levels and yield reduction. To study the relationships between disease severity and grain yield, 4 selected fields in 2008 and 5 selected fields in 2009 were intensively sampled. For 2008, intensive sampling was done in two dryland winter wheat fields and one spring wheat field in Choteau County and one irrigated spring wheat field in Phillips County. In 2009, intensive sampling occurred on two dryland winter wheat fields, one each in Phillip and Chouteau Counties; two dryland spring wheat fields, one each in Glacier and Blaine Counties; and a repeated sampling of the irrigated spring wheat field from Phillips County that had been sampled in 2008. For each field selected, intensive sampling involved collecting 20 crown and grain samples from 20 arbitrarily selected sites along a 600m transect within the field. For each sampling site, heads were collected from 1m² to determine yield and 60 tillers collected from the corresponding area being analyzed for FCR and CRR pathogen populations by the qPCR methodology described above. For this paper pathogen populations are equated to disease severity. The grain was harvested from collected heads using a Vogel thresher (Bill's Welding, Pullman, WA), cleaned, and weighed.

Environmental and spatial characterization of field infection. All fields were georeferenced through Google Earth® software. In cases where no precise GPS location for the field could be determined, coordinates were matched to the nearby geographical place names recognizable in the spatial database. In addition to locations, elevation for each GPS field position was registered. For each survey location, data for average growing degree days between May and August, average annual temperatures and precipitation, growth period temperature and precipitation (April to August), general soil types, and soil

water holding capacity were extracted with ArcViewGIS (version 9.0, ESRI, Redland, CA) from the geographical information system (GIS) database MAP *Atlas* 6.0 (11). All environmental data were analyzed on either an individual pathogen species basis or as a group of species according to their environmental association using a Spearman-Rank coefficient of correlation.

Data analysis. DNA copy numbers obtained through qPCR for each disease of interest were $\log(x+1)$ transformed to reduce variance instability within the data sets. In addition, zero values were trimmed from data sets when comparisons between results of qPCR and isolation methods were made using correlation coefficients and linear regressions. Across years, Levene' test of homogeneity of variance found variances homogenous and therefore the data from both years was combined. Pearson and Spearman-Rank coefficients of correlation and linear regressions were performed to examine various relationships between disease/pathogen populations as determined by qPCR or conventional methods and other measured parameters using the PROC COR and PROC REG procedures of SAS[®] software (Version 9.2, Inc. Cary, NC, USA). Standard chi-square test was used to compare frequencies of infested fields by county obtained through isolation methods and qPCR assay for each disease. Multiregression analyses were conducted to assess additive effects or interaction between populations of FCR and CRR as quantified by qPCR and grain yield for the extensively sampled fields, considering each individual field and for a generalized model where the field factor was a fixed effect.

In order to determine significant spatial patterns for the different diseases and fungal species determined through qPCR and plating, geospatial statistics were used. All fields (with or without FCR and CRR pathogens) were included in the analysis, and tests for spatial autocorrelation were undertaken using Moran's I statistic z score. This statistic provides an evaluation of whether the observed distribution pattern for an exhibited fungal species is clustered, dispersed, or random. All latitudinal and longitudinal coordinates for the field locations were corrected to a decimal degree and tests for each fungal species were implemented within the spatial statistics component from the PROC VARIOGRAM of SAS[®] 9.2 version (SAS Institute).

Relationships between the extracted environmental variables from the GIS database and population of both diseases obtained by qPCR or percentage of infection for each pathogen of interest were analyzed using the PROC COR (SAS Institute). Spearman-Rank correlations were considered to avoid variance differences and distribution effect of analyzed variables. Additional, Kruskal-Wallis sum rank tests and non-parametric multiple comparisons for the differences in ranks with a Wilcoxon rank-sum tests and Benjamini-Hochberg (BH) correction method were conducted to determine differences in level of plant infection among the general soil type classifications for Montana by using the packages "Rcmdr" and "PairwiseCI" of the R-Software (www.r-project.org).

Associations between crop production factors and values obtained from the qPCR quantification were analyzed using a bivariate distance vector in a permutation multivariable analysis of variance (PerMANOVA) through the adonis function from the package "vegan" of the R-software. This function permits the analysis of univariate or

multivariate data using any distance measure and linear model. The calculated statistic (*pseudo-F*) is calculated, like a traditional F-statistic, as the sum of the squared distances among groups divided by the sum of the squared distances within groups relative to the overall sum of the pairwise distances (1,36). This statistical method uses Euclidean distance permutations to generate the MANOVA by assuming the same distribution for all the groups or variables analyzed. Non-parametric Wilcoxon-Mann-Whitney or Kruskal-Wallis tests were considered to determine significant differences between two or more groups in the crop factor variables by using the Package “Rcmdr” of R-Software.

RESULTS

Fungal populations as determined by traditional isolation methods. The field incidence rates for FCR and CRR pathogens were 51% and 92%, respectively as determined by conventional methods of isolation. *Fusarium culmorum* was detected in 36% of the fields and *F. pseudograminearum* was detected in 31% of the fields with both pathogens being present in 16% of the fields. Overall these two pathogens were isolated from 20% of the tillers examined. Populations as determined by rates of isolation showed *F. culmorum* was the dominant FCR pathogen in Glacier, Toole and Blaine counties; while *F. pseudograminearum* was dominant in Phillips and Teton Counties (Table 1). The third FCR complex species, *F. graminearum* was not detected. Across the two years, the three species (*F. culmorum*, *F. pseudograminearum* and *F. graminearum*) were absent from 45 of the sampled fields, which includes all of the fields in Judith Basin and Fergus Counties (Table 1). In contrast to *Fusarium* crown rot, the

CRR related pathogen, *B. sorokiniana* was detected in all counties sampled in the survey (Table 1) and was absent from just 7 fields, all located in either Chouteau or Liberty Counties. Using selective media, *B. sorokiniana* was isolated from 15% tillers examined (Table 1). The highest populations, as indicated by isolation frequencies, were in Fergus, Judith Basin, and Blaine Counties. Numerous other known wheat pathogens were isolated. Those detected in a significant percentage of the fields included *F. acuminatum* (32%), *F. avenaceum* (16%), *F. equiseti* (55%), *Microdochium nivale* (21%), *F. oxysporum* (19%), *F. sporotrichioides* (19%), *F. redolens* Wollenweb. (27%), *F. dimerum* Penz. (24%), *F. solani* (10%); *Gaeumannomyces graminis var. tritici* (4%) and *Oculimacula* species (*O. yallundae* or *O. acuformis*) (49%). The isolates of *Oculimacula* were predominately from winter wheat fields (62.5% winter wheat vs 35.3% spring wheat) and the Take-all pathogen was only found in three irrigated fields located in Teton County and one irrigated field located in Phillips County. Other genera of fungi isolated including: *Alternaria*, *Torula*, *Curvularia*, *Trichoderma*, *Rhizoctonia*, *Penicillium*, *Nigrospora*, *Epicoccum*, *Trichotecium*, and several unidentified non-sporulating cultures. All of these were rare and isolated at frequencies of less than 3% of the total samples (data not shown).

Fungal populations as determined by qPCR methods. FCR and CRR pathogens were present in 57% and 93% of the fields as determined by real-time qPCR, respectively. These values were not significantly different from those determined by traditional culturing methods (FCR $\chi^2 = 1.964$, $P = 0.992$; CRR $\chi^2 = 0.041$, $P = 1.00$). Average DNA copy number for *Fusarium* spp. and *B. sorokiniana* were 148,497 and

18,669, respectively (Table 2). As determined by qPCR, members of FCR complex were not detected in fields from Judith Basin and Fergus Counties. In contrast, DNA copy numbers for the FCR complex were at their highest in fields sampled in Toole and Glacier Counties (Mean = 340,577 and Mean = 250,580). Twelve percent of the fields had copy numbers in excess of 400,000 for the FCR assay (range = 415,770 - 1,925,597; Mean = 770,719). In comparison, populations of *B. sorokiniana* were relatively homogeneous as determined by qPCR, except in Glacier and Teton Counties where populations were particularly low (Means = 6,397 and 5,476, respectively; Table 2). Fifteen percent of the fields had particularly high levels of *B. sorokiniana* with DNA copies numbers >40,000 copies (44,790-90,243 copies; Mean = 59,900; Table 2). In 2008, DNA copies numbers for the FCR complex and for *B. sorokiniana* were negatively correlated with each other at both the field and county level (Spearman rank coefficients: $r = -0.319$, $P = 0.044$ and $r = -0.798$, $P = 0.005$, respectively). For 2009, these relationships were not present (Spearman rank coefficients: $r = -0.194$, $P = 0.171$; $r = -0.243$, $P = 0.498$, respectively).

Results from qPCR and traditional isolation methods were positively correlated with each other for the two pathogen systems (Pearson coefficients: FCR, $r = 0.714$, $P = <0.001$; CRR $r = 0.525$, $P = <0.001$; Fig. 2a and 2b).

Assessment of intensively sampled fields. For the nine intensively sampled fields, relationships between yield and the populations of both the FCR complex and *B. sorokiniana* as indicated by log DNA copy numbers were analyzed (Table 3). Among these, the FCR complex populations had significant negative correlations with yield in

two dryland winter wheat and one-spring wheat fields. For the 20 sampled sites in the dryland winter wheat fields in Chouteau (CH1) and Phillips Counties (PH5) FCR complex populations ranged from 0 to 3,454,704 and 690 to 1,255,929 DNA copy numbers of the *tri5* gene, respectively. When yields were regressed against population, a FCR population of 400,000 DNA copies was equated to a yield loss of 24.6% in the field in Chouteau (CH1) and 34.9% in the field locate in Phillips county (PH5). The irrigated spring wheat field sampled during two years in Phillips County (PH3) had a marked increase in FCR complex populations in the second year, which was negatively correlated with yield in that year (Table 3). In this case, the population of the FCR complex ranged from 9,059 to 2,417,753 DNA copies and a population of 400,000 DNA copies was associated with a 21.0% decrease in yield. One additional spring wheat field from Blaine County in 2009 (BL5) showed a positive correlation between *Fusarium* populations and yield (Pearson coefficient: $r = 0.524$; $P = 0.0177$). However, the removal of two extreme outliers from this analysis resulted in an insignificant relationship (Pearson coefficient: $r = 0.326$ and $P = 0.1862$). For *B. sorokiniana*, log DNA copy numbers were negatively correlated with yield in the irrigated spring wheat field sampled in Phillips County in both years of the study, and one dryland spring wheat crop in Glacier County in 2009 (Table 3). For the 20 sampled sites in the irrigated field in Phillips County (PH3) in 2008 and 2009, populations of *B. sorokiniana* ranged from 26,048 to 196,289 and 16,965 to 196,004 DNA copy numbers of the *gpd* gene for each year, respectively. When yield were regressed to population of *Bipolaris*, a population of 40,000 DNA copy numbers was associated with a decrease in yield of 3.2% and 12.5% for the irrigated field in

Phillips County (PH3) in 2008 and 2009, respectively, while a 31.0% decrease in yield was determined for the dryland field from Glacier County (GL1); where population of *Bipolaris* ranged between 2,243 to 87,898 DNA copy numbers. A multiple regression analysis did not detect any effect on grain yield for the interaction between populations of FCR and CRR when field was considered as a fixed effect ($R^2 = 0.796$, $P = 0.619$), although in two fields (CH1 and PH3 during 2008), which were analyzed individually by using multiple regression, interaction was significant at the 10% level of significance.

Assessment of spatial distribution and environmental factors. The high variability observed in population levels as detected by qPCR and traditional isolations methods motivated efforts to analyze spatial patterns for both diseases using a Moran's I z score statistic. This resulted in significant spatial autocorrelations being detected for populations of both the FCR complex and *B. sorokiniana* as represented by DNA copy numbers (FCR complex, $z = 9.76$, $P < 0.001$; CRR populations $z = 3.51$, $P < 0.001$). Field incidence levels, as determined by isolation methods, auto-correlated for *F. culmorum* ($z = 11.74$, $P < 0.001$), *B. sorokiniana* ($z = 4.11$, $P < 0.001$), and for the combined *F. culmorum* and *F. pseudograminearum* incidences ($z = 8.40$, $P < 0.001$). These results implied that the spatial distributions for each pathogen were in highly clustered, nonrandom patterns. Incidence for *F. pseudograminearum* was not significantly auto-correlated ($z = 1.20$, $P = 0.232$) and this implied that populations of this pathogen were dispersed randomly among the fields in the surveyed area. Because of the spatial autocorrelation results, environmental variables including growing degree days, elevation and average and maximum temperatures and precipitation were examined for

associations with define areas of disease concentration. From these analyses, it was found that FCR complex populations as measured by qPCR were negatively correlated with annual temperatures (Spearman rank coefficient: $r = -0.271$, $P = 0.009$) while *B. sorokiniana* populations as measured by qPCR were positively correlated with elevation (Spearman rank coefficient: $r = 0.228$, $P = 0.030$), growing degree days (Spearman rank coefficient: $r = 0.230$, $P = 0.029$), and average and maximum temperatures observed in June (Spearman rank coefficients: $r = 0.254$, $P = 0.015$, $r = 0.229$, $p = 0.029$, respectively), and July (Spearman rank coefficients: $r = 0.260$, $p = 0.012$; $r = 0.207$, $P = 0.048$, respectively), and with maximum temperature in August (Spearman rank coefficient: $r = 0.231$, $P = 0.027$). Populations of *F. culmorum* as determined by traditional isolation methods were negatively correlated with annual temperature (Spearman rank coefficient: $r = -0.414$, $P < 0.001$) and average and maximum temperatures observed during June (Spearman rank coefficients: $r = -0.269$, $P = 0.009$, $r = -0.258$, $P = 0.001$, respectively), July (Spearman rank coefficients: $r = -0.2834$, $P = 0.007$; $r = -0.267$, $P = 0.010$, respectively), and August (Spearman rank coefficients: $r = -0.279$, $P = 0.007$; $r = -0.347$, $P < 0.001$, respectively). Field incidences of *F. pseudograminearum* were positively correlated with elevation (Spearman rank coefficient: $r = 0.212$, $P = 0.037$), growing degree days (Spearman rank coefficient: $r = 0.210$, $P = 0.046$), soil water holding capacity (Spearman rank coefficient: $r = 0.221$, $P = 0.035$), average temperatures in August (Spearman rank coefficient: $r = 0.236$, $P = 0.024$), and maximum temperatures observed during summer months ($P < 0.10$). Field

incidence level for *B. sorokiniana* as determined by traditional media isolation did not correlate with any environmental parameter (data not shown).

In addition to these environmental variables, general soil types were analyzed as disease factors using Kruskal-Wallis sum rank test and non-parametric means comparison (Table 4). Soil types were significantly different for log DNA copy numbers for the FCR complex as quantified by qPCR ($\chi^2 = 18.42$, $P = 0.005$). Wheat samples grown in silty soil type *Gb5* from Fergus County and near to Stanford in Judith Basin County did not have FCR infections different from samples grown in the other soil types examined. Log DNA copy numbers for *B. sorokiniana* were not significantly different among the different soil types ($\chi^2 = 10.97$, $P = 0.089$). *F. culmorum* and *F. pseudograminearum* populations as quantified by plating method were significantly different ($\chi^2 = 18.35$, $P = 0.005$; $\chi^2 = 14.07$, $P = 0.029$, respectively). Clayey and shallow clay of *Gb4* soil type from the northern portion of Glacier County had high incidence of *F. culmorum* (58.3%), while the clayey and shallow clay soils *Pb2* type in the irrigated fields in Teton County and the *Ap1* from Phillips County were more associated with *F. pseudograminearum* (17.5%). The lowest incidence for *B. sorokiniana* was observed in the *Pb2* soils from Teton County (5.8%), however no differences were observed among soil types ($\chi^2 = 12.33$, $P = 0.055$; Table 4).

Assessment of Cropping Factors. Cropping factors including tillage, crop rotations, glyphosate herbicide treatments, fungicide seed treatment, nitrogen fertilizer rates and wheat types were analyzed by PerMANOVA for their impacts on log DNA copy numbers for the pathogens as determined by qPCR. The only significant effect was for

wheat type, where DNA copy numbers for the FCR complex populations were significantly different (*pseudo F* = 1.413, $P < 0.001$) in spring wheat (Mean = 285,662 copies) than in winter wheat (Mean = 63,929 copies). No statistical differences were detected for *B. sorokiniana* populations.

DISCUSSION

For this survey, *B. sorokiniana* was found to be the most widespread pathogen species within the “Golden Triangle” of Montana. This result is comparable to surveys reported in Canada (5,18,27,61) and the US (28,58,68,69) but are significantly different than distributions reported in the Pacific Northwest where CRR was only of secondary concern (55). Perhaps, the high incidence of *B. sorokiniana* may be partially explained by the use of *Bipolaris* selective media for this study, which increased the overall isolation rates for *Bipolaris* relative to the non-selective media by 2.7%. None the less, with a 12.3% isolation rate on PDA, *B. sorokiniana* would still have been highly prevalent across “the Golden Triangle”. While *Bipolaris* was more widespread than the FCR complex, the population sizes were often an order of magnitude smaller as quantified by qPCR. Whether the results of the two qPCR assays are comparable in this manner or whether protocols bias the result towards the FCR complex is unknown. Certainly the hallmark infection court for the CRR pathogen is the subcrown internode and unlike the FCR complex (17,34), it is not noted for aggressively colonizing the lower tiller. For that reason, the first internodes used for this study are probably a biased sample when comparisons are made between these pathogen species. With that said,

severe CRR infections, which are likely to significantly affect yield, generally extend beyond the crown into the first internode tissues and so these tissues should act as a good point for measuring CRR severity and the impacts of CRR on wheat production in Montana.

Among the complex of species associated with the FCR complex in Montana, *F. culmorum* dominated. Its populations were highly structured which suggests they may have reached equilibrium with their environmental and ecological constraints across the region. This pattern was similarly expressed for *B. sorokiniana*, where despite an almost ubiquitous distribution, its populations were found structured based on environmental parameters, such as elevation, which has been associated with *B. sorokiniana* distribution in another survey (63). Environmental associations with *F. culmorum* showed the fungus preferred the cooler areas of “the Golden Triangle” particularly in the northern regions and along the Rocky Mountain front. This conforms to previous observations of this pathogen being a cool weather pathogen (12,13,40). Similarly, the observation that *F. pseudograminearum* prefers warmer conditions (13) was also confirmed by this survey where the proportion of infection by *F. pseudograminearum* was positively correlated with maximum temperature during the summer months. In contrast to *F. culmorum*, populations of *F. pseudograminearum* showed a non-clustered, random distribution suggesting that this pathogen is still colonizing the region and has yet to become bound by environmental and/or ecological constraints. This adaptation of *F. pseudograminearum* to warm conditions in Montana could be used in the future to assess changes associated with global warming.

It is interesting to note that FCR was not found in 45 fields. This absence from a considerable number of fields showed the high variability of FCR populations throughout the area. For most fields, this absence may or may not indicate a lack of endemicity for the pathogen complex. However, the universal absence of the FCR complex from 16 fields sampled in Judith Basin and Fergus Counties may very well indicate that for whatever reason these pathogens are not endemic to these counties. Within “the Golden Triangle”, these counties are rather unique within the surveyed region for another reason and that is that they sit on top of the Judith Bench with soils generally characterized as silt-loams (Argiborolls-CalciBORolls). Also, Fergus and Judith Basin counties are part of a different agroecoregion than that present in the North Plains of Montana, with higher precipitation and lower temperatures and evapotranspiration rates compared to the dominant agroecological region present in other surveyed counties (39). The impact of agroecological zones in the distribution and prevalence of crown and root rot pathogens has been clearly observed in another survey done by Tunali et al (63), who showed that incidence of *Fusarium* species are varied among eleven agroecological zones in Turkey. In addition, growers in Fergus and Judith Basin counties area typically annually-crop their fields and use longer crop rotations than growers in most of the remaining areas surveyed (data not presented). While this study could find no link between disease and rotation or cropping intensity, soil types and some environmental variables were significantly associated with FCR and may potentially explain the absence of FCR in Judith Basin and Fergus Counties. Curiously, *F. graminearum*, the third member of this complex, was never isolated from any of the samples collected across the region. Besides

being a devastating floral pathogen, *F. graminearum* has been shown to be an aggressive crown rot pathogen capable of holding its own with the other two members in the FCR complex (15). In recent years, this pathogen has caused localized head blight outbreaks within the southern and eastern portions of the state (10) and it will be interesting to follow its populations to see if they can become established in areas not conducive to head blight.

Among the assorted other fungi isolated from field samples, *Oculimacula* spp. were dominant. The isolation rates for these species exceeded the combined incidence rate for *F. culmorum* and *F. pseudograminearum*. Until now, eyespot and the pathogens that cause it *O. yallundae* and *O. aciformis* were considered uncommon and relatively unimportant to Montana, with the disease only occasionally being identified in winter wheat grown in mountain valleys or a few wetter areas in the eastern portions the state (45). However, *Oculimacula* spp are serious problems in winter wheat grown in areas where winters are cool and wet like the Pacific Northwest where significant yield losses may occur (37). While it is not addressed by this study, eyespot and its corresponding pathogens warrant re-evaluation for their importance here in Montana. Surprisingly, a significant number of *Oculimacula* isolations were from spring wheat where it is not considered a significant problem due to the pathogens being favored by cold-weather. However in light of these findings, this may need to be reconsidered as well.

Assorted other fusaria (besides the FCR complex) were isolated from tiller material at rates lower than *B sorokiniana* and *Oculimacula* spp.. Among them, *F. equiseti*, *F. acuminatum*, *F. redolens*, and *F. dimerum* were detected in over 20% of the fields

sampled. Of these *F. equiseti*, *F. acuminatum* and *F. dimerum* are considered to be less pathogenic (58) and their effects on wheat crops are not well characterized. The fourth fusaria, *F. redolens* was considered conspecific with *F. oxysporum* (38), but recently has been considered a separate clade from the *F. oxysporum* complex (3,7). *F. redolens* is known to affect asparagus (3), onion (51), peas and beans (8), but *F. redolens* has been not described as member in the FCR complex and infecting wheat before. High distribution of this fungus may be associated with the recent incorporation of dry peas in crop rotations for this area, but additional studies will be required to identify whether this association occurs, since the samples size was too low and no pathogenicity tests were done in this study to make any strong statements on this issue. Assorted other fusaria were found at lesser frequencies and are likely to play only minor roles in the crown and root rot complexes.

DNA copies for the FCR complex were particularly high in 12% of the fields sampled (in excess of 400,000 copies). These levels of infection expressed in DNA copy numbers of *tri 5* gene have been correlated with yield losses and high disease severity scores for FCR in the first internode (29). For intensively sampled fields, these levels of the pathogen were associated with losses of 24.6 and 34.9% for the dryland fields and 21% for the second year of wheat recrop in an irrigated field. While attributing losses to disease survey results is always tricky, it would be safe to assume that these heavily infested fields were severely affected by FCR and the growers are experiencing significant FCR related losses. Like the FCR complex, populations of *B. sorokiniana*, as expressed in DNA copy numbers, were in excess of 40,000 copies in 15% of the fields.

Losses associated with these populations were less than those of attributed to FCR with losses of 3.2 and 12.5% for both years in the irrigated field respectively, and 31.0% for a dryland field. From these numbers it is hard to assess which disease is causing the largest losses in the area. While losses are greater for individual fields affected by severe FCR, CRR is more widely dispersed and causing more generalized losses for the region. As an aside it should be noted that these two diseases were negatively correlated with each other in the first year of the study and not in the second. Negative correlations between these diseases have been reported by others at survey level (18) which would suggest in the one case if these pathogens are separating due to niche differentiation then the diseases could be addressed individually but if they are antagonistic, that control of one may simply result in replacement by the other and only limited yield gains would be recognized by disease control. Considering that antagonism between fusaria pathogens and *B. sorokiniana* has been described under controlled conditions (23,33,47,60), population replacement between pathogens seem likely to occur. However, antagonistic responses or interaction between fusaria and *Bipolaris* pathogens were not clearly defined in this survey at field level and antagonism between both pathogens has not been determined in experimental setting under field conditions by using the qPCR assays (unpublished data). Perhaps, negative relationships between pathogen populations in a field is regulated by dynamics of competition for colonizing the different wheat tissues or/and displacement between each pathogen depending of the environmental conditions favoring each other.

The DNA copies for the FCR complex were higher in spring wheat than winter wheat. These results confirm early work conducted in PNW where FCR was more severe in spring wheat than in winter wheat (54). Other crop factors such as growth type, tillage, crop rotation, seed treatments, nitrogen rates, water availability and use of glyphosate did not show any relationship to either FCR or CRR. These results contrast with others where pathogen abundance and disease severity for both FCR and CRR were influenced by crop rotation, cropping history, tillage system and use of glyphosate (19,20,21,22,24). Fernandez's work (19,20,21,22,24) was done in a more homogenous environment than occurred in this study, where topology and environment varied greatly across counties. In addition, the sampling in the Fernandez's studies were more robust as well. For these reasons, results for this study are considered less conclusive with respect to cropping factors and not contradictory to the assorted work of Fernandez.

To our knowledge, this is the first survey to assess crown and root rot of wheat using real-time qPCR. Incidences and populations as determined the qPCR assays were not significantly different from those obtained using traditional isolation methods commonly used in the past (4,12,14,17,18,19,20,25,26,44,46,53,55,57,58,63,65,66), suggesting that qPCR is an adequate tool to assess FCR and CRR in future surveys. In some respects, the qPCR assays appear to be superior to previous methods, particularly in providing a greater range of assessment for pathogen populations and corresponding disease severities. This appears to have led to a greater sensitivity for pathogen detection as both pathogens were detected in more fields with qPCR than with traditional isolation methods. In addition, these measures were not subject to interspecies interference that

can dominate culturing methods and which necessitated our switching *Bipolaris* assessment to a selective media. Additionally, differences in cost between the two methodologies are not actually so different. The cost of a field sample analyzed by qPCR was around \$14, considering DNA extraction and run in triplicate for FCR and CRR pathogens, while using the conventional plating in two growth medias (PDA-CLA and selective CRR media) the cost per field sampled was around \$8.2. However, when time of labour to process 20 field samples were considered, the time for qPCR analysis of the two pathogens required 21 hours, while the conventional method of identification and isolation spent ~38 hours of work. Then, when the difference in values of labour was around \$10/hr for 20 field samples were considered, the traditional method was \$170 dollars higher than the qPCR method.

For this survey, the qPCR assays were non-specific, detecting the FCR complex and the genus *Bipolaris*. These general assays are useful when generalized results are wanted like for FCR or where only one species from the said genus is present and pathogenic to the crop in question such as *B. sorokiniana* and common root rot of wheat in Montana. When these conditions are not met, other tools such as traditional isolations and/or specific PCR assays are needed. Already, specific PCR assays are available for *F. culmorum*, *F. pseudograminearum*, *F. graminearum* (2,49,50), *F. avenaceum* (49), *F. poae* (41), *F. acuminatum*, (67), and *F. oxysporum* (16) and others are likely to be developed in the near future as the demand increases.

In previous work, DNA copy numbers for the FCR complex were found to be at their highest at harvest and if crowns were left in situ these copy numbers dropped off steadily

until the spring of the next year (30). For this reason, samples taken for this study were taken within two weeks or less of harvest to minimize the effects of copy number instability. This self-imposed restriction was probably the largest drawback to the use of qPCR for surveys as it limited sampling to the busiest time of the year, a time when assistance is least available and without assistance, the short duration limited the number of fields sampled and the intensity of the sampling. How timing affects outcomes for competing methods is unknown and beyond the scope of this study. Certainly, it is likely that the isolation frequency for the FCR complex and *B. sorokiniana* would be maximized by the limiting sampling to within a few days of harvest. Similarly crown discoloration and/or bleaching probably vary relative to days before or after harvest. Would the effects of these traditional methods be similar to those that occur for qPCR is something worth exploring in the future?

This is the first report on the qPCR assay for *Bipolaris* developed for this study. Primers and probe from the glyceraldehyde-3-phosphate dehydrogenase-like gene sequence used in this assay were tested on 23 species likely to occur within Montana's survey and did not amplify any of these species. The assay's results corresponded well to results obtained using traditional isolation methods, which showed that assay was useful for these circumstances. Another closely related species, *B. maydis*, which is not common to this area, did amplify with the assay at levels similar to those seen with *B. sorokiniana* DNA. This was not unexpected as sequence information on other species within the genus is not available making species specific design challenging at best. At this time *B. maydis* is not prevalent within Montana, but it may become more common as

corn production spreads into the region. At that time, this assay may not be as useful tool for examining CRR in the field.

This survey is the first systematic assessment of FCR and CRR distribution and prevalence conducted in the area denominated “the Golden Triangle” of wheat crop production in the state of Montana. From this survey it was shown that CRR is the most widespread disease within “the Golden Triangle” production area of central Montana and while FCR and the pathogens that cause it are more variable throughout the region. In particular, FCR was not found in the silty loam fields of Judith Basin and Fergus counties. When severe both diseases cause significant losses. For CRR this could occur in 15% of the fields while for FCR this occurred in 12% of the fields. This is the first time qPCR was used to survey wheat fields for FCR and CRR and results from these analyses were comparable with traditional isolation methods but had key advantages and disadvantages that may need to be addressed. Analysis of environmental parameters, showed *F. culmorum* incidence is favored by cooler summers while *F. pseudograminearum* is favored by warmer summers as previously reported (12,13,40). Finally, populations of the FCR complex and *B. sorokiniana* negatively correlated with each other in 2008. While these correlations did not occur in 2009, reports from others suggest that this may be a real phenomenon that needs to be explored because of the practical control implications that it may represent (18,23,33,47,60). This possible antagonism or niche exclusion dynamic between these two diseases and associated pathogens may have practical implications. Development of fungicide seed treatments, cultivar resistance or changes in tillage practices to reduce FCR or CRR may not result in

the yield gains that one might expect as reduction losses due to one disease, because this yield gains would be partially compensated by increasing losses due to the other disease.

ACKNOWLEDGMENTS

This research was supported in part by funds from the Montana Wheat and Barley Committee and Montana Agricultural Experiment Station. We also appreciate the support and assistance of the county extension agents of the Montana State University Extension Service in the area surveyed. Thank are also given to the growers who collaborated with this project and Dr. Diane Cooksey for sharing with us the Map *Atlas* 6.0 software used in this study.

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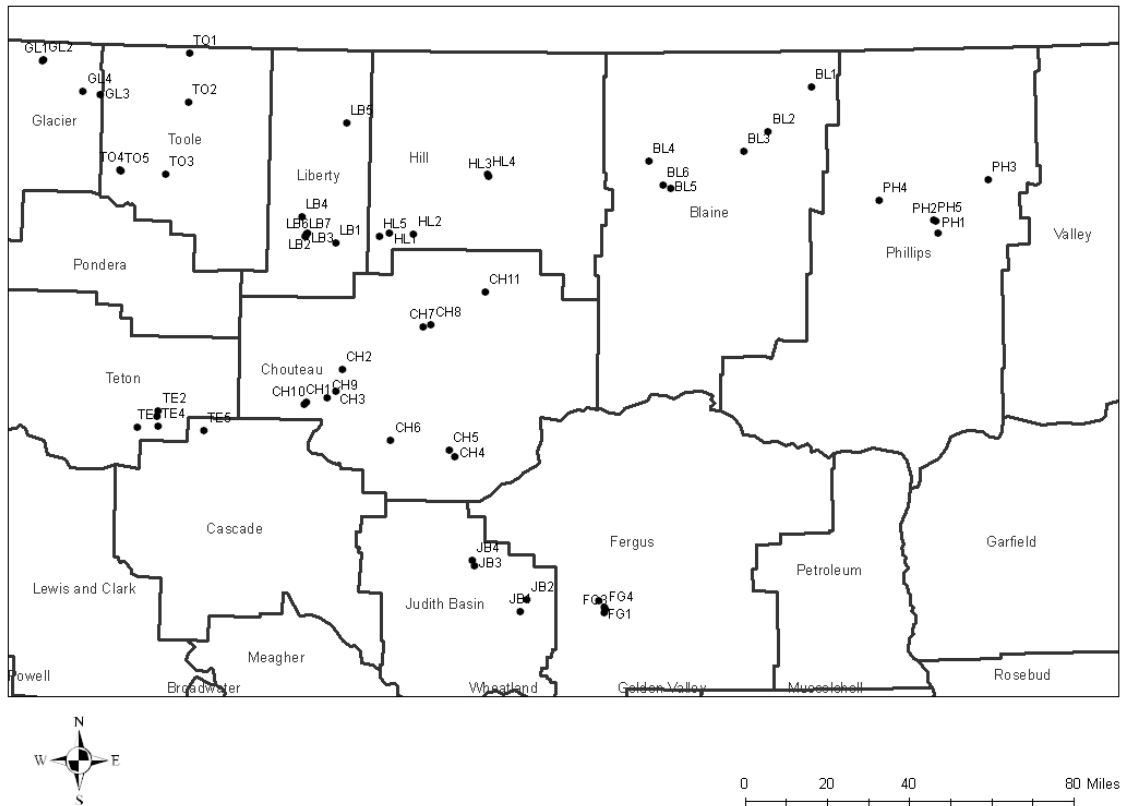
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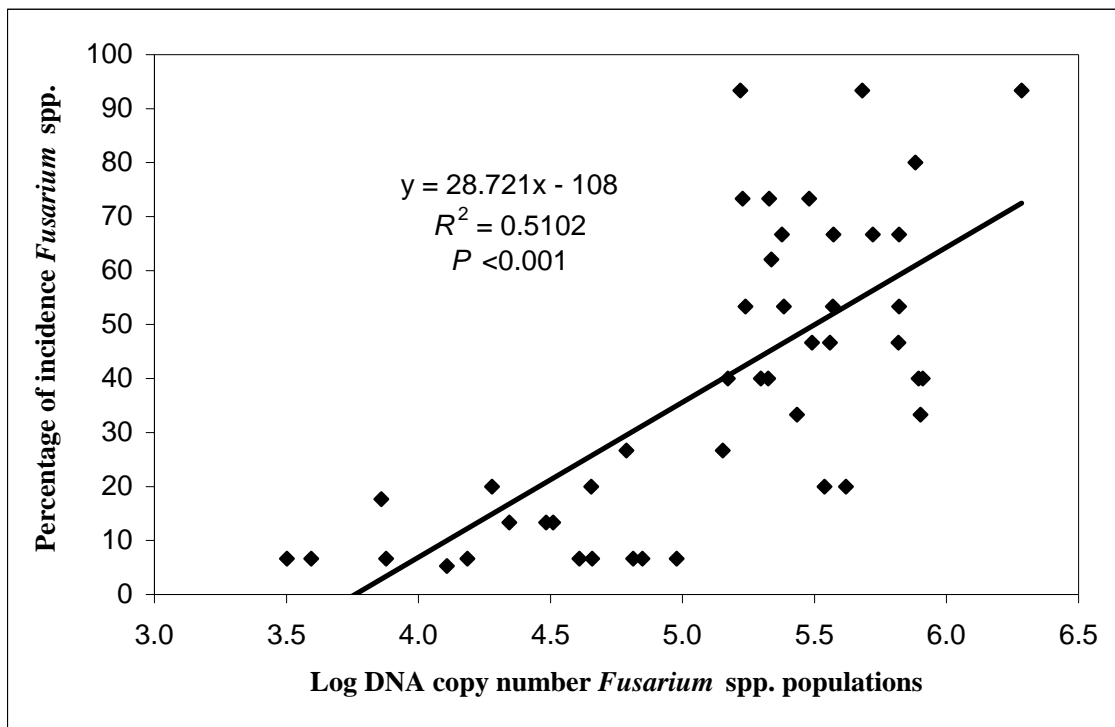
Fig. 1. Spatial distribution of survey sites¹ in ten Montana counties sampled during 2008 and 2009.



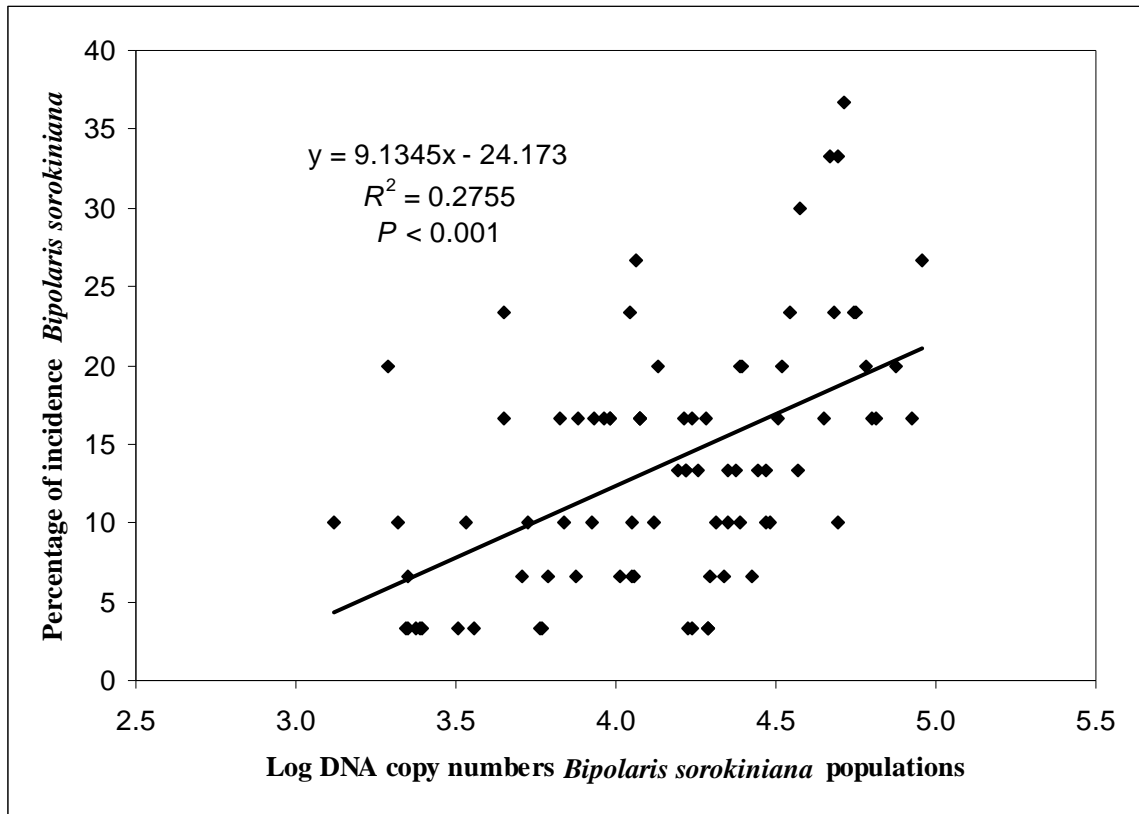
¹ Sampled fields coded by using the initial letters for each county where the field was located and the number assigned to each field in the county prior to taking the samples.

Fig. 2. Relationships between percentage of infected tillers as determined by traditional culturing methods and pathogen populations as determined by quantitative real-time PCR assays for *Fusarium* crown rot¹ (a) and common root rot² (b) pathogens. Zero values were trimmed from data sets to avoid variance restrictions. The resulting analyses were conducted across 46 and 83 fields for the *Fusarium* crown rot complex and *Bipolaris sorokiniana*, respectively.

a) Relationship for *Fusarium* crown rot (FCR)



b) Relationship for common root rot (CRR)



¹ Percentages of incidences by *Fusarium* spp. were based on the frequencies of isolation of *Fusarium culmorum* and *F. pseudograminearum* obtained by plating 3mm internode segments in PDA and re-isolation in Carnation leaf-agar (CLA). FCR TaqMan[®] probe was used to quantify *F. culmorum*, *F. pseudograminearum*, and *F. graminearum* populations in wheat internodes based in quantification of the DNA copy number of the *tri5* gene.

² Percentages of incidences by *Bipolaris sorokiniana* were based on the frequencies obtained by plating 3mm segment of the first internode on a *Bipolaris sorokiniana* selective media (57). CRR TaqMan[®] probe was able to quantify *Bipolaris sorokiniana* populations in wheat internodes based in quantification of the DNA copy number of the *gpd* gene.

Table 1. Incidence on a percent of infected tiller basis for pathogens causing Fusarium crown rot (FCR) and common root rot (CRR) as determined by plate culturing method of 3mm wheat internode segments in ten counties surveyed during 2008 and 2009 in Montana.

County	Sampled fields	Plated tillers ¹	Incidence (%) ²			
			<i>Fc</i>	<i>Fpg</i>	<i>Ftotal</i>	<i>Bs</i>
Judith Basin	8	120	0	0	0	19
Fergus	8	120	0	0	0	28
Phillips	8	120	10	17	27	16
Blaine	9	135	14	10	24	19
Chouteau	16	240	11	7	18	7
Liberty	11	165	4	10	13	18
Hill	9	135	1	4	5	17
Toole	9	135	23	5	28	12
Glacier	7	105	63	6	69	12
Teton	6	90	12	21	33	13
Total tillers	91	1365	174	103	277	210
Incidence (%)			13	8	20	15

¹ Total number of tillers plated in PDA and re-isolated in Carnation leaf-agar (CLA) for FCR pathogens or a selective *Bipolaris sorokiniana* media for CRR (57). Tillers have been used previously in a real-time quantitative PCR analysis of both diseases and fifteen internode segments were sampled per field in each one of the already mentioned nutritive media.

² Pathogens species causing FCR were *Fusarium culmorum* (*Fc*), *F. pseudograminearum* (*Fpg*), and *B. sorokiniana* (*Bs*) for CRR. Sum of total tillers infected by *F. culmorum* and *F. pseudograminearum* (*Ftotal*) are also shown.

Table 2. Average DNA copy number populations of Fusarium crown rot (FCR) and common root rot (CRR) determined by using real-time quantitative PCR for fields located in ten counties surveyed during 2008 and 2009 in Montana.

County	Sampled fields	DNA copy numbers	
		<i>Fusarium</i> spp. ¹	<i>B. sorokiniana</i> ²
Judith Basin	8	0	20,322
Fergus	8	0	22,050
Phillips	8	188,876	24,424
Blaine	9	222,831	32,377
Chouteau	16	161,594	12,607
Liberty	11	100,903	24,358
Hill	9	62,598	24,424
Toole	9	340,577	13,421
Glacier	7	250,580	7,234
Teton	6	157,010	5,476
Average populations		148,497	18,669
Total fields	91		

¹ FCR TaqMan[®] probe was able to quantify *Fusarium culmorum*, *F. pseudograminearum*, and *F. graminearum* populations in wheat internodes based in quantification of the DNA copy number of the *tri5* gene.

² CRR TaqMan[®] probe was able to quantify *Bipolaris sorokiniana* populations in wheat internodes based in quantification of the DNA copy number of the *gpd* gene.

Table 3. Pearson correlation coefficients between grain yield (g / m²) and log DNA quantities (copy number) of Fusarium crown rot (FCR) pathogens and common root rot (CRR) pathogen in 20 sampled sites (1.0-m²) for nine fields extensively sampled in North Central and Central region of Montana during the crop season 2008 and 2009.

Field ¹	Wheat Type	Cropping system	Year	Dominant pathogens ²	Pearson coefficient of correlation ³ (P value)	
					<i>Fusarium</i> spp. ⁴	<i>B. sorokiniana</i> ⁵
CH1	Winter wheat	Dryland	2008	<i>Fpg/Bs</i>	-0.488 (0.029)	-0.236 (0.315)
CH2	Winter wheat	Dryland	2008	<i>Fpg/Bs</i>	-0.210 (0.374)	-0.033 (0.889)
CH6	Spring wheat	Dryland	2008	<i>Fc-Fpg/Bs</i>	0.194 (0.410)	-0.249 (0.2879)
PH3	Spring wheat	Irrigated	2008	<i>Fpg/Bs</i>	0.232 (0.324)	-0.693 (<0.001)
CH9	Winter wheat	Dryland	2009	<i>Fc-Fpg/Bs</i>	-0.195 (0.408)	0.218 (0.354)
PH5	Winter wheat	Dryland	2009	<i>Fpg/Bs</i>	-0.462 (0.040)	0.174 (0.462)
BL5	Spring wheat	Dryland	2009	<i>Fpg/Bs</i>	0.326 ⁶ (0.1862)	0.009 (0.969)
GL1	Spring wheat	Dryland	2009	<i>Fpg/Bs</i>	-0.076 (0.749)	-0.422 (0.0637)
PH3	Spring wheat	Irrigated	2009	<i>Fpg/Bs</i>	-0.524 (0.012)	-0.637 (0.003)

¹ Fields were coded by putting the initial letter for each county where the field was located and the number assigned to each field in the county previously to take the samples in a regional survey conducted on the field. The irrigated spring wheat crop field coded PH3 was sampled during the two years of research.

² Dominant pathogens species were *Fusarium culmorum* (FC), *F. pseudograminearum* (Fpg) for FCR, and *B. sorokiniana* (Bs) for CRR. Dominance species in each field were determined by plate culturing method from data collected during a two years survey conducted in the North and Central regions of Montana.

³ Correlations among variables were considered significant at $P < 0.05$.

⁴ FCR TaqMan[®] probe was able to quantify *F. culmorum*, *F. pseudograminearum*, and *F. graminearum* populations in wheat internodes based in quantification of the DNA copy number of the *tri5* gene.

⁵ CRR TaqMan[®] probe was able to quantify *Bipolaris sorokiniana* populations in wheat internodes based in quantification of the DNA copy number of the *gpd* gene.

⁶ Value reported is the result of two outliers being removed from the data set. With the additional data points $r = 0.524$ and $P = 0.018$.

Table 4. Averages disease infections expressed as DNA copy number as determined by qPCR and percentage of incidence as determined by conventional isolation methods for Fusarium crown rot (FCR) and common root rot (CRR), and their associated pathogens observed in surveyed fields in seven different soil types in Montana (2008-2009).

Soil type ¹	Sampled fields	DNA copy numbers		Percentage of incidence ⁴		
		FCR ²	CRR ³	<i>Fc</i>	<i>Fpg</i>	<i>Bs</i>
<i>Gb5</i>	12	0.0 b ⁵	23,884	0.0 c	0.0 c	16.4
<i>Sg2</i>	23	123,224 a	18,063	15.4 b	9.3 bc	9.6
<i>Gb4</i>	4	298,737 a	8,624	58.3 a	0.0 c	15.9
<i>Sg1</i>	16	139,162 a	26,738	10.8 b	0.4 c	15.6
<i>Ap1</i>	8	150,902 a	25,713	3.3 bc	17.5 ab	17.5
<i>Og2</i>	8	152,990 a	22,323	8.3 bc	5.8 bc	9.6
<i>Pb2</i>	4	173,053 a	5,833	6.7 bc	28.3 a	5.8
Total fields	75					
<i>P</i> -value ⁶		0.005	0.089	0.005	0.029	0.055

¹ General soils of Montana: *Gb5* = Argiborolls-Calciborolls; *Sg2* = Argiborolls-Paleargids-Natrargids; *Gb4* = Argiborolls; *Sg1* = Argiborolls- Haploborolls; *Ap1* = Torrifluvents-Salorthids; *Og2* = (Mollisols) Argiborolls- Haploborolls; *Pb2* = Calciborolls-Calciorthids.

² FCR TaqMan[®] probe was able to quantify *Fusarium culmorum*, *F. pseudograminearum*, and *F. graminearum* populations in wheat internodes based on DNA copy number of the *tri5* gene.

³ CRR TaqMan[®] probe was able to quantify *Bipolaris sorokiniana* populations in wheat internodes based on DNA copy number of the *gpd* gene.

⁴ Pathogens species causing FCR were *Fusarium culmorum* (*Fc*), *F. pseudograminearum* (*Fpg*), while in CRR was *B. sorokiniana* (*Bs*).

⁵ Means followed by the same letter on each column were not significantly different according to a non-parametric multiple comparisons for the difference in ranks with a Wilcoxon test and Benjamini-Hochberg (BH) adjustment ($\alpha=0.05$). Mean separations

were done on copy numbers $\log(x+1)$ transformed for the DNA copy number of the *tri5* and *gpd* genes for FCR and CRR, respectively.

⁶ *P*- values obtained after to conduct Kruskal-Wallis rank sum tests for each variable.

CHAPTER 4

Population dynamics between *Fusarium pseudograminearum* and *Bipolaris sorokiniana* in spring wheat stems by using real-time qPCR

Contribution of Authors and Co-Authors

Manuscript in Chapters 4

Chapter 4:

Co-Author: Alan T. Dyer, Andy C. Hogg, and Barry J. Jacobsen

Contributions of Dr. Alan T. Dyer helped design and implement the field experiments and contributed with his insight in the statistical processing of the data. M.Sc. Andy C. Hogg collaborated with his expertise in developing a probe to quantify *Bipolaris sorokiniana* infection. Dr. Barry J. Jacobsen provided financial support and was the primary chair of the study. Dr. Dyer and Dr. Jacobsen worked on all parts of the manuscript through its successive revisions, reading and approving the final manuscript.

Manuscript Information Page

- Authors: Ernesto A. Moya, Alan T. Dyer, Andy C. Hogg, and Barry J. Jacobsen.

- Journal name: Plant Disease

- Status of the manuscript

Prepared for submission to peer-reviewed journal

Officially submitted to a peer-reviewed journal

Accepted by a peer-reviewed journal

Published in a peer-reviewed journal

- Publisher: The American Phytopathological Society

- Data of submission (for submitted manuscript)

- Date manuscript will appear (for accepted work)

- Issue in which manuscript appears in (for published works)

Population dynamics between *Fusarium pseudograminearum* and *Bipolaris sorokiniana* in spring wheat stems by using real-time qPCR

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ABSTRACT

Fusarium pseudograminearum and *Bipolaris sorokiniana* are the causal agents of Fusarium crown rot and common root rot of wheat respectively and cause significant losses worldwide. Population dynamics at the seedling stage between these two pathogens has been studied in the past, but there is no information about potential competition at late stages of wheat development. In three location years using cv. Hank spring wheat the effect of *F. pseudograminearum* and *B. sorokiniana* inoculum applied singly or in mixtures at seeding was measured using seedling stand, grain yield, and pathogen populations in the first internode at heading, milk and harvest stages of wheat development using real-time quantitative Polymerase Chain Reaction (qPCR). High and low rates of *F. pseudograminearum* inoculum reduced *Bipolaris* populations in the field trials ($P < 0.05$), but *B. sorokiniana* inoculations did not affect *Fusarium* populations. Fungal populations of both pathogens increased from heading until harvest and *Fusarium* colonized wheat stems earlier than *Bipolaris*. Despite the antagonism between their respective populations, *Fusarium* and *Bipolaris* did not prevent infection by each other in

the first internode of wheat stems. In comparison with naturally infested fields, inoculations increased incidence of infection and coinfection relative to natural settings observed for both pathogens ($P < 0.05$). At the seedling stage, both fungi alone or combined reduced the seedling stands when compared to a non-inoculated control for the three location years ($P < 0.05$). Grain yield and *Fusarium* populations were inversely correlated ($P < 0.05$), while *B. sorokiniana* populations did not show an effect on yield.

INTRODUCTION

Fusarium crown rot (FCR), and common root rot (CRR) diseases of wheat and other cereal agro-ecosystems are perennial problems that cause significant losses in the Northern Great Plains of North America and other semiarid regions of the world (28). In Montana and the Pacific Northwest, FCR of small grains is primarily caused by *Fusarium culmorum* (W. G. Sm.) Sacc., *F. pseudograminearum* (O'Donnell & T. Aoki; group I) (= *Gibberella coronicola*), and *F. graminearum* Schwabe (group II) (= *G. zaeae* (Schwein.) Petch) (15,28). In other regions, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ellis & Everh., *F. equiseti* (Corda) Sacc., and *Microdochium nivale* (Fr.) Samuels & Hallett (= *F. nivale* (Fr.) Sorauer) have been included in the crown rot disease complex (28). *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. Ex Dastur) is the causal agent of CRR and with FCR is part of a complex causing dryland seedling, foot, crown, and root rot in cereals crops in the Northern Great Plains and Canadian Prairies (10,12,13).

Fusarium populations and *B. sorokiniana* affect wheat and other cereals in every development stage from seedling to grain fill (20,28). Among the *Fusarium* species causing crown rot in Montana, *F. culmorum* causes the greatest losses to seedling blight while *F. pseudograminearum* and *F. graminearum* cause more severe late season disease (8,39). The adoption of no-till and conservation tillage system practices in wheat-fallow production systems has led to an increase of *F. pseudograminearum* populations in recent years (28,37). *F. pseudograminearum* is a strictly residue born pathogen that depends on infesting late season tillers for survival between cropping systems (30,37). It has been hypothesized that this is increasing the selection pressure on the pathogen to capture residues in order to survive the prolonged non-cropping period (crop/fallow cropping system) of Montana (8). The effects of tillage practices on *B. sorokiniana* are variable with *Bipolaris* being favored by conventional-till in barley crop productions (13) and wheat (7,12,26,32,45), while another study shows higher infection where stubble is retained than where it is removed (49). Recent surveys in Montana and Canadian Prairies have shown negative correlation between populations of FCR and CRR pathogens (10, Moya et al. 2010 as cited in this issue).

Given an increase in both FCR and CRR incidence and the negative association between the pathogens, it is important to understand the potential for antagonism between them. Dual inoculations studies between FCR and CRR pathogens have been conducted since the 1930's (Hynes 1938 cited by 22) to address potential interaction between these pathogens. Ledingham's work (22) determined an antagonistic effect on seedling emergence of wheat plant simultaneously inoculated with *F. culmorum* and *B.*

sorokiniana. Tinline (44) reported that prepossession of the internode by *B. sorokiniana* does not prevent subsequent invasion by the fusaria, but that prepossession by the fusaria greatly reduces subsequent infection by *B. sorokiniana* in studies of single or combination inoculation of wheat with *B. sorokiniana*, *F. culmorum*, and *F. acuminatum*. Scardaci and Webster (34) identified antagonism between *F. graminearum* and *B. sorokiniana* when they were co-inoculated in barley, resulting in lower levels of seedling blight and root rot. These authors also determined that when the pathogens were inoculated in sequence, one 21 days before the other, the pathogen inoculated first was re-isolated most frequently, indicating the importance of prior colonization and possession of substrate. Fernandez et al. (11) showed *F. acuminatum* inoculation significantly augmented the effect of *B. sorokiniana* infection when wheat seedling plants were inoculated with both fungi. However, it is important to understand that all of these studies were carried out using autoclaved soil to avoid masking the interaction of the cereal root rot pathogens by other microflora. This obviously does not simulate the environment confronted by these two pathogens in a crop field. Under field conditions, co-inoculations with *F. culmorum* and *B. sorokiniana* did not affect wheat emergence or yield (22) but data on the relative population interactions or the impact on late season crown and root rot was not reported.

The incidence of FCR caused by *F. pseudograminearum* is increasing in several areas of wheat production worldwide (2,29,33,38,39,40,46) and *B. sorokiniana* is widespread in the cereal agro-cropping systems worldwide (12,13,14,41,42,46,48). However, no studies assessing the interaction between populations of these two fungi in the ecological

niche of the wheat stems has been conducted and no studies assessing how the population dynamics change during different wheat development stages has been addressed.

Moreover, almost all studies of population interaction between these two diseases have been conducted and assessed at the seedling stage not at later stages of cereal development. Significant components of yield loss from the FCR and CRR disease occur during the post seedling growth stages and colonization of stem tissues post heading may be critical to identify differences in pathogen survival and inoculum potential.

Assessing pathogen population dynamics in wheat tissues has historically been done using isolation and culturing of targeted pathogen species. This methodology is labor-intensive and complicated by many factors such as competition and antagonism from other organisms in plant tissue, needed expertise in identifying species, media selectivity that favors isolation of certain fungi, and assay sensitivity (16). Additionally, investigation of the interdependence among pathogen species involved in the stem base of wheat is limited using plate cultures to confirm the presence or absence of individual species on individual tillers (31). With the advent of real-time polymerase chain reaction (qPCR), researchers have developed assays to accurately quantify population of FCR and CRR (15,16,43, Moya et al. 2010 as cited in this issue). Pettitt et al. (31), assessing competition and interdependence between *Fusarium* species in different fields, suggested that confirmation of interactions could probably be best achieved by using qPCR. Assessments of competition and interaction with co-inoculated experiments between ectomycorrhizal fungi and some plant pathogens by using real-time PCR have been

conducted recently and those studies have shown the utility of applying this technique to enumerate the populations of multiple pathogens in mixed plants infections (17,47).

Because both FCR and CRR incidence and severity are increasing under conservation tillage systems and because negative associations have been documented between the causal pathogens, it appeared important to assess their potential interaction within field situations particularly at post heading growth stages. To address this need, this study examined the disease and the pathogen community dynamics between aggressive pathogenic isolates of *F. pseudograminearum* and *B. sorokiniana* within inoculated wheat plants at seedling and at heading, milk and harvest stages of wheat development under field conditions using qPCR. These results were then compared with observations made on individual tillers collected from naturally infested commercial spring and winter wheat fields.

MATERIAL AND METHODS

Pathogen isolates and wheat cultivar. Field trials were conducted by assessing the population interaction between *F. pseudograminearum* isolate 2228 (Fpg 2228) and *B. sorokiniana* isolate 2344 (Bs2344) within the hard red spring wheat cv. Hank (Barkley Ag Enterprises, LLP [Bozeman MT]). Fpg 2228 was collected in Loma, MT (Liberty county) and it was chosen because it is an aggressive isolate and reduced yield on wheat (8), while Bs 2344 was collected in Carter, MT (Chouteau county), and it was chosen for the same characteristics as described for Fpg 2228 (Dyer, unpublished data). For both fungi, oat kernel inoculum was produced as described by Mathre and Johnston (27).

Field trials between *F. pseudograminearum* and *B. sorokiniana*. During the summer of 2008 and 2009 three experiments were conducted to assess the population dynamics between *F. pseudograminearum* and *B. sorokiniana* within spring wheat during seedling (Feekes stage 1), heading (Feekes stages 10.1 to 10.5), milk (Feekes stage 10.54), and harvest (Feekes stage 11) stages. Trials were conducted at the MSU-Arthur H. Post Research Farm (Bozeman, MT), during the years 2008 and 2009, and in 2008 one trial at the MSU-Central Agricultural Research Center in Moccasin, MT. Soil at the Bozeman sites is classified as Amsterdam silt loam, while the soil at the Moccasin site is classified as Judith clay loam.

In both locations and years, the experimental unit was a plot eight rows wide, seeded with rate of 200 seed per 3-m row with 30-cm centers. Plots of 3-m long were sown on May 5, 2008 and May 18, 2009 in Bozeman and April 28, 2008 in Moccasin. Plots were inoculated in-furrow at planting with Fpg 2228 and Bs 2344 colonized oat kernel inoculum with three different inoculation rates of Fpg 2228 and Bs 2344 forming 3x3 factorial combinations that was replicated 6 times for 54 plots. In-furrow inoculation rates were of 0 (none), and 1 (low), and 3 (high) g of colonized oat kernel inoculum per linear meter of row for both fungi. Field sites were top dress fertilized previous to seeding at rates of 67.2 kg of nitrogen/ha in Moccasin, 51.6 kg of nitrogen/ha in Bozeman during 2008 and 78.6 kg of nitrogen/ha in 2009. Weeds were controlled at both location and years by hand hoeing to prevent herbicide-pathogen interactions.

Plant emergence was assessed at the two-leaf stage (Feekes stage 1) between 22 to 25 days post planting. Total number of emerged seedlings was counted on the rows 2, 4,

and 6 on May 27, 2008 and June 02, 2009 in Bozeman, and May 20, 2008 in Moccasin. Thirty tillers were randomly chosen from each plot at heading stage (Feekes stages 10.1 to 10.5), milk stage (Feekes stage 10.54), and harvest (Feekes stage 11) for DNA extractions and then analyzed by qPCR. During heading and milk stages sampling times, stems were collected from the 2nd and 7th rows, and the rows 3 to 6 on the plot were left for yield evaluations. Stem collection at the heading stage was done on July 11, 2008 and July 16, 2009 in Bozeman and on July 08, 2008 at Moccasin. Milk stage stem collection was conducted on July 18, 2008 and August 06, 2009 in Bozeman, and July 21, 2008 in Moccasin. Stem collection in harvest stage was done after harvesting from the central four rows of the plot on August 27, 2008 and September 16, 2009 in Bozeman, while in Moccasin stems were collected on August 22, 2008.

The four rows of each plot in Moccasin was harvested with a Wintersteiger plot combine (Wintersteiger USA Inc, Salt Lake City, UT), while the four center rows of each plot in the experiment in Bozeman were harvested for yield with a small bundle rice binder (Mitsubishi Agricultural Machinery Co., Ltd., Tokyo, Japan) and threshed using a Vogel thresher (Bill's Welding, Pullman, WA).

DNA isolation. To isolate pathogen DNA within wheat tillers, the leaf tissue was removed from the 30 collected tillers and 3 mm stems sections were cut from the base of the first internode of each tiller. Ten 3 mm tissue sections were placed in a tube along with 100 mg garnet and two ceramic balls, and then three tubes were used for each field plot sample for each date of assessment. Bulked DNA was then extracted from the tiller sections using the Fast-DNA kit (QBiogene Inc., Irvine, CA) as per the manufacturer's

instructions and following modifications previously described by Hogg et al. (15). The resulting DNA samples were diluted 1:10 with molecular grade water for qPCR analysis.

Quantification of pathogens from first internode infections. Real-time qPCR used to quantify populations of *B. sorokiniana* and *F. pseudograminearum* within plant tissues used probes dual-labeled with the 6- carboxyfluorescein (6-FAM) fluorescent reporter dye and the 6-carboxyltetramethylrhodamine (TAMRA) fluorescence quencher. The primers/probe combinations used for quantify *F. pseudograminearum* populations was previous described (43). For *B. sorokiniana*, primers and the probe were designed using reported sequences of glyceraldehyde-3-phosphate dehydrogenase-like (*gpd*) gene sequence for isolate ND93-1, Gene Bank accession no. EF513209.1 as was described by Moya et al. (2010) in this issue. The primer-probe combination used for *B. sorokiniana* was previously shown to be specific to this specie (Moya et al. 2010 as described in this issue). All qPCR assays were performed using the Rotor gene Q (QBiogene Inc., Irvine, CA) and a two-step protocol with the following thermocycling parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The reaction mixes for qPCR contained (25 µl) 12.5 µl of Universal TaqMan Master Mix (PE Applied Biosystems), 2.5 µl of 2 µM TaqMan probe, 2.25 µl of a 1:1 mixture of 20 µM forward and reverse primers (Integrated DNA Technologies, Inc. Coralville, IA), 2.75 µl of molecular grade water, and 5 µl of DNA sample. Each run included four 10-fold-dilution standards, ranging from 6.6 million copies down to 6,600 copies. For *Fusarium* and *B. sorokiniana* the DNA standards used for absolute quantification during qPCR was a cloned fragment of the *tri5* and *gpd* genes taken from *F. culmorum* isolate 2223 (15) and

B. sorokiniana isolate 2234, respectively, and the process of quantification followed the protocol as described by Moya et al. (2010) in this issue. In this article, the amount of DNA copy numbers of the *tri5* gene was considered as the quantified population of *Fusarium* spp. of the FCR complex (*F. culmorum*, *F. pseudograminearum*, and *F. graminearum*), while the amount of DNA copy numbers of the *gpd* gene was considered the quantified population of *B. sorokiniana*.

Evaluation of *Fusarium* spp. and *B. sorokiniana* infections in the first internode of individual wheat tillers. DNA quantification of *Fusarium* spp. and *B. sorokiniana* populations in the first internode of individual tillers was conducted to assess the dynamics between the two fungal species in experimentally inoculated wheat tillers and tillers collected from naturally infected fields. In 2008, twenty symptomatic tillers were obtained from the spring wheat field experiment inoculated with FCR and CRR pathogens at Bozeman and from naturally infested spring wheat (Shonkin, MT), and a winter wheat (Carter, MT) naturally infected with both diseases. During 2009, 20 tillers showing symptoms of the disease were obtained from the inoculated spring wheat field experiment in Bozeman and from naturally infested spring wheat (Cut Bank, MT) and winter wheat (Malta, MT). In total 20 tillers were collected and assessed from naturally infected spring and winter wheat fields and co-inoculated plot fields each year. Tillers of the spring wheat field trials were chosen after harvest from the plots inoculated with a mix of high level of Fpg 2228 and Bs 2234 (3 g of infected oat kernels per linear meter of each fungus) from the experimental sites at Bozeman and from symptomatic disease tillers chosen from the naturally infested commercial crop wheat fields obtained a week

before harvest. From each stem, a 3 mm section from the base of the first internode was cut and the DNA extracted and quantified for populations *Fusarium* species and *B. sorokiniana* following the methodology described above.

Statistical analyses. Multifactor ANOVAs considering locations (Moccasin 2008, Bozeman 2008 and 2009), and inoculation treatments (3x3 possible inoculum combinations of Fpg 2228 and Bs 2234) and interaction between these two factors were conducted for plant emergence, DNA copy numbers of each assessed gene quantified for each date of assessment (heading, milk and harvest stage), and grain yield. If the factor location was significant ($P < 0.05$) for a variable response, this variable was analyzed separately through a randomized complete block design analysis for each field trial. Multifactor ANOVAs and randomized complete block design analysis were performed by the procedure PROC GLM (Statistical Analysis System (SAS) Institute Inc., version 9.2, Cary, NC) and the least significant difference (LSD) range test was used to compare means ($P < 0.05$). Prior to analysis, DNA copy numbers quantified through qPCR were $\log(x + 1)$ transformed and their variances were analyzed for homogeneity (Levene test, $P > 0.05$). After the multifactor ANOVAs were conducted, an analysis of covariance was performed on DNA copy number quantified through qPCR in each wheat growth stage by using PROC MIXED with the REPEATED statement for repeated measures (SAS Institute), where several covariance matrices were evaluated and the independent structure of covariance between each date of assessment was chosen because it had the lowest Akaike information criterion (AIC) score with the fewest number of covariance parameters. This implied that DNA copy numbers quantified at each different stage were

independent from one time of assessment to the next, and then this situation allowed using an univariate analysis split plot in time to assess competition between both pathogen population in wheat tillers, where the independent variables were different inoculum concentration of each fungus (high, low, and none), fungal population (*Fusarium* populations and *B. sorokiniana* population), and date of assessment (heading, milk stage, and harvest). Univariate analysis split plot in time for the randomized complete block design was performed by the procedure PROC GLM and LSD test was used to compare means ($P < 0.05$). Relationship between grain yield and level of infection in each disease assessment date (seedling counts, heading, milk stage, and harvest) were evaluated through correlation and regression analyses by using PROC COR and PROC REG (SAS Institute), respectively.

Evaluation of the *Fusarium* spp. and *B. sorokiniana* population infection in individual wheat tillers were conducted on the populations obtained from the forty tillers collected from naturally infected wheat fields and co-inoculated plot fields during the two year of research. Populations of each pathogen observed in wheat tillers during each year were grouped to meet requirements of the statistical test. Number of tillers infected by FCR and CRR pathogens, co-infected tillers, and no infected by the pathogens in each group was determined. Comparisons of observed and expected proportions of each described category of infection among fields were conducted through chi-square tests of homogeneity ($P < 0.05$) and expected proportion of each category within each field was conducted through a chi-square good-of-fitness test ($P < 0.05$). Wilcoxon signed rank tests with continuity correction were used to compare population of both pathogens

populations as quantified by qPCR for the observation combined between the two years from the tillers collected from naturally infected wheat fields and co-inoculated plot fields. The package “Rcmdr” of the R software (www.r-project.org) was used to conduct these non-parametric analyses.

Dependency between *Fusarium* and *B. sorokiniana* populations quantified by qPCR at harvest for plots from each location and for the individual tillers collected from the inoculated and naturally infected fields were determined through correlation analyses using PROC COR (SAS Institute). Spearman correlations were considered to avoid distribution effect of analyzed variables.

RESULTS

Seedling counts were significantly affected by the inoculation with both fungi at the three trial locations (Table 1, $P < 0.001$), and interaction between inoculation treatment combinations and location was observed ($P < 0.001$). Across locations, *Fusarium*, *Bipolaris* and combined *Fusarium* and *Bipolaris* inoculations reduced seedling counts by 19.2, 11.9 and 26.7% respectively. In general, use of inoculum of both Fpg 2228 and Bs 2344 used alone or combined reduced in average seedling counts for Moccasin by 30.7%, for Bozeman 2008 by 14.8%, and for Bozeman 2009 by 19.8% when compared to the non-inoculated plots. Locations showed different numbers of emerged seedling among trials ($P < 0.001$), with the field trial at Bozeman 2008 showing 16.6% and 35.6% more emerged seedlings than at Bozeman 2009 and at Moccasin 2008 field trials respectively. The Bozeman 2009 field trial had 22.7% more seedlings than the field experiment in

Moccasin. At the Bozeman 2008 and Moccasin trials, the treatment inoculated with high rates of both pathogens had the greatest reduction in seedling counts (24.8 and 57.2%, respectively), but this treatment was not different from the combination of low rates of Fpg 2228 and high rates Bp 2344 in the Bozeman 2008 trial. In the Bozeman 2009 field trial, the treatments with high rates of Fpg 2228 alone or mixed with high or low rates of Bs 2344 caused the greatest reductions in seedling counts (32.7%, 29.9% and 26.6% of reduction, respectively). All field trials showed that treatments inoculated with high rates of Fp 2228 and Bs 2344 alone or combined with other rates of both pathogens inoculum were significantly different of the non-inoculated control ($P < 0.001$) and had the greatest effects on seedling stands.

Populations of *Fusarium* spp. and *B. sorokiniana* as expressed by DNA copy numbers of the *tri5* and *gpd* genes respectively in the three different late stages of plant growth (heading [Feekes stages 10.1 to 10.5], milk [Feekes stage 10.54], and harvest [Feekes stage 11]) were significantly different between them ($P < 0.001$) and between locations ($P < 0.001$). Therefore results for each field trial and plant stage were analyzed individually to fully observe the dynamics involved. For Moccasin, low and high rates of Fpg 2228 inoculum had significantly increased *Fusarium* spp. populations relative to the non-inoculated plots ($P < 0.001$; Fig. 1a-1). Populations of *Fusarium* spp. were not affected by either rate of Bs 2234 inoculum (Fig 1a-2). The high rate Fpg 2228 inoculum resulted in a reduced population of *B. sorokiniana* in the first internode of tillers ($P < 0.001$; Fig. 1a-1). The high and low rates of *Bipolaris* populations were not different from the non-inoculated plots (Fig 1a-2). The population of *Fusarium* and *Bipolaris* at the Moccasin

location were different at the three late stages of wheat development ($P < 0.001$, Fig. 2) with the lowest level of *Fusarium* spp. populations observed during heading (125,346 DNA copies *tri5* gene), and highest level of this pathogen were observed at harvest (3,168,844 DNA copies *tri5* gene) (Fig. 2; Table 2). *B. sorokiniana* populations were similar during heading (5,889 DNA copies *gpd* gene) and milk stage (11,012 DNA copies *gpd* gene), but increased markedly at harvest (192,642 DNA copies *gpd* gene). Also, significant reductions of *B. sorokiniana* populations were observed in plots single-inoculated with Fpg 2228 at milk and harvest stages when means comparison among individual inoculum treatments were analyzed (Table 2).

The 2008 field trial at Bozeman showed similar patterns of ecological competition between the populations of the two fungi as was described for Moccasin. Plots inoculated with *Fusarium* increased the population of the pathogen relative to the non-inoculated plot, and this increase was associated with reduction of *B. sorokiniana* populations (Fig 1b-1). Populations of *B. sorokiniana* in non-inoculated plot were not different from the high and low inoculated rates of Bs 2344 ($P = 0.104$) and populations of *Fusarium* were not affected by presence of *Bipolaris* inoculum (Fig 1b-2). Comparison among wheat development stages showed the same level of *Fusarium* infection between heading and milk stages and significantly higher populations at harvest (Fig. 2; Table 2). *B. sorokiniana* had low populations during heading stage as observed in Moccasin, but high populations occurred during milk stage. The effects of Fpg 2228 inoculum on *Fusarium* populations were most dramatic in Bozeman 2008 plots and were much more modest in Moccasin and at Bozeman in 2009. For example, populations of

Fusarium in Bozeman 2008 were in average 15 times greater as indicated by DNA copies of *tri5* gene than at Moccasin at heading (Table 2). Plots only inoculated with high rates of Fpg 2238 showed a clear reduction of *B. sorokiniana* population during heading and harvest, while this phenomenon was only observed at milk and later harvest stages in Moccasin.

Although the population dynamics displayed in Bozeman in 2009 were less dramatic than in the other two trials, they followed similar patterns. *Fusarium* inoculations positively affected *Fusarium* populations and negatively affected *Bipolaris* populations while *Bipolaris* inoculations did not affect either (Fig 1c-1; Fig. 1c-2). For 2009 in general, *Fusarium* and *Bipolaris* populations were moderate, never attaining the level observed in 2008 trial (Table 2). Populations of *Fusarium* were lower at heading and similar at milk stage and harvest stage, while *B. sorokiniana* populations increased from heading to harvest (Fig. 2). Significant differences among inoculated treatments and the control non-inoculated were not observed for the Bozeman 2009 site (Table 2).

Fusarium colonized of wheat tillers earlier than *Bipolaris* populations. Progression of the disease in the field trial showed that at heading, *Fusarium* had an average of 36.8% of the final populations observed at harvest already established in the stems, while *B. sorokiniana* populations at heading had established only 4.5% of the population finally observed at harvest. For *Fusarium* populations, at heading were 3.9%, 64.8% and 105% of the final population observed at harvest in the field trials in Moccasin and Bozeman 2008 and 2009, while *Bipolaris* populations were 3.0%, 8.0% and 3.8%, respectively.

Grain yield over the three location-years showed significant differences for the different inoculum treatment combinations used for both fungi ($P < 0.001$). Average grain yields were 1162.7 kg/ha in Moccasin, 1842.7 kg/ha in Bozeman 2008, and 3345.7 kg/ha in Bozeman 2009 trial. For the Bozeman 2008 field trial, plots inoculated with *Fusarium* had significantly lower grain yields and those yields were significantly different from both the non-inoculated control and the treatments inoculated only with *B. sorokiniana* ($P < 0.001$). Inoculation with only high or low rates of *B. sorokiniana* inoculum did not reduce grain yield in comparison to the non-inoculated plots. These patterns were not observed in Moccasin and Bozeman 2009 field trials, where grain yields were not significantly different among the inoculum treatments. During 2008, the field plots in Bozeman were affected by an unusual hailstorm during grain fill (early milk stage, Feekes stage 10.54) and environmental conditions were drier and hotter compared to the long-term averages registered in that location.

The relationships between seedling establishment and grain yield was only significant for the Bozeman 2009 site ($r = 0.385$; $P = 0.004$), while fungal populations vs. grain yield were only negatively correlated and significant for *Fusarium* populations in the Bozeman 2008 and the Moccasin trials ($r = -0.624$, $P = <0.001$; $r = -0.384$, $P = 0.004$, respectively). A significant linear relationship existed between *Fusarium* populations and yield grain in Bozeman 2008 ($R^2 = 0.505$; $P = <0.001$). *Fusarium* population and grain yield were not significantly correlated for the Bozeman 2009 field trial. *Bipolaris* populations did not affect yield in the three field trials (Moccasin $P = 0.738$; Bozeman 2008 $P = 0.382$;

Bozeman 2009 $P = 0.544$) and effects of *Bipolaris* population on grain yield could be not determined by these field trials.

Dependency between copy numbers of *Fusarium* spp. of the FCR complex and *B. sorokiniana* at plot level only showed a significant inverse correlation for both populations at Bozeman 2008 trial (Spearman rank coefficient: $r = -0.387$, $P = 0.004$), while the Bozeman 2009 trial had a positive correlation between both diseases (Spearman rank coefficient: $r = -0.387$, $P = 0.004$) and the Moccasin 2008 trial was not significant.

In 2008, weather was relatively warm and dry in Bozeman compared to the long-term averages registered in that location. Precipitation was 83.8, 67.6, 32.5 and 17.8 mm and average temperatures were 12.5, 19.6, 26.8 and 25.9°C for May, June, July, and August, respectively. Weather conditions in Moccasin were cooler compared to Bozeman and were wet at beginning of the trail and relatively drier during summer months. Precipitation was 109.7, 74.7, 11.4 and 22.6 mm and average temperatures were 9.8, 13.6, 19.3 and 19.4 °C for May, June, July, and August, respectively. In comparison with 2008, weather in 2009 was relatively wetter and cooler in Bozeman. Precipitation was 40.9, 66.5, 70.9 and 38.4 mm and average temperatures were 17.1, 17.8, 23.7 and 23.7°C for May, June, July, and August, respectively.

Evaluation of Fusarium spp. and B. sorokiniana infections in the first internode of individual wheat tillers. High coinfection of the individual lower internode by *Fusarium* spp. and *B. sorokiniana* were observed in co-inoculated tillers collected along 2008 and 2009 from the experimental sites in Bozeman compared to individual tillers collected from naturally infected fields (Fig. 3). The proportion of lower internodes single

infected, co-infected and non-infected by *Fusarium* and *Bipolaris* as detected by qPCR were similar for the naturally infested spring and winter wheat ($\chi^2 = 2.177, P = 0.537$). When naturally infested lower internodes spring or winter wheat were compared to the inoculated spring wheat the chi-square showed a significant effect of inoculation (spring wheat vs. coinoculated $\chi^2 = 17,135, P = <0.001$; winter wheat vs. coinoculated $\chi^2 = 26.876, P = <0.001$). In the inoculated plots at Bozeman *Fusarium* was identified from 95% of the lower internodes compared to naturally infested fields where infections were detected in 60% of spring wheat and 45% of winter wheat lower internodes. For *Bipolaris*, the inoculated plots at Bozeman showed that this fungus was identified from 75% of the lower internodes compared to 52.5% of spring wheat and 55% of winter wheat lower internodes from naturally infested fields. Wilcoxon signed rank test with continuity correction showed that population of *Fusarium* spp. as expressed by DNA copy numbers of the *tri5* gene observed in individual lower internodes for tillers collected from the co-inoculated plots in Bozeman (mean = 188,210 DNA copy numbers *tri 5* gene, SD = 235,124, range = 0-909,154) were not different from the FCR populations observed in naturally infested tillers collected from spring wheat fields ($V = 279, P = 0.123$) but different from those collected from winter wheat fields ($V=85, P <0.001$), while the spring wheat tillers showed higher FCR populations than the winter wheat tillers ($V= 357, P = 0.011$ [spring wheat fields: mean = 108,575 DNA copy numbers *tri 5* gene, SD = 165,185, range = 0-729,407; winter wheat fields: mean = 24,121 DNA copy numbers *tri 5* gene, SD = 62,779, range = 0-285,228]). Populations of *B. sorokiniana* as expressed by DNA copy numbers of the *gpd* gene were not different between spring and

winter wheat naturally infected fields ($V=229$, $P=0.717$ [spring wheat fields: mean = 518 DNA copy numbers *gpd* gene, $SD=1,663$, range = 0-8,625; winter wheat fields: mean = 404 DNA copy numbers *gpd* gene, $SD=1,096$, range = 0-6,294]), while population of CRR in the Bozeman experimental sites (mean = 6,562 DNA copy numbers *gpd* gene, $SD=13,492$, range = 0-25,868) were different from the CRR populations quantified for spring wheat ($V=81$, $P<0.001$) and winter wheat fields ($V=118$, $P=0.001$).

Dependency between copy numbers of *Fusarium* spp. of the FCR complex and *B. sorokiniana* in individual tillers showed a significant and negative correlation for the tillers collected from the coinoculated plots (Spearman rank coefficient: $r=-0.713$, $P<0.001$) and for the tillers showing infection by one or both diseases collected from the naturally infected fields (Spearman rank coefficient: $r=-0.507$, $P<0.001$). Infected tillers collected from both spring wheat and winter wheat naturally infested fields had a negative correlation (spring wheat Spearman rank coefficient: $r=-0.480$, $P=0.011$; winter wheat Spearman rank coefficient: $r=-0.613$, $P<0.001$). The same was observed for coinoculated tillers collected during different years from the experimental site in Bozeman, but the effect was more noticeable during 2008 crop season (Spearman rank coefficient: $r=-0.886$, $P<0.001$) because tillers infected by both pathogens were only significant at 10% during 2009 (Spearman rank coefficient: $r=-0.388$, $P=0.091$). Negative correlations for tillers obtained from natural fields were only significant when tillers showing no infection detected by qPCR were removed.

DISCUSSION

For this project, increasing rates of *F. pseudograminearum* inoculum were associated with increasing populations of *Fusarium* and corresponding decrease in *B. sorokiniana* populations within the lowest internode of wheat stems. Because the high indigenous populations of *B. sorokiniana*, it was not possible to evaluate the reciprocal association. The practical implication of this implied antagonism between these pathogens may be that developing control for FCR may not result in the yield gains that one might expect since reduction in FCR related losses brought about by control of FCR would be partially compensated for by increasing in losses due to CRR. How much compensation could be expected is hard to know. From data collected under natural field situation for this project, almost 50% of the crown rot samples displayed co-infection in which control of the FCR would likely be replaced by an increase in common root rot damage. Whether the reciprocal compensating dynamic occur is an important question that needs to be addressed. Past studies with the closely related *F. culmorum* suggest that *B. sorokiniana* may not have a reciprocal effect on *Fusarium* populations (34,44) and therefore suppression of CRR may yield results more directly proportional to this disease's importance in the wheat agroecosystem.

Why *Fusarium* populations suppress *B. sorokiniana* in the lower internode is unknown. Direct antagonism mediated by antibiosis between *B. sorokiniana* and *F. pseudograminearum* mycelium has been not observed under *in vitro* conditions (36). Nevertheless, Ledingham (22) reported inhibited germination of *B. sorokiniana* spores by the closely related species *F. culmorum*. Other reports have linked the mycotoxin

deoxinivalenol (DON) with reduction of gene expression in *Trichoderma atroviride* (25). However, *in vitro* experiments using filter discs amended with DON (100 µg of DON / µl), we could not show reduced growth of *B. sorokiniana* isolate 2234 (data not shown). Within wheat tissue, both Tinline (44) and Scardacci and Webster (34) reported *F. culmorum* and *F. graminearum* infection, respectively, prevented later *Bipolaris* infections. If this exclusion dynamic dominates these interactions under natural field conditions one would expect a deficit in co-infections under field conditions. This was not observed in the trials reported here, but more study of its potential appears warranted. Another explanation for the dynamics observed would be that resource competition was involved. Inverse dependence between both pathogen populations was observed at harvest in the plot in Bozeman 2008 and for tillers collected from naturally infected and co-inoculated sites, which suggested a competition dynamic between the pathogens for the first internode. This dynamic for both pathogen populations would be occurring at tiller and plot levels, but also occurs at regional levels as has been reported by Fernandez and Jefferson in the Canadian Prairies (10) and observed by us in a Montana survey (Moya et al. 2010 as cited in this issue). If resource competition is involved, one would expect classic zero sum dynamics to be involved with associated prediction of community stability and compensatory population dynamics. These were not tested within these trials but should be examined in the future. High colonization of lower internodes by *Fusarium* populations at the heading or milk stage was demonstrated in these studies, while *Bipolaris* populations were not seen increasing within lower internode until harvest stage. These results are in concordance with the observation of

Tinline (44) and Scardacci and Webster (34), who determined that other *Fusarium* spp. preempt *Bipolaris* infection. This phenomenon could be suggesting a “priority effect”, where an early colonist negatively affects the performance of later arrivals through preemption of shared resources (1,35). This phenomenon has been described recently playing a major role in dynamics of early colonization of pine seedlings by ectomycorrhizal (EM) fungi (18,19).

In addition to microbe-microbe interactions, the reduction in the population of *B. sorokiniana* by *Fusarium* populations could have been affected through indirect antagonistic interactions mediated by the plant, as it was suggested for some ectomycorrhizal interactions (18). Several studies have revealed evidence of pathogenesis-related (PR) proteins induced by *F. pseudograminearum* and *B. sorokiniana* in wheat plants (4,5,6,23,24). The reduction of *Bipolaris* populations could be caused by elicitation of a resistance response by the fungal populations in the wheat plant, which presumably do not affect *F. pseudograminearum*, but are capable of attenuating *B. sorokiniana* colonization of roots, crowns, or culms. Recently, this model was suggested to explain the interaction between *Phytophthora medicaginis* Drechs. and *Aphanomyces euteiches* Drechs. in alfalfa (47).

The results from individual tiller evaluation showed that use of pathogen inoculation resulted in greater incidence of infection by *Fusarium* and *Bipolaris* and therefore greater incidence of coinfection than that observed in natural settings. This also suggests that the competitive effects witnessed in the field trials are less common under natural field conditions. Differences observed between inoculated trials and naturally co-infested

fields provides us with a warning that these studies must examine interaction dynamics in the natural setting in order to place the dynamics into their proper agroecological perspective. From the agronomical point of view, these results showed that a high level of inoculum in field trials would overestimate the incidence and level of antagonism present in naturally infected fields.

During the establishment of the crop, co-inoculations reduced seedling stands more than single inoculations with either pathogen. In addition, an increase in inoculum levels was associated with greater reduction in the emergence for the three trials. These results were in accordance with the results obtained by the classical mixed experiments conducted by Hynes (cited by 9 and 22), who demonstrated that disease expression at seedling stage was considerably enhanced by co-inoculation with *B. sorokiniana* and *F. culmorum*. However, these results were different from the results of Ledingham's (22) field experiments, where combined inocula of *B. sorokiniana* and *F. culmorum* did not lower seedling emergence as much as the more aggressive of the single pathogen inoculations. Statistical interactions between concentration of inoculum from Fpg 2228 and Bs 2344 were not observed (data not shown). This situation suggests that the reduction in seedling counts by combination of inoculum would be associated with the increased inoculum levels represented by the combined inocula and not due to filling complementary niches. In contrast to seedling stand, yield was only reduced by inoculation with *Fusarium* in 2008. Inoculation with *B. sorokiniana* did not reduce yield significantly over the non-inoculated control and co-inoculations did not show any additional effect on yield above that seen with individual inoculation with *Fusarium*.

According to these results, *Fusarium* spp. populations could be considered a more aggressive late season pathogen and better colonizers of lower internodes than *B. sorokiniana* on wheat. Unfortunately, late season populations of *Bipolaris* were only marginally increased by inoculations and therefore their relative effect on *Fusarium* populations and other parameters such as yield cannot be well ascertained by the results of this study.

This study only examined community dynamics at the base of the first stem internode and the competitive relationship elsewhere in the plant were not addressed. Infection by *B. sorokiniana* has long been associated with infections of the subcrown internode and culm bases (9,34,44) and this has been associated with yield losses (21). For this reason, the assessment of rot at the subcrown internode has been commonly used to define CRR damage (10,12,13,41,42,48,49,50). If *B. sorokiniana* has some adaptation favoring its colonizations of subcrown internodes, sampling of the first stem internode could bias the population dynamics observed by this study toward *Fusarium*. Whether the dynamic between these two pathogens be reversed if the focus of population studies was on the sub-crown internodes is something worthy of further study.

The effect of environmental factors on population dynamics of *Fusarium* and *Bipolaris* were observed by this study. Prevailing environmental conditions accelerated or delayed the progression of *F. pseudograminearum* and *B. sorokiniana* across field trials. This was observed at Bozeman 2009 site, where inoculation did not result in significant increases in pathogen populations due to uncommonly high summer rainfall and lower temperature that did not favor the disease development. In contrast, warmer and drier

conditions observed during summer months for both locations in 2008 favored FCR (3,28), and CRR development (48) and inoculations were more effective and population dynamics were better observed as the result.

In conclusion, the competition dynamics between both *F. pseudograminearum* and *B. sorokiniana* showed that rates of *F. pseudograminearum* inoculum were associated with reduction of *Bipolaris* populations in the first internode in the field trials. This may have implications for disease control strategies in the future as control of FCR may lead to replacement by CRR. The reciprocal dynamics are unknown. Population of *B. sorokiniana* may or may not reduce *Fusarium* populations as high native populations of *Bipolaris* obscured these dynamics in these experiments and prevented conclusion being drawn. Both *Fusarium* and *Bipolaris* populations in the wheat stems increased from heading through harvest with *Fusarium* preemptively colonizing lower internodes relative to *Bipolaris*. Despite effects on their community dynamics, *Fusarium* and *Bipolaris* did not prevent infection by the other in the first internode of wheat plants. Inoculation significantly increased incidence of infection and co-infection relative to natural settings and may have increased the levels of competition observed relative to natural setting. These trials demonstrated the utility of qPCR technique to examine the population dynamics of multiple pathogens in mixed plant infections under field conditions and showed the importance of comparing the level of infection developed in inoculated experimental trial with real world agro-ecological systems managed by growers. These results demonstrate the important role competition plays amongst these two pathogenic fungi on wheat plants. Additional studies are warranted to understand

this interplay within different disease systems and how variability within species such as *F. pseudograminearum* and *B. sorokiniana* affect their interspecies competition.

ACKNOWLEDGMENTS

This research was supported in part by funds from the Montana Wheat and Barley Committee and the Montana Agricultural Experiment Station. Thank are also given to Bob Johnston, David Wichman and Jeff Johnston for their support and assistance during the experimental work.

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Table 1. Number of emerged wheat seedlings in 3 linear meters of row at 2 leaf stage for different inoculation treatment combinations² with *Fusarium pseudograminearum* isolate 2228 (Fpg) and *Bipolaris sorokiniana* isolate 2344 (Bs) observed in field trials conducted at Moccasin 2008 and Bozeman 2008 and 2009.

Treatments ²	Number of emerged seedling on 3 linear meters			
	Locations			
	Moccasin 2008	Bozeman 2008	Bozeman 2009	Average
High Fpg	81.4 d ¹	150.0 cd	103.0 e	111.4
Low Fpg	113.9 b	161.1 b	135.2 ab	136.6
High Fpg-Low Bs	74.6 d	148.9 cd	112.3 cde	111.9
Low Fpg-High Bs	81.0 d	135.4 ef	123.2 bcd	113.1
High Fpg-High Bs	57.2 e	130.7 f	107.3 de	98.3
Low Fpg-Low Bs	94.8 c	155.5 bc	130.2 bc	126.8
High Bs	113.2 b	143.4 de	133.7 b	130.0
Low Bs	125.5 a	159.1 b	136.8 ab	140.4
Non-inoculated	133.8 a	173.8 a	153.0 a	153.4
Average	97.2 c ²	150.9 a	125.8 b	
Factor	P-value ⁴			
Treatments (Trt)	<0.001	<0.001	<0.001	<0.001
LSD ⁵	8.765	11.680	8.176	
Location (Loc)				<0.001
Interaction (Trt*Loc)				<0.001

¹ Means followed by the same letter were not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

² Inoculation treatments were 3, 1 or 0 g of inoculated oat per linear meter on the row applied in-furrow during seeding.

³ Overall average for each field trial. Means followed with a same letter in the row are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

⁴ *P*-values obtained from multifactorial ANOVA for each particular factor assessed from three fields trials, where Trt = Treatments; Loc = Location; Trt * Loc = Interaction between treatment and location.

⁵ LSD = Least significant difference values calculated for doing mean separation on observation in each location.

Table 2. Average populations of *Fusarium* crown rot (FCR) and common root rot (CRR) observed with different inoculation treatment combinations⁷ of *Fusarium pseudograminearum* isolated 2228 (Fpg) and *Bipolaris sorokiniana* isolated 2344 (Bs) as determined through quantification of the DNA copy numbers of *tri5* and *gpd* genes using qPCR in different growth stages in three field trials assessing population dynamic of both diseases in Montana (2008-09).

a) Moccasin 2008 field trial

Treatments ⁷	Heading (Feekes stages 10.1-10.5)				Milk Stage (Feekes stage 10.54)				Harvest (Feekes stage 11.0)			
	<i>Fusarium</i> spp. ¹		<i>B. sorokiniana</i> ²		<i>Fusarium</i> spp.		<i>B. sorokiniana</i> .		<i>Fusarium</i> spp.		<i>B. sorokiniana</i> .	
High Fpg	180,892	ab ³	5,231	ab	362,775	ab	174	c	3,664,980	a	71,176	d
Low Fpg	201,148	a	10,992	ab	1,210,492	ab	13,041	ab	3,590,127	a	106,643	cd
High Fpg-Low Bs	180,309	ab	608	bc	1,118,194	ab	8,476	ab	3,611,226	ab	71,463	cd
Low Fpg-High Bs	133,351	a	1,669	bc	1,112,669	ab	14,717	ab	2,965,398	ab	139,068	bc
High Fpg-High Bs	146,629	ab	4,477	c	689,963	ab	5,653	b	4,217,087	ab	72,038	cd
Low Fpg-Low Bs	97,341	ab	2,755	ab	2,035,667	a	1,796	ab	3,381,753	ab	127,393	bc
High Bs	59,669	ab	14,520	a	716,158	b	29,502	ab	2,708,285	ab	412,561	a
Low Bs	95,545	ab	9,645	ab	420,791	ab	12,237	a	1,935,909	b	338,509	abc
Control	33,229	c	3,107	ab	303,753	ab	13,511	ab	2,444,835	ab	394,931	ab
Average	125,346		5,889		885,607		11,012		3,168,844		192,642	
Probability ⁴	0.026		0.026		0.376		0.0023		0.364		0.0011	
CV ⁵	20.68		29.07		11.72		31.83		3.51		9.30	
LSD ⁶	1.07		1.04		0.73		1.13		0.26		0.54	

b) Bozeman 2008 field trial

	Heading (Feekes stages 10.1-10.5)		Milk Stage (Feekes stage 10.54)		Harvest (Feekes stage 11.0)	
--	--------------------------------------	--	------------------------------------	--	--------------------------------	--

Treatments	<i>Fusarium</i> spp.		<i>B. sorokiniana</i>		<i>Fusarium</i> spp.		<i>B. sorokiniana</i>		<i>Fusarium</i> spp.		<i>B. sorokiniana</i>	
High Fpg	1,685,492	a	1,426	b	1,848,249	a	30,182		4,557,233	a	18,633	d
Low Fpg	1,977,089	a	3,832	ab	1,521,482	a	30,516		2,462,132	a	41,624	bc
High Fpg-Low Bs	4,031,837	a	2,039	ab	2,024,488	a	73,411		3,538,403	a	53,034	cd
Low Fpg-High Bs	1,581,756	a	10,239	ab	828,158	a	51,929		3,844,913	a	62,257	bc
High Fpg-High Bs	2,922,329	a	4,523	ab	2,923,457	a	39,466		3,890,661	a	57,846	bc
Low Fpg-Low Bs	4,411,819	a	2,121	a	2,769,614	a	109,561		6,668,623	a	75,382	bc
High Bs	94,652	b	15,110	a	17,024	b	64,804		308,841	b	172,505	a
Low Bs	31,156	b	12,280	a	42,610	b	95,627		489,410	b	172,523	a
Control	112,524	b	9,831	a	31,165	b	97,407		220,183	b	110,420	ab
Average	1,872,073		6,822		1,334,027		65,878		2,886,711		84,914	
Probability	< .0001		0.0261		< .0001		0.2296		< .0001		< .0001	
CV	21.06		26.88		19.01		17.26		6.41		8.70	
LSD	1.27		1.00		1.15		0.90		0.46		0.47	

c) Bozeman 2009 field trial

Treatments	Heading (Feekes stages 10.1-10.5)		Milk Stage (Feekes stage 10.54)		Harvest (Feekes stage 11.0)	
	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>	<i>Fusarium</i> spp.	<i>B. sorokiniana</i>
High Fpg	559,887	689	205,490	8,436	383,815	19,661
Low Fpg	382,687	148	286,056	6,275	248,907	12,451
High Fpg-Low Bs	501,415	126	282,167	6,866	430,623	24,644
Low Fpg-High Bs	354,851	1,943	240,686	8,144	335,740	21,564
High Fpg-High Bs	373,884	805	269,962	6,819	414,334	18,146
Low Fpg-Low Bs	271,922	1,044	205,117	7,727	330,581	17,993
High Bs	335,420	1,005	144,299	15,119	329,558	22,272
Low Bs	132,544	785	221,324	6,752	320,458	27,394
Control	247,825	1,038	188,235	8,179	203,103	24,983
Average	351,159	842	227,037	6,212	333,013	21,012
Probability	0.1249	0.5008	0.5832	0.6641	0.1698	0.0988
CV	12.47	38.49	5.83	11.57	4.07	4.77
LSD	0.700	1.02	0.3568	0.503	0.26	0.20

¹ Populations of FCR caused by *F. culmorum*, *F. pseudograminearum* or *F. graminearum* were quantified based in the amount of DNA copy number of the *tri5* gene.

² CRR populations were quantified based in the amount of DNA copy number of the *gpd* gene of *B. sorokiniana*.

³ Means followed by the same letter on each column were not significantly different according to Fisher's protected LSD ($\alpha=0.05$). Mean separations were done on copy numbers $\log(x+1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

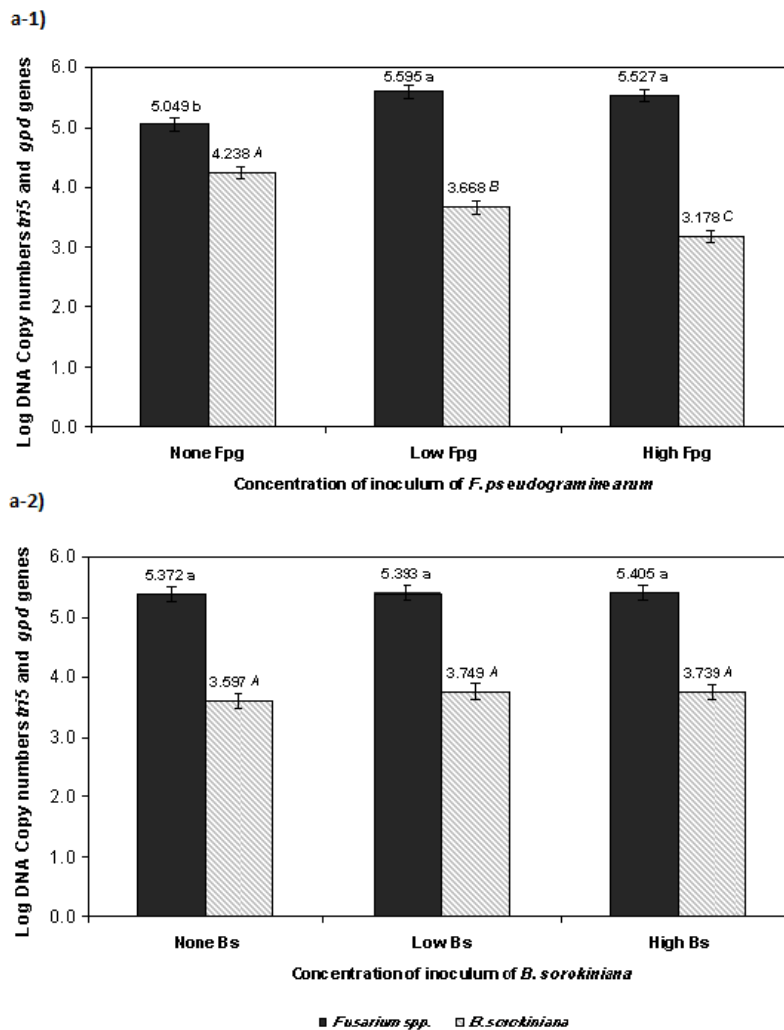
⁴ Probability value calculated for copy numbers $\log(x+1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

⁵ CV = Coefficient of variation for copy numbers $\log(x+1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

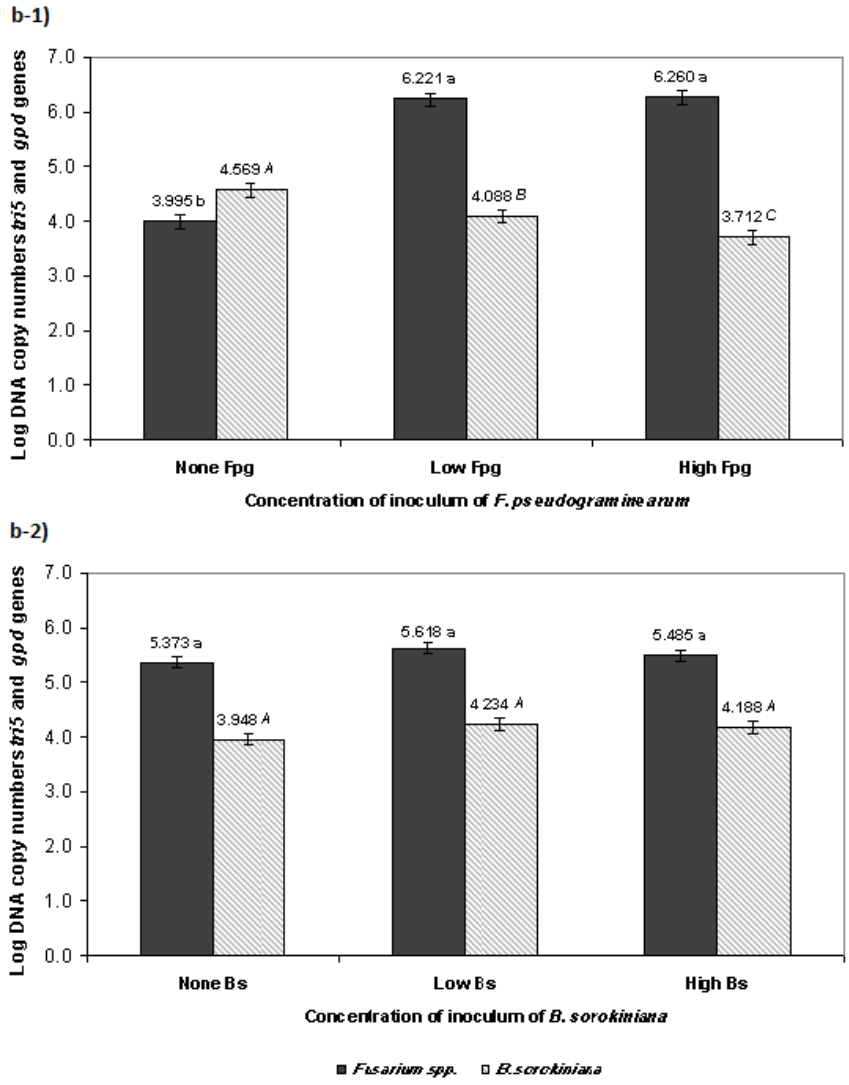
⁶ LSD = Least significant difference values calculated for doing mean separation on copy numbers $\log(x+1)$ transformed for the DNA copy number of the *tri 5* and *gpd* genes.

⁷ Inoculation treatments were 3, 1 or 0 g of inoculated oat per linear meter on the row applied in-furrow during seeding.

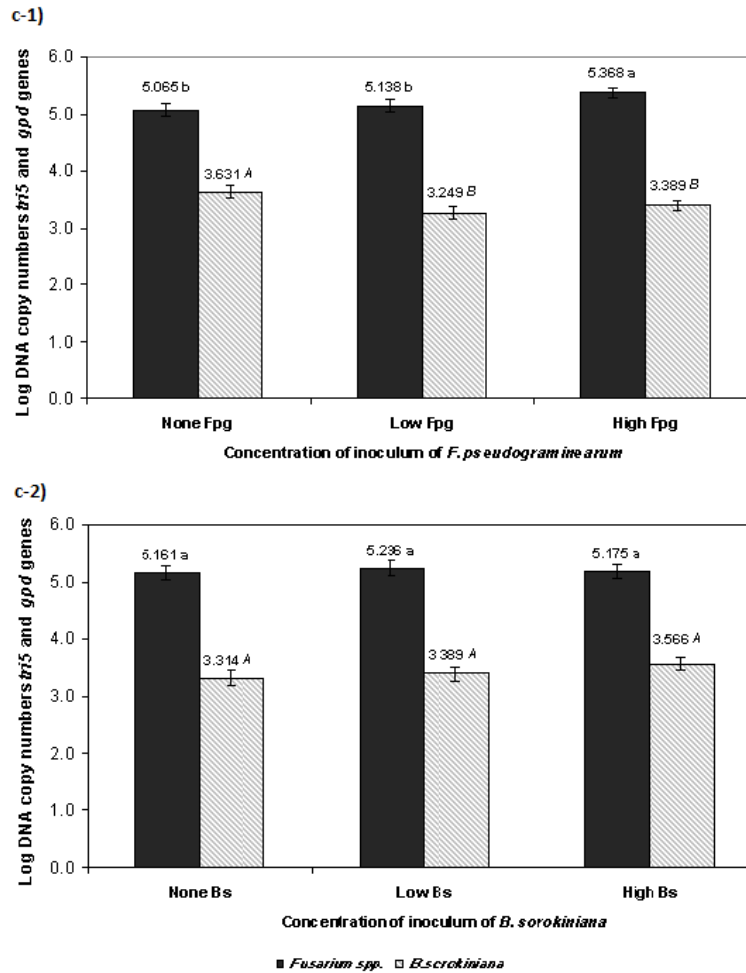
Fig. 1. Average populations of *Fusarium* spp and *Bipolaris sorokiniana* determined through qPCR quantification of DNA copy numbers² of the *tri5* and *gpd* genes respectively for different inoculum levels³ of *Fusarium pseudograminearum* isolate 2228 (Fpg) and *Bipolaris sorokiniana* isolate 2344 (Bs) in a field trial conducted in Moccasin 2008 (a), in Bozeman 2008 (b) and Bozeman 2009 (c).



b) Bozeman 2008 field trial



c) Bozeman 2009 field trial

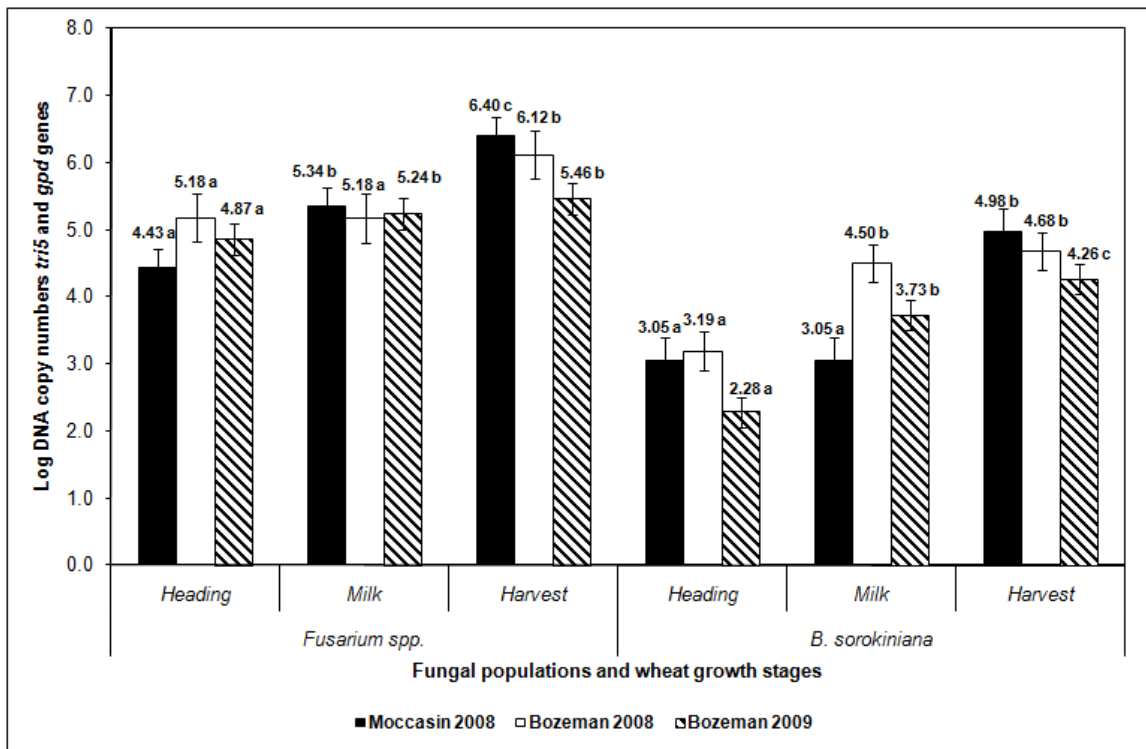


¹ Means followed by the same letter on each bar of the same color were not significantly different according to Fisher's protected LSD ($\alpha=0.05$). Bars on the top of each column are the standard error for each sample.

² DNA copy numbers quantified through qPCR for each gene were $\log(x + 1)$ transformed.

³ Inoculation treatments were 3, 1 or 0 g of inoculated oat per linear meter on the row applied in-furrow during seeding.

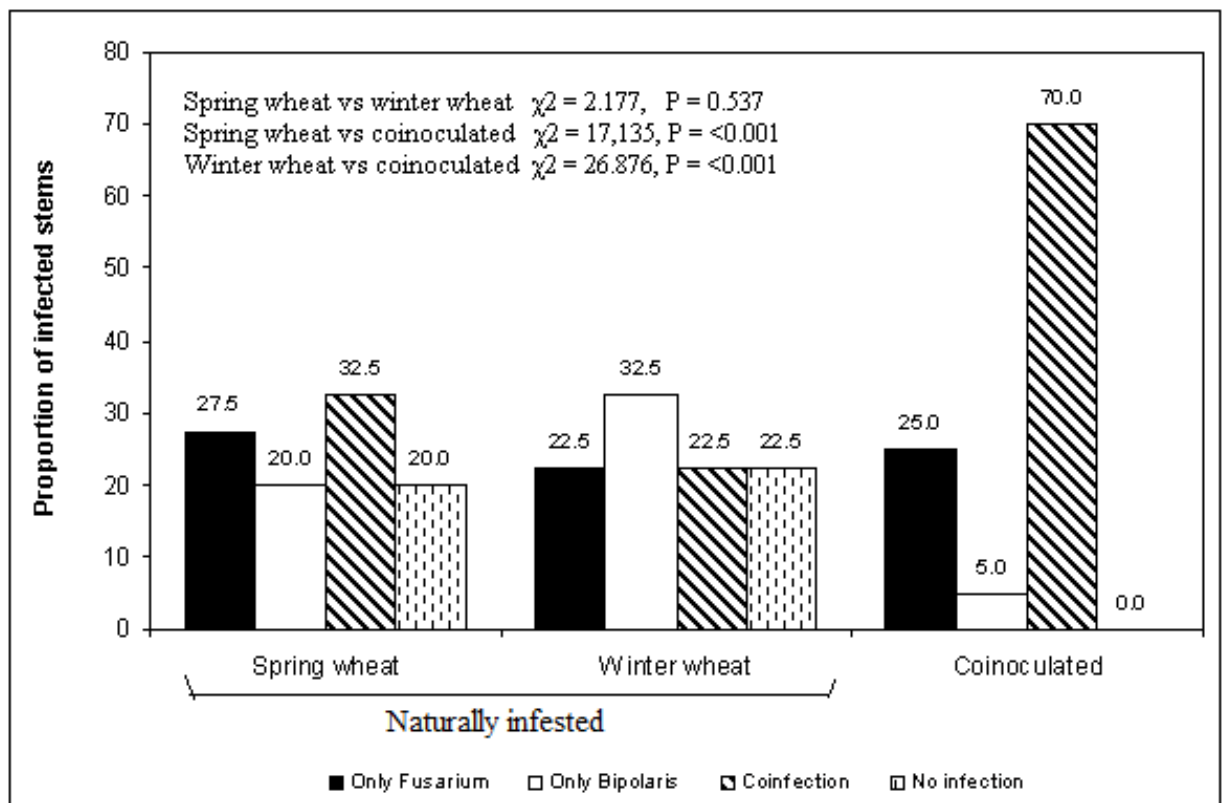
Fig. 2. Overall average populations of *Fusarium* spp and *Bipolaris sorokiniana* determined through quantification of the DNA copy number² of the *tri5* and *gpd* genes using qPCR for three wheat growth stages in Moccasin 2008 and Bozeman 2008 and 2009 field trials assessing population dynamic of both fungi with different inoculums rates of *F. pseudograminearum* isolate 2228 and *B. sorokiniana* isolate 2344.



¹ Means followed by the same letter on each bar of the same color and fungus specie were not significantly different according to Fisher's protected LSD ($\alpha=0.05$). Bars on each column are least significant differences (LSD) calculated for each field trial and fungus specie.

² DNA copy numbers quantified through qPCR for each gene were $\log(x + 1)$ transformed.

Fig. 3. Proportion of forty individual lower internodes infected by *Fusarium* crown rot species (*F. culmorum*, *F. pseudograminearum*, and *F. graminearum*), common root rot caused by *Bipolaris sorokiniana*, co-infected with both fungi, and not infected for single stems collected from naturally infested spring wheat and winter wheat fields¹ and from the coinoculated plots² in the Bozeman trials during two years as determined by qPCR (2008-09).



¹ Forty symptomatic tillers from naturally infested spring wheat fields were collected in Shonkin in 2008 and Cut Bank in 2009, while forty symptomatic tillers from naturally infested winter wheat fields were collected in Carter in 2008 and Malta in 2009.

² Forty symptomatic tillers from co-inoculated plots with 3 g of inoculum of *Fusarium pseudograminearum* isolate 2228 and *Bipolaris sorokiniana* isolate 2344 (Bs) per 1.0

linear meter were collected from experimental spring wheat sites in Bozeman in 2008 and 2009.

CHAPTER 5

Integrated management of *Fusarium* crown rot of wheat using fungicide seed treatment, cultivar resistance, and induction of systemic acquired resistance (SAR).

Contribution of Authors and Co-Authors

Manuscript in Chapters 5

Chapter 5:

Co-Author: Barry J. Jacobsen

Contributions of Dr. Barry J. Jacobsen were to assist design of the glasshouse and field experiments and he was the primary chair of the study. Also, Dr, Jacobsen worked on all parts of the manuscript through its successive revisions, reading and approving the final manuscript.

Manuscript Information Page

- Authors: Ernesto A. Moya and Barry J. Jacobsen.
- Journal name: Biological Control
- Status of the manuscript
 - Prepared for submission to peer-reviewed journal
 - Officially submitted to a peer-reviewed journal
 - Accepted by a peer-reviewed journal
 - Published in a peer-reviewed journal
- Publisher: Elsevier
- Data of submission (for submitted manuscript)
- Date manuscript will appear (for accepted work)
- Issue in which manuscript appears in (for published works)

Integrated management of *Fusarium* crown rot of wheat using fungicide seed treatment, cultivar resistance, and induction of systemic acquired resistance (SAR).

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Abstract

Fusarium Crown Rot (FCR) of wheat (*Triticum aestivum* L.) is a perennial problem for wheat producers worldwide. Difenoconazole–mefenoxam (0.65g/100g seed) (Dividend RTA, Syngenta, Greensboro, NC) fungicide seed treatment reduced FCR severity 29-50% while seed treatment with *Bacillus pumilis* isolate 314-16-5 and *T. harzianum* (T-22, Bioworks, Victor, NY) provided control statistically different than the untreated or seed treated with *Bacillus* isolate L324-92 or *Pseudomonas fluorescens* isolate 2-79 (L324-92 and 2-79 were obtained from D. Weller, USDA/ARS) on the cultivar Hank in glasshouse trials. Assessment of resistance in inoculated glasshouse trials and innate activity levels of three pathogenesis-related (PR) proteins in apoplastic fluids of five non-inoculated spring wheat cultivars determined lowest disease severity on cv. Volt and the highest levels of endochitinase and β -1, 3-glucanase activity compared to the cultivars Utopia, MT0550, Hank and Knudson ($P < 0.05$). Induction of SAR with foliar applications of *Bacillus mycooides* isolate BmJ (1.5×10^8 cfu/ml) or acibenzolar-*S*-methyl (1.0mM) (ASM [Actigard, Syngenta]) on the cultivars Hank, Knudson and Volt reduced the severity of

FCR by 10% compared to a water control ($P < 0.05$). BmJ application increased concentrations of peroxidase and endochitinase, while ASM increased β -1, 3-glucanases levels in cultivars Volt and Hank compared to water controls ($P < 0.05$). Integration of the management tools, difenoconazole-mefenoxam seed treatment, cultivar resistance, and SAR induction, showed integration of all three management tools did not reduce disease severity more than use of cultivar resistance plus fungicide seed treatment or SAR induction in glasshouse trials. In a dryland field trial, integration of all three management tools reduced disease severity and FCR populations more than individual tools ($P < 0.05$). However, SAR induction provided similar control to difenoconazole–mefenoxam seed treatment on both Hank and Volt in an irrigated field trial.

Key words: biological control, *Fusarium* crown rot, seed treatment, systemic acquired resistance, *Bacillus mycooides* isolate BmJ, Integrated management .

1. Introduction

A diverse group of *Fusarium* spp. damage small grain cereals by rotting the seed, seedlings, roots, crowns, basal stems, and in some case, infect the heads or spikes and grains (Paulitz et al., 2002). *Fusarium* Crown Rot (FCR) of wheat (*Triticum aestivum* L.) is a perennial problem that occurs in most cereal-producing regions of the world (Burgess et al., 2001; Paulitz et al., 2002; Cook, 2010). Losses associated to FCR have been described ranging between 3 and >50% in fields in the Pacific Northwest (PNW) (Cook,

1968; Smiley et al., 1996; Smiley et al., 2005). FCR is most commonly caused by *Fusarium culmorum* (W. G. Sm.) Sacc., *F. pseudograminearum* (O'Donnell & T. Aoki; group I), and *F. graminearum* Schwabe (group II), (Paulitz et al., 2002; Cook, 2010). Several other *Fusarium* species have been included as causal agents in the crown rot disease complex but these are thought to be of less importance (Fedel-Moen and Harris, 1987; Smiley and Petterson, 1996; Cook, 2010). Adoption of conservation tillage practices has been linked to increased FCR severity by increasing the amount of inoculum survival in crop residues (Wildermuth et al., 1997; Smiley et al., 1996; Paulitz et al., 2002; Akinsanmi et al., 2007).

Different strategies have been suggested for management of FCR. Cook (2010) recommended the use of clean and chemically disinfected seed, adjusting the date of seeding, proper fertilization, tillage to hasten infected residue decomposition, crop rotations avoiding other host crops (primarily cereal crops and grasses), use of cultivars with resistance to the pathogens or to water stress. Among these strategies, fungicide seed treatments are recommended for FCR management. For example emergence of winter wheat in fields tended to be superior for seed treated with difenoconazole (Dividend[®]), alone or mixed with metalaxyl (Smiley and Patterson, 1995). However, efficacy of chemical control is limited to early stages of the wheat growth cycle, since fungicide seed treatments do not maintain their efficiency much beyond the seedling stage (Balmas et al., 2006). Current resistance in commercial cultivars to FCR is only partial and losses can be severe when climatic conditions are favorable for disease development even on resistant cultivars (Burgess et al., 2001; Strausbaugh et al., 2005).

Seedling and adult-plant tolerance (partial resistance) to some FCR complex pathogens have been reported (Collard et al., 2005; Bovill et al., 2006), and is associated with reduced damage to crown and stem base tissue (Wildermuth et al., 2001).

Biological control agents (BCAs) have shown promise for the control of FCR (Huang and Wong, 1998; Dal Bello et al., 2002; Johansson et al., 2003; Luongo et al., 2005; Khan et al., 2006; Singh et al., 2009). Two basic approaches have been considered for use of BCAs in the control FCR: *i*) Manipulation of microbial antagonists to increase the rate of mortality of survival structures of *Fusarium* spp. in cereal residues (Wong et al., 2002; Luongo et al., 2005; Singh et al., 2009), and *ii*) seed treatment to protect the plant with BCAs (Huang and Wong, 1998; Dal Bello et al., 2002, Johansson et al., 2003; Khan et al., 2006). Seed treatments with *Burkholderia (Pseudomonas) cepacia* (Huang and Wong, 1998) pseudomonads and *Pantoea* sp. (Johansson et al., 2003), *Stenotrophomonas maltophilia*, *Bacillus cereus* and isolates of *Trichoderma harzianum* (Del Bello et al., 2002) have significantly reduced FCR infections caused by different *Fusarium* species on wheat and have increased grain yield and seedling stand in field experiments (Huang and Wong, 1998; Johansson et al., 2003). Also, Khan et al. (2006) working with pseudomonad seed treatment against *F. culmorum* reported the induction of a wheat class III plant peroxidase gene, suggesting that part of the biocontrol activity of these bacteria could be due to the induction of systemic acquired resistance (SAR) in host plants.

Induction of SAR by chemicals or BCAs has proven to be a valuable tool in plant disease control (Vallad and Goodman, 2004; Jacobsen et al., 2004). While wheat germplasm with a high level of resistance to FCR has not been described, the induction of

defense genes through application of methyl jasmonate or benzo(1,2,3)thiadiazole-7-carbothionic acid-*S*-methyl ester (BTH, Bion, Syngenta) enhanced resistance and delayed symptom development (Desmond et al., 2006). Several BCAs have been shown to induce SAR and Induced Systemic Resistance (ISR) (Kloepper et al., 1992; Vallad and Goodman, 2004; Bargabus et al., 2002, 2003, 2004; Walter et al., 2005). An example of a BCA that induces SAR is *Bacillus mycooides* isolate BmJ, which has provided control of diseases caused by fungal, bacterial, and viral pathogens in plant species including sugar beet (Bargabus et al., 2002, 2003, 2004), cucumber (Neher et al., 2008), tomato, melon, potato, and *Arabidopsis thaliana* (Neher, 2008). BmJ is able to induce PR-proteins through a salicylic acid (SA)-independent but NPR1-dependent pathway in sugar beet (Bargabus-Larson and Jacobsen, 2007) and NPR1 and jasmonic acid (JA)/ethylene dependent pathways in *A. thaliana* (Neher, 2008). This novel signaling defense described for BmJ may activate the defense response to necrotrophic fungal pathogens such as that mediated through JA pathway described by Desmond et al. (2006; 2008).

Jacobsen and Backman (1993) stated the importance of integrating host resistance, biological and cultural controls in integrated management systems both as alternatives and supplements to pesticides. Combinations of agents that induce resistance with fungicides or biological control agents or with disease resistant hosts can provide effective disease control, especially in situations where achieving acceptable disease control is difficult (Walter et al., 2005; Jacobsen et al., 2004). Since FCR is difficult to control and immunity-type host-disease resistance is not available, this paper addresses evaluation of an integrated pest management program for FCR. The biological control

agents, *Trichoderma harzianum* (T22, Victor, Geneva, NY), *Pseudomonas fluorescens* isolate 2-79, *B. pumilis* isolate 341-16-5, and *Bacillus* isolated L324-92 were compared to difenoconazole-mefenoxam seed treatment for control of FCR. The level of FCR resistance and the innate activity level of three PR-Proteins on five spring wheat cultivars were assessed by greenhouse experiments as well as the level of induction of SAR with *B. mycooides* BmJ and acibenzolar-*S*-methyl (ASM) was investigated. Finally, the integration of host plant resistance, fungicide seed treatment and BCA induced resistance was evaluated relative to FCR control in both glasshouse and field experiments.

2. Materials and Methods

2.1 Assessment of seed treatments for control of Fusarium crown rot of wheat.

2.1.1 Preparation of pots

Pots (10² cm) containing 400 g of pasteurized soil substrate MSU mix (1/3 sand, 1/3 peat, and 1/3 topsoil plus wetting agent [Aquagrow 2000, Aquatrols, Cherry Hill, NJ]) were used in glasshouse experiments. Pathogen inoculated pots were inoculated with *F. culmorum* isolate 2279 using a 100 g macroconidia infested soil place in a layer immediately below the planted seeds. Soil above and below this layer was pasteurized soil. The soil infestation was done by mixing pasteurized soil substrate MSU mix with the suspension of macroconidia in a tumbler machine to obtain concentrations of 10,000

macroconidia per gram of soil (1.0×10^6 per pot). Macroconidia were obtained by growing the fungus in 25% Potato Dextrose Broth for 10 days followed by filtering through cheesecloth. The number of macroconidia was determined by counting using a hemacytometer.

2.1.2 Preparation of seeds and seed treatments

Seeds (cv. Hank) were disinfected for 30 s by immersion in 70% ethanol followed by two rinses with sterile distilled water. Seeds were air dried for 3 hours in fumehood at room temperature 23 ± 1 C. Seed were untreated or treated with: *Bacillus* sp. L 324-92 (isolate courtesy Dr. David Weller, USDA/ARS, Pullman, WA); *Pseudomonas fluorescens* strain 2-79 (isolate courtesy Dr. David Weller, USDA/ARS, Pullman, WA); *Bacillus pumilis* MSU 341-16-5; *Trichoderma harzianum* Rifai strain KRL-AG2 (T-22, BioWorks Inc., Victor, NY); and difenoconazole (3.21%) + mefenoxam (0.27%) (Dividend[®] XL RTA, Syngenta Crop Protection, Inc., Greensboro, NC). All seeds treated with BCAs and untreated (UTC) were treated with a 0.5% aqueous suspension of carboxy-methyl-cellulose (CMC). *Bacillus* and *Pseudomonas* treatments used bacterial cells from 72 hour old cultures grown on Tryptic Soy agar (TSA). Ten ml of the CMC solution was applied on the plate with the BCAs, scraped, returned to a test tube, stirred vigorously for 5 seconds, and then the suspension applied to 100 g seeds. Populations of BCA's on seeds were approximately 1.0×10^8 c.f.u. per seed as determined by dilution-plating assay done after treatment. *T. harzianum* (T-22 formulation) was applied at 1.5 g

/ 100 g of seeds. Difenoconazole-mefenoxam was applied in a dose of 0.65 g / 100 g of seeds. BCAs and fungicide seed treatments were applied to 100 g of seed and mixed in plastic bags for 3 min. Treated seeds with the described treatments were air-dried for 20 hrs at $23^{\circ}\text{C}\pm 2^{\circ}\text{C}$ before planting. Seed were planted at 2-cm deep in 10 cm pots prepared as described in 2.1.1.

2.1.3 Assessment of *Fusarium* crown rot severity

Plants were grown in the glasshouse at $24/15^{\circ}\text{C}\pm 2^{\circ}\text{C}$ day /night under 14 hours of light 10 hours of night conditions from planting until Feekes stage 11. Day length was supplemented to a 14 hours photoperiod using 1000 Watt Metal Halide Lamps. Pots were watered three times per week and fertilizer was applied weekly by adding water-soluble Peters Professional solution (15-15-15+trace elements, Scott-Sierra Horticultural Products Company, Marysville, OH) during watering and injected at rate of 50ppm of N. At harvest, FCR severity was determined by using a crown rot rating (CRR) scale of 1 to 4 for the first internode of each plant, where: 1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored as described by Hogg et al. (2007). A disease severity score (DSS) was calculated by summing the number of plants in each CRR category multiplied by the value of each category and dividing this sum by the total number of plants x 4 (the number of categories), then multiplying by 100 to create a DSS for each pot. The resulting DSS provides a score from 25–100 and takes into consideration that a symptomless clean stems could have significant *Fusarium*

populations and observations made at the first internode have correlated well with yields and *Fusarium* populations (Hogg et al., 2007).

Experiments were repeated three times. A completely randomized block design for the treatments with 5 replications was used and the three experiments were analyzed by using a split plot design analysis where the treatments were the main plot and each experiment the subplot.

2.2 Evaluation of susceptibility of five spring wheat cultivars to *Fusarium* crown rot caused by *Fusarium culmorum* isolate 2279

2.2.1 Preparation of plant material

The spring wheat cultivars Utopia (*Triticum durum*), Hank, Volt, MT 0550, and Knudsen were used. Prior to seeding, the seeds were disinfected for 1 min with 0.054% sodium hypochlorite, rinsed twice with sterile distilled water and air dried for 3 hrs at 23°C±2°C. Pots (10 cm²) with a capacity of 400 g of pasteurized MSU soil mix were used and four seeds were placed equidistant and 2.0 cm deep. Cultivars Knudsen and the line MT 0550 were derived from crosses with Chinese variety Sumai 3 and have resistance to *Fusarium* head blight (FHB). Cultivar Volt also shows resistance to FHB (<http://www.sarc.montana.edu/mwbc/>). The cv. Utopia was used as a check because it is highly susceptible to FCR (Hogg et al., 2007).

2.2.2 Inoculation procedure

Pots were inoculated 15 days post planting (dpp) with macroconidia of *F. culmorum* isolate 2279 (Dyer et al., 2009) using a perforated microcentrifuge tube with eight lateral holes, which was inserted in the center of the pot soil and equidistant from the emerged plants (Fig. 1). The soil inoculation was done by applying a suspension of 1.0×10^6 macroconidia suspended in 20 mL of distilled water with a pipette. Macroconidia were obtained by growing *F. culmorum* in Mung bean liquid media for 10 days (Bai et al., 2000). The media was filtered through cheesecloth and the number of macroconidia was determined by counting using a hemacytometer. Mung bean liquid media was preferred in this experiment, because this gave higher production of *F. culmorum* macroconidias than Potato Dextrose Broth.

Plants were grown in a glasshouse until Feekes stage 11 as described in 2.1.3 and during this time pots were maintained on trays and watered by infiltration from the bottom every two days with 220-mL of water per pot. Fertilizer was applied weekly as described in 2.1.3. Mature stems were assessed for FCR severity by using the crown rot rating (CRR) scale and disease severity score (DSS) described previously.

The experiment was repeated twice. A completely randomized block design with 6 replications for the cultivars was used and the two experiments were analyzed by using a split plot design analysis where the cultivars were the main plot and each experiment the subplot.

2.3 Evaluation of innate concentrations of Pathogenesis Related Proteins (PR-Proteins) of five spring wheat cultivars

2.3.1 Preparation of plant material

The five spring wheat cultivars described above were used to determine the innate apoplastic activity levels of the three PR-proteins: peroxidase, β -1, 3-glucanase, and endochitinase. PR-proteins were assessed to determine if these compounds had some relationship with the expression of resistance of the evaluated cultivars mentioned above. Seeds were disinfected as is described in 2.2.1. Plants were grown in pots (10 cm²) containing of 400 g of pasteurized MSU soil mix and five seeds were placed equidistant and planted 2.0 cm deep.

2.3.2 Collection of apoplastic fluid (AF)

Crowns and leaves from plants 21 dpp from each pot were cut in 30 mm long pieces, thoroughly rinsed in distilled water, and then vacuum infiltrated in apoplast buffer (4.88 g MES and 58.44 g NaCl / L, pH 6.2) for 13 min using a waterjet vacuum pump. The infiltrated tissues were dried on blotting paper, inserted vertically in a 3.0 ml syringe, which was placed inside of a 50 ml Falcon centrifuge tube (Becton Dickinson Labware, Franklin Lakes, NJ) with a 1.5 mL centrifuge tube placed in the bottom, and centrifuged at 2100 rpm at 4°C for 12 min. After centrifugation, the AF collected from the samples was again centrifuged at 8000 rpm for 1 min to eliminate chloroplast contamination. The AF was frozen immediately at -21°C and maintained at this temperature until use.

2.3.3 Protein concentration

Total protein concentration was assayed by the method of Bradford (1976) using the Bio-Rad protein assay dry reagent (Bio-Rad, USA), with bovine serum albumin as the protein standard.

2.3.4 Apoplast peroxidase activity

A modification of the method of Moloi and Van der Westhuizen (2006) was used. The assay mixture (250 μL) contained 2 μL AF, 15 μL 8.2mM H_2O_2 , 25 μL 50mM guaiacol, 85 μL dd H_2O and 125 μL 80mM potassium phosphate buffer (pH 5.5). The increase in absorbance was measured at 470 nm for 5 min at 25°C against a blank containing all the reagents except for the AF. The specific enzyme activity of the protein samples was determined by comparison to the horseradish peroxidase II standards (Calbiochem., Darmstadt, Germany [(5.6 x 10⁻³ to 5.6 x 10⁻⁴ units)]) and corrected considering the protein contents of the enzyme extracts.

2.3.5 Apoplast β -1, 3-glucanase activity

A modification of the method developed by Fink et al. (1988) and Moloi and Van der Westhuizen (2006) was used. The assay mixture contained 2 μL AF, 50 μL 2 mg / ml laminarin, and 48 μL 50mM sodium acetate buffer (pH 5.0) and they were placed in a PCR plate well. After incubation at 37°C for 16 h, 100 μL of Somogyi reagent (Somogyi, 1952) was added and incubated at 95°C for 10 min in a PCR machine. After cooling, 100

μL of Nelson's reagent (Nelson, 1944) was added. The absorbance was measured at 540 nm. The blank and the standards were subjected to the same procedure. The specific enzyme activity of the protein samples was determined by comparison to the laminarinase standards of *Trichoderma* spp. (Sigma-Aldrich, St. Louis, MO [1.0×10^{-2} to 5.0×10^{-2} units]) and corrected with the protein content.

2.3.6 Apoplast endochitinase activity

A modification of the method of Fujita et al. (2006) and Hung et al. (2002) was used. The assay mixture contained 1.5 μL AF and standards, 120 μL of 0.001% glycol chitin (0.40 mL 0.01% dissolved in 40 mL of 50 mM of Acetate buffer pH 5.0), and they were placed in a PCR plate well. After incubation for 16 h at 37°C the products of endochitinase activity were estimated using the Imoto and Yagishita method (1971). The absorbance was measured at 420 nm. The blank and the standards were subjected to the same procedure. Activity of the protein samples was determined by comparison to the chitinase standard from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO [0.6 to 0.2 units]) and corrected with the protein content.

The experiment was repeated twice. A completely randomized design with 8 replications (2 subsamples per pot) for each cultivar was used and the statistical design analysis was conducted as is described in 2.2.2.

2.4 Evaluation of Pathogenesis Related Proteins (PR-Proteins) activity on Hank and Volt spring wheat induced plants with foliar application of BmJ and ASM

Seeds of the spring wheat cultivars Hank and Volt were disinfected as is described in 2.2.1. Three seeds per pot were planted in 10 cm² pots containing 400 g of pasteurized MSU soil mix at deep of 2.0 cm, and grown for 16 days in a growth chamber (Convion model EF 74, Winnipeg, Manitoba, Canada), and maintained at 25±1°C day/20±1°C night and 14 h of light (30,000 lux) and 10 h of dark. Plants were maintained on trays and watered by infiltration each three days with 2 liters of water per 12 pots as described in 2.2.2.

2.4.2 Application of inducers of SAR

Twelve dpp, plants of each cultivar were sprayed with suspension of ddH₂O with 0.01% Tween 20 containing *B. mycooides* isolate BmJ at dose of 0.35 g of the commercial product BmJ WP (Montana Microbial Products, Inc, Missoula, MT, 3 x 10¹⁰ spores per gram) in 1 L, 1.0 mM ASM (commercial formulation Actigard, Syngenta, Greensboro, NC) solution, or only distilled water with 0.01% Tween 20. Plants were sprayed until runoff and maintained separated inside of the growth chamber until foliage was dry.

Plant samples consisting of the stem and leaves of both cultivars and from each treatment were taken four days after the inducers were sprayed to collect of apoplastic fluid, following the procedure described in 2.3.2. The AF's collected were diluted 1:10 and used to determine the apoplastic levels of peroxidase, β-1, 3-glucanase, and chitinase. Total protein concentration was assayed by the method of Bradford (1976)

described in 2.3.3. Intercellular peroxidase and endochitinase activities were assessed as is described in 2.3.4 and 2.3.6, respectively.

Intercellular β -1,3-glucanase activity was assessed following the microplate-based carboxymethylcellulose assay described by Xiao et al. (2005), and modified by Neher (2008), because this is a simpler and quicker method than that described in 2.3.5. The assay mixture contained 30 μ L of 1 % laminarin (from *Laminaria digitata*, Sigma-Aldrich, St. Louis, MO) and 1 % CM-Pachyman (Megazyme International Ireland Ltd, Ireland) in 50 mM sodium acetate buffer (adjusted to pH 4.0 with glacial acetic acid) and 30 μ L of each sample and the standard, which were combined in a PCR plate well and sealed with a silicone sealing mat. After incubation for 4 h at 30°C in a thermocycler the product was mixed with 60 μ L of dinitrosalicylic acid reagent (DNS) consisting of 30 mM dinitrosalicylic acid (Alfa Aesar, Ward Hill, MA), 14.9 mM phenol, 2.8 mM sodium sulfite, 0.5 M sodium-potassium-tartrate (EMD Chemicals Inc., Darmstadt, Germany), and 0.2 mM NaOH were added to the solution and the PCR plate was incubated again for 5 min at 95 °C as described above. Subsequently 100 μ L of solution were transferred to a 96 well flat-bottom assay plate and the absorbance was measured at 540 nm with a SpectraMax plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The blank and the standards were subjected to the same procedure. Activity of the protein samples was determined by comparison to the laminarinase (from *Trichoderma* sp., Sigma-Aldrich, St. Louis, MO) standard (0.001 to 0.00001 units) and corrected with the protein content.

The experiment was repeated twice. A completely randomized design with four replications in a factorial arrangement was used, where cultivar and treatment were considered the factors.

2.5 Evaluation of control on Fusarium crown rot by induction of Systemic Acquired Resistance by Bacillus mycooides isolate BmJ and Acibenzolar-S- methyl ester (ASM) in three spring wheat cultivars

2.5.1 Preparation of plant material and inoculation procedure

The spring wheat cultivars Hank, Volt, and Knudsen were used in this experiment. Previously to seeding, the seeds were disinfected as described in 2.2.1. The inoculation method was the same described in 2.2.2

2.5.2 Application of inducers of SAR

Plants were induced by spraying plants with four leaves, 3 days before inoculation with suspension of ddH₂O with 0.01% Tween 20 containing BmJ at 1.5×10^8 CFU per ml, 1.0 mM ASM (commercial formulation Actigard, Syngenta) solution, or only distilled water. Plants were sprayed until runoff (~1.7 mL / plant) and maintained separated inside of the glasshouse until foliage was dry. Sprayings were repeated at 19, 26 and 33 days post planting (dpp). Plant inoculation was conducted at 15 dpp as described in 2.2.2.

Plants were grown until Feekes stage 11 as previously describe in 2.1.3. During this time pots were maintained on trays and watered by infiltration every two days with 220-mL of water per pot. Fertilizer was applied weekly as described in 2.2.2.

The experiment was repeated three times. A completely randomized design with six replications in a factorial arrangement was used, where cultivar and treatment were considered the factors. FCR severity was assessed as described in 2.1.3.

2.6 Glasshouse integrated disease management experiments on Fusarium crown rot in spring wheat

To assess integrated control of FCR fungicide seed treatment, cultivar resistance and induction of SAR were evaluated alone or in combination in two glasshouse and two field experiments. The glasshouse experiments were done in experiments separated in time under growing conditions described in section 2.1.3. Pots were prepared and inoculated with a layer of inoculum below the seed such as was described in 2.1.1. Seed treatments were: untreated seeds or seeds treated with difenoconazole-mefenoxam (Dividend[®] XL RTA) as described at 2.1.2. SAR induction was achieved through two sprayings of *B. mycooides* isolate BmJ as described in 2.4.2. Sprayings were done at 21 dpp and 15 days later. Treatments without BmJ application were sprayed with double distilled water plus 0.01% Tween 20. Cultivars used were the susceptible cultivar Hank and the moderately resistant cultivar Volt. Treatments were untreated seed without inoculation, and the

following inoculated treatments: untreated seed with and without SAR inducer spraying, and a treated seed with and without spraying of BmJ.

Glasshouse experiments used plants grown until Feekes stage 11 under conditions described in section 2.1.3, and during this time pots were maintained as described in 2.1.3. FCR severity was determined as described in 2.1.3.

The experiment was repeated twice. A completely randomized design in a factorial arrangement with 10 replications was used, where cultivar and treatments were considered the factors.

2.6.1 Assessment of the effect of an integrated disease management on Fusarium crown rot by using a fungicide seed treatment, cultivar resistance, and induction of SAR in spring wheat in field experiments.

Field experiments were done during the 2009 crop season at the MSU Southern Agricultural Research Center at Huntley, MT under dryland conditions and in an irrigated commercial production field in Manhattan, MT. Both fields had a history of FCR problems and were naturally infested. The same treatments described in 2.6 excluding the untreated non-inoculated treatment. The experiment at Huntley was seeded at 53.55 kg/ha, 3.5 cm deep on April 23, 2009 in chemical fallow spring wheat stubble using a Hedge 1000 small plot drill (Hege Equipment, Inc. Colwich, KS). The soil type was Fort Collins Clay Silt Loam and plots received 26.4 cm natural precipitation (April to August). Plots were 3.66 m x 1.40 m with four seeded rows. Thirty units of nitrogen/ha

as urea was broadcast applied pre-plant. The plots were sprayed preplant with pyrasulfotole (3,3%) + bromoxynil Octanoate (13,4%) + bromoxynil heptanoate (12.9%) at 0.804 L/ha (Huskie, BayerCropScience, Research Triangle Park, NC) + pinoxaden (5,05%) at 1.20 L/ha (Axial XL, Syngenta, Greensboro, Crop Protection Inc., NC) + R-11 Surfactant (292.8 ml/ha, Wilbur-Ellis Company, Fresno, CA) + ammonium sulfate (AMS [1,12 kg/ha]) on May 11, 2009 to control weeds. The experiment in Manhattan was sown on May 21, 2009 in an irrigated field cropped to wheat in 2008. Residue management in the experimental site was done by chopping the residues to disperse straw, followed by harrowing to disperse residue windrows, and finally the field was cultivated with disc plow and followed by a single irrigation to germinate volunteer seed during fall of 2008. In spring 2009, prior to seeding the field site was tilled with a chisel plow for residue incorporation. Each plot had six rows of 4.9 m length x 0.305 m width spacing between rows (8.97m² per plot). Experiments were seeded with a hoe drill machine at rate of 67.2 kg/ha. The field received 21.7 cm natural precipitation (May to August) and was irrigated as needed (11.4 cm from July to September) using a center pivot and irrigation was discontinued during flowering during two weeks (July 17 to August 01) to reduce risk of FHB infection. Additionally, plots were sprayed aerially with tebuconazole (38.7%) at 1.023 L/ha (Folicure[®], BayerCropScience, Research Triangle Park, NC) in 50 L/ha water diluent on July 21, four days after anthesis to provide control of Fusarium head blight. Plants also were treated early in June with azoxystrobin (7.0%) + propiconazole (11.7%), (Quilt[®], Syngenta Crop Protection Inc., Greensboro, NC) to control Tan spot and Septoria leafspot in a ground application as a tank mix with

herbicides. Weed control was done with MCPA (40%) at 0.355 L/ha (MCPA Ester[®]) and clodinafop-propargyl (22.3%) at 0.290 L/ha, (Discover[®], Syngenta Crop Protection Inc., Greensboro, NC) at seedling stage. Fertilization was done pre-plant with a mix of 50-20-60 of N-P-K fertilizers (985.9 kg/ha) and injection of foliar liquid fertilizer through center pivot irrigation at dough development stage (28-0-0-3 at 12.32 kg/ha in 93.50 L of water).

Spraying of BmJ was done in the same dose described in 2.6 for both experiments at 100 ml of spray volume per plot. The experiment in Huntley was treated with BmJ on May 15 (22 dps, GS12 two leaf stage) and May 27 (34 dps, GS14-16~ 4-6 leaf stage). The Manhattan experiment was treated with BmJ on June 9 (19 dps, GS13-14, 3-4 leaf stage) and June 24 (34 dps, GS 23-24, 3 to 4 tillers).

The four rows of each pot in Huntley were harvested at Feekes stage 11.4 on August 17th, with a Wintersteiger plot combine (Wintersteiger USA Inc, Salt Lake City, UT), while the three center meters from the four middle rows of plot in the experiment in Manhattan were harvested for yield with a small bundle rice binder (Mitsubishi Agricultural Machinery Co., Ltd., Tokyo, Japan) and threshed using a large Vogel thresher (Bill's Welding, Pullman, WA) on September 24th.

After harvest 50 tillers randomly selected from the center four rows were scored visually for FCR using the scale as is described in section 2.1.3. Additionally, thirty tillers from each plot were randomly selected and used for quantitative real-time polymerase chain reaction (qPCR) TaqMan[®] assay that detects *F. culmorum*, *F.*

graminearum, and *F. pseudograminearum*. This qPCR procedure followed the protocols developed by Strausbaugh et al. (2005) and Hogg, et al. (2007).

Both experiments used four repetitions in a randomized complete block design.

2.7 Statistical analysis

Analysis of variances (ANOVA) or multifactorial ANOVAs were performed for the disease ratings and yields obtained according the experimental designs for each experiment by the procedure PROC GLM (Statistical Analysis System (SAS) Institute Inc., version 9.2, Cary, NC). Protected least significant difference (LSD) range test was used to compare means ($P < 0.05$). Levene's test for homogeneity of variance of the treatments was used to determine whether experiments could be combined in an ANOVA. Levene's tests were performed with PROC GLM (SAS Institute).

3. Results

3.1 Assessment of control of Fusarium crown rot on Hank spring wheat using seed treatments

Assessment of the seed treatment performance for control of FCR in glasshouse experiments (Fig. 2) showed that in average the highest levels of severity were observed on the untreated control (UTC) and seeds treated with *Bacillus* spp. L 324-92 or *P.*

fluorescens 2-79. These biological seed treatments had a numerically lower but not significant different level of severity than UTC ($P < 0.05$). *Bacillus pumilis* isolate 314-16-5, T-22 and difenoconazole-mefenoxam seed treatments had statistically lower DSS than the UTC ($P < 0.05$). The greatest reduction in the DSS was for difenoconazole-mefenoxam seed treatment with a disease reduction of nearly 50%. These experiments served as the basis for selecting the difenoconazole-mefenoxam seed treatment for integrated control program.

3.2 Evaluation of susceptibility of five spring wheat cultivars to Fusarium crown rot caused by Fusarium culmorum isolate 2279

Based in glasshouse trials and inoculations with *F. culmorum* isolate 2279, cultivars differed in susceptibility to FCR (Fig. 3; $P < 0.05$). Volt showed the lowest level of severity among the assessed cultivars, while cvs. Utopia and MT 0550 were the most susceptible. The cultivars Hank and Knudsen were intermediate in susceptibility, although Knudsen was statistically equivalent to Volt in susceptibility ($P < 0.05$). These experiments served as the basis for selecting the commercial cultivars Hank and Volt as the susceptible and resistant cultivars for integrated experiments.

3.3 Evaluation of innate concentrations of peroxidase, endochitinase, β -1, 3-glucanase activities of five spring wheat cultivars

Specific enzyme activity in apoplastic fluid for peroxidase, endochitinase, β -1, 3-glucanase in non-inoculated wheat plants showed significant differences among the spring wheat cultivars assessed 21 dpp ($P < 0.05$; Table 1). Wheat cultivars MT 0550 and Knudsen had higher peroxidases activity than cultivars Volt, Hank, and Utopia ($P < 0.05$). For endochitinases, Volt showed the highest level of activity while the lowest level of activity of this PR-Protein was observed in Utopia and with Knudsen, Hank and MT 0550 being equivalent ($P < 0.05$), although Hank and MT 0550 were not different from Utopia. β -1, 3-glucanase activity was highest in cultivar Volt followed by MT 0550, Hank and Knudsen, while Utopia showed the lowest level of activity, which was not different of the activity observed in cultivar Knudsen. These experiments served as the basis to select commercial cultivars with differential PR-Protein activity potential used in later experiments. The cv. Volt had the highest endochitinase and β -1, 3-glucanase activities while having the lowest peroxidase activity; cv. Knudsen had the highest peroxidase activity and intermediate endochitinase and β -1, 3-glucanase activities, while cv. Hank had a relatively low innate activity for all three PR-Proteins measured.

3.4 Evaluation of Pathogenesis Related Proteins (PR-Proteins) activity on Hank and Volt spring wheat induced plants with foliar application of BmJ and ASM

There were no significant differences between induced specific activities of apoplastic peroxidase and endochitinase for the two cultivars while both cultivars differed

significantly with respect to induced β -1, 3-glucanase ($P < 0.05$; Table 2). On average, BmJ only increased specific activity of peroxidase and endochitinase when compared to the control, although peroxidase activity between ASM and BmJ were not significantly different. The two inducers also differed in induction of β -1, 3-glucanase with only ASM showing specific activity for this PR-protein in both cultivars (Table 2.). β -1, 3-glucanase activity observed between the two experiments was significantly different ($P < 0.001$), but a test for homogeneity of variance showed that the observations of each experiment could be combined ($P = 0.889$).

3.5 Evaluation of control on Fusarium crown rot by induction of Systemic Acquired Resistance by Bacillus mycooides isolated BmJ and Acibenzolar-S- methyl ester in three spring wheat cultivars

The cultivars Hank, Knudsen, and Volt were chosen to assess the FCR control by SAR inducers based on their resistance to FCR and innate peroxidase, endochitinase, β -1, 3-glucanase expressions. Results of the experiments showed different levels of infection among experiments ($P < 0.001$), although the same amount of macroconidia and method of inoculation were used. However, a test of homogeneity of variance determined that the observations of each experiment could be combined ($P = 0.917$). Both SAR inducers reduced significantly the DSS by 9.7% when compared to the treatment control ($P < 0.001$; Table 3). Moreover, among the cultivars, Volt showed the lowest severity to FCR, followed by Knudsen and Hank ($P = 0.015$) with the cultivar Hank having the highest

FCR DSS and showing the greatest response to SAR induction as measured by disease control. Statistical interaction between cultivar and treatment was not observed ($P = 0.402$).

3.6 Integrated disease management experiments on Fusarium crown rot in spring wheat

The collected data from the two glasshouse experiments conducted to assess the integrated management program showed statistical difference between both experiments ($P < 0.001$), but after a test of homogeneity of variance both experiments could be combined for a general analysis ($P = 0.816$). DSS were significantly different between the cultivars Hank and Volt ($P < 0.001$). Cv. Hank had in average higher FCR disease severity than cv. Volt. DSS for the treatments were significantly different among them ($P < 0.001$), and all treatments reduced the level of infections of FCR compared to the untreated control (UTC) (Table 4). Pots without inoculation (control healthy) showed some level of infection on the plants but this treatment was significantly different from the inoculated treatments. BmJ reduced FCR severity by 11.3% when compared to the UTC. Difenoconazole-mefenoxam seed treatment in average reduced the DSS by 15.0%. Integration of all three management tools did not reduce disease severity more than use of cultivar resistance plus fungicide seed treatment or SAR induction in these glasshouse trials. Statistical interaction between cultivar and treatment was not observed ($P = 0.741$).

3.6.1 Assessment of the effect of an integrated disease management on Fusarium crown rot by using a fungicide seed treatment, cultivar resistance, and induction of SAR in spring wheat in field experiments

Field experiments carried out to assess the effect of the integrated management program on FCR in spring wheat cultivars showed in a multifactorial ANOVA analysis that all variable responses (grain yield, DSS, and DNA quantification) presented significant differences between the two field experiments ($P < 0.001$). Then, with the objective to observe the location effect on the treatments, mean comparisons were performed separately for each field trial (Table 5). In both field trials, treatments did not affect grain yield for either cultivar or for the cultivars averaged together. The cv. Hank had higher grain yield than cv. Volt in the dryland conditions at Huntley ($P < 0.001$), while both cultivars had similar grain yield under irrigated conditions at Manhattan and averaged 30.9% more yield than in Huntley. DSS was significantly reduced by the integrated treatment at Huntley and by all treatments at Manhattan for the cv. Volt ($P < 0.05$). DSS for cv. Volt was lower at both locations ($P < 0.05$) and treatments did not have any significant effect on DSS for cv Hank. Treatment that integrated SAR induction with BmJ and seed treatment with difenoconazole-mefenoxam presented the higher reduction of FCR disease severity at Huntley, while at Manhattan, integration did not reduce DSS more than use each practice alone. DSS analysis of FCR disease determined that integration was associated with lowest number of symptomatic stems in

the highest disease category (Category 4; $P = 0.025$, data not shown). Using real time PCR that quantified the DNA copies of FCR pathogens demonstrated that DNA copy numbers were reduced by fungicide seed treatment, BmJ spraying and the integrated BmJ-fungicide seed treatments at both field experiments ($P < 0.05$). However, in the experiment at Huntley, the integrated treatment was significantly lower than BmJ spraying used alone and similar to the fungicide seed treatment, while at the Manhattan location DNA copy number was reduced equally by all treatments.

4. Discussion

These experiments demonstrated that cultivar resistance, both biological and chemical seed treatments, and SAR induced by either BmJ or ASM can individually reduce DSS. Bio-based seed treatments were not effective in reducing FCR disease in organic certified trials on the cv. Big Sky and Promontory at Big Sandy, MT and Bozeman, MT during a two year study (data not shown), despite *T. harzianum* and *B. pumilis* 314-16-5 seed treatments showing reduction of DSS in pot experiments. BCAs typically affect a narrow spectrum of diseases on specific crop hosts (Jacobsen and Backman, 1993), and this characteristic could explain the lack of effective control from *P. fluorescens* 2-79 and *Bacillus* spp. L324-92, previously demonstrated to control take-all caused by *Gaeumannomyces graminis* var. *tritici* on wheat (Weller, 1983; Kim et al., 1997). *B. pumilis* 314-16-5 has been used as seed treatment for the control of sugar beet damping-

off caused by *Pythium ultimum*, *Rhizoctonia solani* and *Aphanomyces cochlioides* in both field and glasshouse experiment (Jacobsen et al., 2004) and this bacterial strain has increased the production of chitinase when sprayed sugar beet leaves (Bargabus, et al., 2004). Induction of chitinase by *B. pumilis* 314-16-5 could explain the higher variability observed with this strain in the glasshouse experiments and higher yield observed for this bacterial strain in a field trial performed in Bozeman, MT during 2007 (data not shown).

T. harzianum Rifai strain KRL-AG2 (T-22) was effective for control of *F. culmorum* under glasshouse conditions, reducing the disease severity in average by 26% compared to the untreated control. However, under field conditions *T. harzianum* was not better than the untreated control in the trial conducted in Big Sandy, MT and Bozeman, MT during 2006 and 2007 (data not shown). This may be due to the high soil pH in field experiments (Big Sandy pH = 7.7-8.5; Bozeman pH=7.6-8.4) compared to the soil mix used in greenhouse experiment (MSU mix pH=6.2-6.8). Jacobsen (personal communication) had observed that *Trichoderma* isolates did not work well in alkaline soils in Montana, and the impact of alkaline soils on the biocontrol activity of *Trichoderma* spp. has previously been recognized (Papavizas, 1985). Difenoconazole-mefenoxam showed the greatest reduction of *F. culmorum* DSS under controlled glasshouse conditions as would be expected based on the work of Smiley and Patterson (1995). The formulation of Dividend[®] XL RTA, is a commercial fungicide seed treatment recommended to control 16 soil-borne and seed-borne diseases on cereals. The results obtained in the pot experiments plus this broad spectrum of action was considered when it was chosen to be part of the integrated disease management program. Under

field conditions, difenoconazole-mefenoxam alone reduced the populations of FCR pathogens in both experiments. Also, effect of this fungicide seed treatment on the disease severity was observed on cv. Volt in the Manhattan field trial.

The cv. Volt showed partial resistance to FCR under controlled glasshouse conditions and in the irrigated field experiment at Manhattan. Volt is hard red spring wheat described as high yielding semi-dwarf wheat suited for production under irrigated conditions. Good yield performance of this cultivar under irrigated condition was observed when yields were compared between experiments in Huntley and Bozeman (41.3% greater yield under irrigated conditions). Volt is also considered to have good tolerance to FHB. Dual resistance of Volt to FCR and FHB contradicts the idea of resistance inversion purposed for the phenomenon where one plant genotype displays a resistance phenotype at one development stage but a susceptible reaction to the same pathogen at another stage (Li et al. 2010). However, resistance inversion was observed on cv. Knudsen and cv. MT 0550, which possess the 1B chromosome from Sumai 3 genome, which gives resistance to FHB, but their performance against FCR was similar or worse than Hank, one of the most susceptible cultivars to FHB and FCR in Montana. Also, the susceptibility to FCR observed in Knudsen and MT 0550 to FCR was in concordance with the work conducted by Xie et al (2006), who noted tissue specific resistance against FHB in cultivars developed from the Chinese Sumai 3 cultivar. Innate peroxidase, endochitinase, and β -1, 3-glucanase activities were variable between cultivars with the cv. Volt having the highest levels of activity for apoplastic endochitinase, and β -1, 3-glucanase. Study of innate PR-protein activity in the apoplastic fluid was considered

because studies with *F. graminearum* infection on wheat spikes have shown that direct contact with the pathogen is not required for induction of defense response genes and that these genes are activated in both susceptible and resistant genotypes (Pristch et al., 2001). Knudsen and MT 0550 had the highest level of peroxidase, while, cv. Hank, highly susceptible to FHB, showed the lowest level of peroxidase. These results were in concordance with the reported by Pristch et al. (2001) in wheat spikes, where water controls had high level of peroxidase gene transcripts in the Sumai 3 cultivar. High levels of endochitinase activity observed in apoplastic fluids from cv. Volt (61% more activity than the more susceptible cultivars assessed) may be related with lower level of severity of FCR observed for this cultivar. High induction of chitinase gene expression in wheat seedling has been associated with resistance against *F. pseudograminearum* (Desmond et al., 2006) and *F. asiaticum* (Li et al., 2009). Innate high levels of endochitinase presented on cv. Volt might also be associated with resistance to FHB, considering that chitinase genes have been expressed in higher-level response to inoculation with *F. graminearum* on the spike (Pristch et al., 2000; 2001). β -1, 3-glucanase activity could be not associated clearly with an innate defense response against FCR, although Volt showed the highest activity level among the assessed cultivars and the most susceptible cv. Utopia had the lowest. Different types of β -1, 3-glucanase have been described on wheat (Muthukrishnan et al., 2001), but this PR-Protein has not been described as playing a major role in reducing FCR or FHB severity (Desmond et al., 2006; Pristch et al., 2000).

ASM and BmJ reduced the DSS of FCR compared to a water control. Treatment with ASM induced greater β -1, 3-glucanases activity in both cultivars and this PR-Protein could be involved in the reduction of FCR severity. The use of ASM induced resistance on wheat has been described by Desmond et al. (2006), where increased defense gene expression was coupled with delayed FCR lesion development observed after one application of BTH (syn. ASM) and where glucanase transcripts (*PR-2*) were consistently greater in a FCR susceptible wheat cultivar treated with BTH and inoculated with *F. pseudograminearum*.

B. mycooides isolate BmJ has been used for biological control of Cercospora leaf spot of sugar beet and *Botrytis* grey mold of *Arabidopsis* through induction of different PR-proteins (Bargabus et al., 2002, 2003, 2004; Neher, 2008) and, in this work, foliar applied BmJ showed the ability to reduce severity of FCR and to induce high concentrations of peroxidase and endochitinase on wheat especially on the cv. Volt. This is the first demonstration of BmJ induced SAR in a monocot. Increased peroxidase activity in wheat after treatment with BmJ could have strengthened the cell wall reducing the ability of *F. culmorum* to penetrate the plant cell, considering that hydrogen peroxidase activity is associated with a physical strengthening of plant walls through peroxidase-catalyzed lignification (Hammerschmidt and Kuc, 1982) and cell wall protein crosslinking (Bradley et al., 1992; Lamb and Dixon, 1997). This strengthening of the physical barrier makes it harder for pathogens to penetrate plant cell by way of enzymatic digestion. High concentration of endochitinase observed on cv. Volt and Hank when was treated with BmJ could also be an important factor involved in the reduction of FCR. Woo et al.

(2006) demonstrated that hydrolytic enzymes chitinase and β -1, 3-glucanase in plants release oligosaccharides from the fungal cell wall that elicit plant defenses. In addition, these enzymes interfere of assembly of the fungal cell wall during elongation when preexisting primers of oligo-N-acetylglucosamines and oligo- β -1,3-glucans are assembled by chitin synthase and β -1,3- glucan synthase, respectively (Mauch et al., 1988). Also, over-expression of chitinase gene and *PR-4* gene in wheat seedlings has also been coupled with high expression of a plant cytochrome P450 gene *CYP709C1*, which is involved in detoxification of exogenous compounds produced during infection by *F. asiaticum* (Li et al., 2009).

The observed variability in the control of FCR after use of SAR induction in glasshouse and field experiments could be not easily explained. During infection, wheat plant defense responses needs to confront the array of enzymes produced by pathogens some of which are able to remove or inactivate those defense responses. For example, catalases and laccases produced by necrotrophic pathogens have been demonstrated to reduce or inactivate active oxygen species (AOS) produced by the plant (Mayer et al., 2001). Active laccases are produced by *F. culmorum* (Kwon and Anderson, 2002) and a significant enhancement of catalase activity production in response to peroxide stress by *F. graminearum* and *F. culmorum* has also been observed (Ponts et al., 2009). Moreover, production of mycotoxins such as deoxynivalenol (DON) has been reported during infection by FCR pathogens (Mudge et al., 2006). This toxin has an inhibitor effect on protein synthesis which could suppress the production of host defense responses as been suggested for Mudge and coworkers (2006). Additionally, DON treatment induces

transcription of AOS and enzymes involved in AOS detoxification in wheat stem (Desmond et al., 2008b; Li et al., 2010), which on the one hand may stimulate programmed host cell death assisting necrotrophic fungal growth, while, on the other hand, the AOS may also be regulating the levels of PR-protein expressions (Desmond et al., 2008b). Also, ammonization and pH modulation observed in apoplastic fluids of wheat seedlings infected with *F. culmorum* may be affecting PR-Protein performance (Aleandri et al., 2007). Thus mechanisms used by the FCR pathogens during pathogenesis can deplete the defenses already present in a wheat cultivar such as Volt or affect the performance of the PR-Proteins induced by treatments with BmJ, ASM or other SAR inducers.

The use of integrated control practices has proven to be the most stable strategy for many diseases (Jacobsen and Backman, 1993). In the case of FCR control, integration of cultivar resistance, fungicide seed treatment, and SAR induction was not able to reduce disease severity significantly when compared to use each of these control practices individually. However, under the dryland field conditions at Huntley, the integration of fungicide seed treatment and SAR induction by BmJ reduced the DSS and decreased the number of stem internodes in the highest disease severity category and the population of FCR pathogen species when they were quantified using qPCR. We can speculate that after the chemical seed treatment had lost its effectiveness, BmJ may induce defense response pathway that could give protection to FCR or other diseases later in the wheat growth cycle.

Disease severity reduction observed through seed treatment, SAR induction, and cultivar resistance alone or in combination did not have effect on grain yield when compared to the control in both field trials. In Manhattan, irrigated conditions probably reduced the plant stress and thus disease effects could be overcome by the plant, considering that FCR severity is increased when water stress is observed late in the crop cycle (Papendick and Cook, 1974; Cook, 1981; Smiley et al., 1996; Burgess et al., 2001; Paulitz et al., 2002). However, while yields were numerically higher in the treated plots no significant differences were detected, suggesting that four repetitions could be too low to unmask real differences in yield between treatments. Heil and co-workers (2000) have shown that treatment of wheat with the plant activator acibenzolar-*S*-methyl reduced both growth and seed production. We did not observe any differences in growth in either field trial. However, it is clear that trying to quantify the allocation costs associated with induced resistance is difficult and this difficulty is exacerbated if the confounding effects of pathogen infection are also considered (Walter and Boyle, 2005). In work by Barbagus et al. (2002) and Jacobsen et al. (2004) on sugarbeet no growth reduction has ever been associated with BmJ treatment and in sugarbeet field trials yields are generally increased by BmJ treatments even in the absence of obvious disease (Jacobsen et al., 2004)

Finally, control of FCR could be improved if common practices such as effective seed treatments are integrated with novel management practices like SAR induction and cultivar resistance as demonstrated in this study. In addition, incorporation of crop rotation and infected residue management could provide additional control of this

widespread and common disease. This research clearly demonstrates that assessment of cultivar resistance to FCR and potential for use of SAR induction with different wheat germplasm could be critical components in developing FCR management programs.

Acknowledgments

This research was supported by funds from the Montana Agricultural Experiment Station project N° 991223. The authors would like to thank Alan T. Dyer, Oliver Neher, Perry Miller, William Grey, and Ken Kephart for their assistance in the field and laboratory work.

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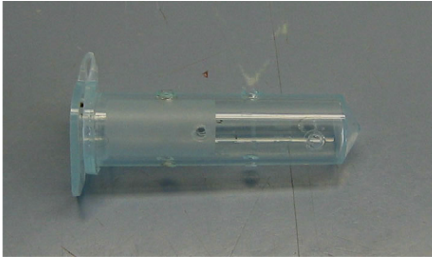
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Fig. 1. Microcentrifuge method of inoculation used as a bioassay to assess cultivar resistance to *Fusarium* crown rot infection and the effect of SAR induction on FCR by using *Bacillus mycooides* isolate BmJ and acibenzolar-*S*-methyl (Actigard, Syngenta Crop Protection, Greensboro, NC).

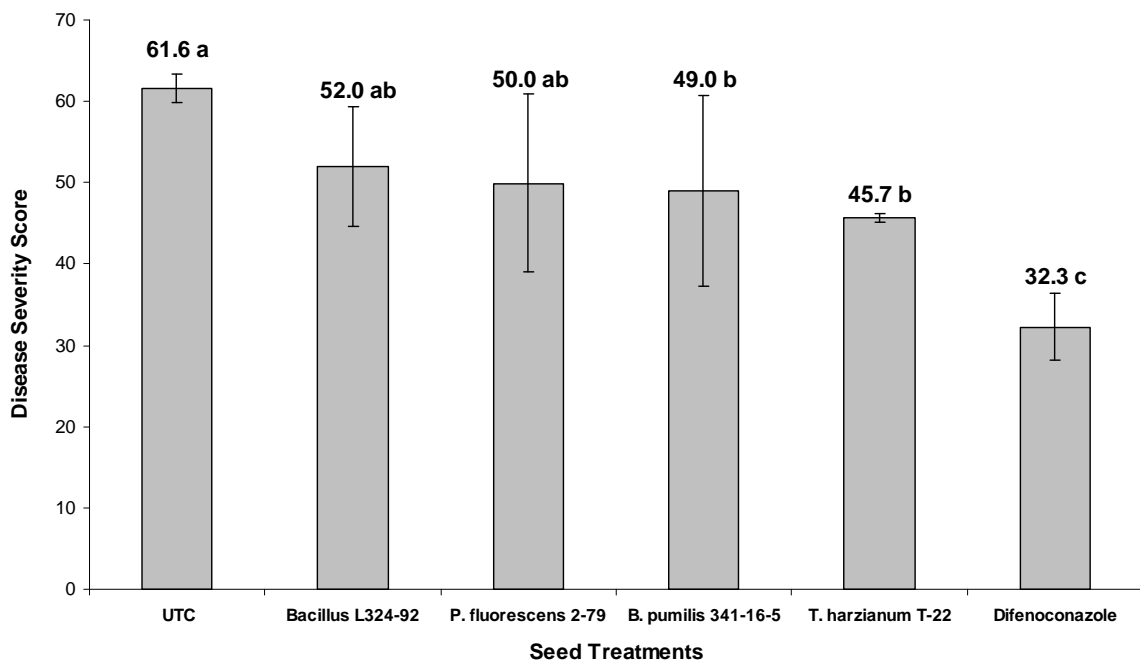


Microcentrifuge tube with eight holes

Microcentrifuge tube inserted equidistant from the four wheat plants in a pot



Fig. 2. Fusarium crown rot disease (FCR) severity scores as affected by seed treatment with *Bacillus* sp. USDA/ARS isolate L 324-92; *Pseudomonas fluorescens* strain 2-79 (USDA/ARS); *Bacillus pumilis* isolate MSU 341-16-5 applied at 1.0×10^8 cfu/seed; *Trichoderma harzianum* Rifai strain KRL-AG2 (T-22, BioWorks Inc., Victor, NY) applied at 1.5 g/100 seed, and difenoconazole-mefenoxam (Dividend RTA, Syngenta Crop Protection) applied at 0.65 g/100g seed on cv. Hank grown to Feekes stage 11 in *Fusarium culmorum* isolated 2279 inoculated glasshouse trials.

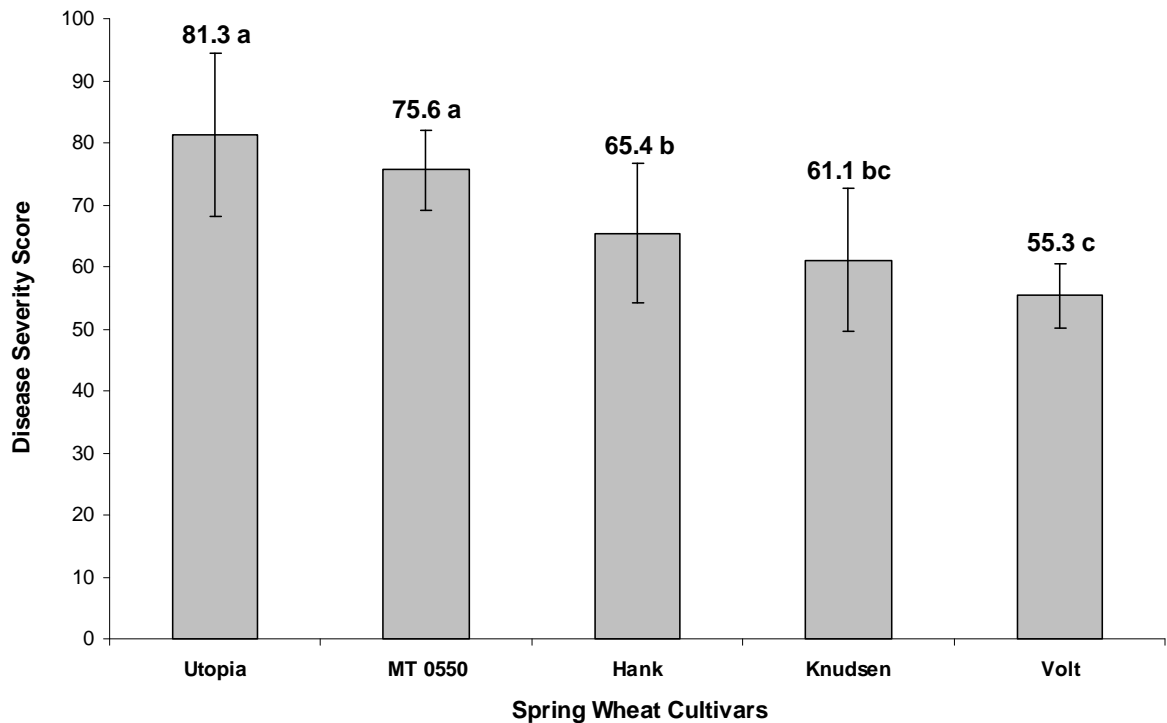


¹ Means followed with a same letter are not significantly different at level of significance of 0.05 according to Fisher's protected LSD.

² Bars correspond to standard deviation for each treatment from three glasshouse trials.

³ Disease severity scores (DSS) of FCR was calculated as: $DSS = [(Class\ 1 * N^{\circ}Plant + Class\ 2 * N^{\circ}Plant + Class\ 3 * N^{\circ}Plant + Class\ 4 * N^{\circ}Plant) / (4 * Total\ number\ of\ plants\ assessed)] * 100$,
where $N^{\circ}Plant$ = number of first internodes of plants rated in one of the 4 classes of severity (1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored).

Fig. 3. Average of *Fusarium* crown rot disease severity scores observed in five spring wheat cultivars inoculated with *Fusarium culmorum* isolated 2279 at 15 days post planting and grown in the glasshouse to Feekes stage 11.



¹ Means followed with a same letter are not significantly different at level of significance of 0.05 according to Fisher's protected LSD.

² Bars correspond to standard deviation for each treatment from two glasshouse trials.

³ Disease severity scores (DSS) of FCR was calculated as: $DSS = [(Class\ 1 * N^{\circ}Plant + Class\ 2 * N^{\circ}Plant + Class\ 3 * N^{\circ}Plant + Class\ 4 * N^{\circ}Plant) / (4 * Total\ number\ of\ plants\ assessed)] * 100$, where $N^{\circ}Plant$ = number of first internodes of plants rated in one of the 4 classes of severity (1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored).

Table 1. Innate specific activity of peroxidase, endochitinase, and β -1, 3-glucanase observed in apoplastic fluids of five spring wheat cultivars grown in pasteurized soil at 21 days post planting.

Cultivar	Activity of PR-protein in apoplastic fluid		
	Peroxidase ¹	Endochitinase ²	β -1, 3-glucanase ³
Utopia	1.2750 b ⁴	0.380 c	0.546 c
MT 0550	2.1186 a	0.426 bc	0.639 b
Hank	1.0849 b	0.437 bc	0.633 b
Knudsen	1.9706 a	0.495 b	0.587 bc
Volt	1.4067 b	0.612 a	0.744 a
LSD	0.4497	0.1119	0.0746
<i>P</i> -value	<0.05	<0.05	<0.05

¹ Specific enzyme activity in units/mg of apoplastic protein⁵/min compared to the horseradish peroxidase II standard.

² Specific enzyme activity in units of endochitinase/mg of apoplastic protein⁵/min compared to chitinase standard from *Streptomyces griseus*.

³ Specific enzyme activity in units of laminarinase/mg of apoplastic protein⁵/min compared to laminarinase standard from *Trichoderma harzianum*.

⁴ Means followed by the same letter are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

⁵ Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

Table 2. Specific activities of apoplastic peroxidase, endochitinase and β -1, 3-glucanase observed in the spring wheat cultivars Hank and Volt following induction by foliar treatment with *Bacillus mycooides* isolate BmJ and Acibenzolar- S – methyl ester (ASM, Actigard, Syngenta Crop Protection, Greensboro, NC) at 16 days post planting⁸.

Treatments	Pathogenesis related protein activities								
	Peroxidase ¹			Endochitinase ²			β -1, 3-glucanase ³		
	Hank	Volt	Average	Hank	Volt	Average	Hank	Volt	Average
Control	1.320	1.219	1.269 b ⁴	0.296	0.281	0.289 b	0.891	0.715	0.803 b
BmJ	1.424	1.694	1.559 a	0.484	0.817	0.651 a	0.945	0.798	0.871 b
ASM	1.492	1.314	1.403 ab	0.396	0.416	0.406 b	1.088	1.073	1.081 a
Average	1.412	1.409		0.392	0.505		0.975 a ⁵	0.862 b	
Factor	<i>P</i> -values ⁷								
Treatment (Trt)	0.049			0.007			<0.001		
Cultivar (Cv)	n/s			n/s			0.009		
Interaction (Trt*Cv)	n/s			n/s			n/s		
Experiment	n/s			n/s			<0.001		

¹ Specific enzyme activity in units/mg of apoplastic protein⁶/min compared to the horseradish peroxidase II standard.

² Specific enzyme activity in units of endochitinase/mg of apoplastic protein⁶/min compared to chitinase standard from *Streptomyces griseus*.

³ Specific enzyme activity in units of laminarinase/mg of apoplastic protein⁶ x 1000 compared to laminarinase standard from *Trichoderma harzianum*.

⁴ Means followed by the same letter in each column are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

⁵ Means followed by the same letter in the row are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

⁶ Protein amount was quantified using Bio-Rad protein assay kit per manufacturer's instructions using bovine serum albumin as standards.

⁷ *P*-values obtained from multifactorial ANOVA for each particular factor assessed in each variable for each PR-Protein assay from two experiments, where Trt = Treatments; Cv = cultivars; Cv * Trt = Interaction between treatment and cultivar; and n/s = no significant differences associated to that factor (*P*-value higher than 0.05).

⁸ Twelve plants per cultivar were treated with both inducers of SAR (BmJ at 0.35 g/1.0-L of the commercial product BmJ WP [Montana Microbial Products, Inc, 3×10^{10} spores per gram] and ASM at 1.0 mM) or only distilled water with 0.01% Tween 20 at 12 days post planting.

Table 3. Average of Fusarium crown rot disease severity scores⁴ for Hank, Knudsen, and Volt spring wheat, where systemic acquired resistance (SAR) was induced by foliar application⁵ of *Bacillus mycooides* isolate BmJ and Acibenzolar- *S* –methyl ester (ASM, Actigard, Syngenta Crop Protection, Greensboro, NC).

Spring wheat cultivar				
Treatment	Hank	Knudsen	Volt	Average
Control	76.8	69.5	68.7	71.7 a ¹
BmJ	62.2	62.7	60.6	61.8 b
ASM	67.6	63.1	55.7	62.2 b
Average	68.9 a ²	65.1 ab	61.7 b	
Treatment (Trt)				<0.001 ³
Cultivar (Cv)				0.015
Interaction (Trt*Cv)				n/s
Experiment				<0.001

¹ Means followed with a same letter in each column are not significantly different at level of significance of 0.05 according to Fisher's protected LSD.

² Overall average for each cultivar. Means followed with a same letter in the row are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

³ *P*-values obtained from multifactorial ANOVA for each particular factor assessed on three glasshouse experiments, where Trt = Treatments; Cv = cultivars; Cv * Trt = Interaction between treatment and cultivar; and n/s = no significant differences associated to that factor (*P*-value higher than 0.05).

⁴ Disease severity scores (DSS) of FCR was calculated as: $DSS = [(Class\ 1 * N^{\circ}Plant + Class\ 2 * N^{\circ}Plant + Class\ 3 * N^{\circ}Plant + Class\ 4 * N^{\circ}Plant) / (4 * Total\ number\ of\ plants\ assessed)] * 100$, where N^oPlant = number of first internodes of plants rated in one of the 4 classes of severity

(1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored).

FCR was established through inoculation with *F. culmorum* isolated 2279.

⁵ Plants were treated with both inducers of SAR (BmJ at 1.5×10^8 CFU per ml and ASM at 1.0 mM) or only distilled water with 0.01% Tween 20 at 12, 19, 26 and 33 days post planting.

Table 4. Average Fusarium crown rot (FCR) disease severity scores⁴ for glasshouse grown plants as affected by SAR induction by foliar application⁵ of *Bacillus mycooides* isolate BmJ (BmJ), difenoconazole-mefenoxam seed treatment (ST), and combination of both treatments on Hank and Volt spring wheat cultivars⁶ inoculated with *F. culmorum* isolate 2279.

Treatments	Spring wheat cultivar		Average
	Hank	Volt	
Untreated control	85.9	66.2	76.3 a ¹
BmJ	77.8	52.2	65.0 b
Seed treatment (ST)	72.2	50.8	61.5 b
ST + BmJ	73.4	51.6	62.5 b
Non inoculated control	58.9	31.8	45.4 c
Average	73.6 a ²	50.6 b	
Treatment (Trt)			<0.001 ³
Cultivar (Cv)			<0.001
Interaction (Trt*Cv)			n/s
Experiment			<0.001

¹ Means followed with a same letter in each column are not significantly different at $P < 0.05$ according to Fisher's protected LSD ($\alpha=0.05$).

² Overall averages for each cultivar. Means followed with a same letter in the row are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

³ P -values obtained from multifactorial ANOVA for each particular factor assessed on two glasshouse experiments, where Trt = Treatments; Cv = cultivars; Cv * Trt = Interaction between treatment and cultivar; and n/s = no significant differences associated to that factor (P -value higher than 0.05).

⁴ Disease severity scores (DSS) of FCR was calculated as: $DSS = [(Class\ 1 * N^{\circ}Plant + Class\ 2 * N^{\circ}Plant + Class\ 3 * N^{\circ}Plant + Class\ 4 * N^{\circ}Plant) / (4 * Total\ number\ of\ plants\ assessed)] * 100$,

where N°Plant = number of first internodes of plants rated in one of the 4 classes of severity (1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored)..

⁵ Plants were treated with BmJ (0.35 g/1.0-L of the commercial product BmJ WP (Montana Microbial Products, Inc, 3×10^{10} spores per gram) at 21 and 36 days post planting.

⁶ Cultivars had differential resistance to FCR with cv. Hank being susceptible and cv. Volt being moderately resistant.

Table 5. Mean grain yield, Fusarium crown rot (FCR) disease severity score², and *Fusarium* spp. DNA copy numbers as affected by an integrated disease management program including induction of systemic acquire resistance with *Bacillus mycooides* isolate BmJ (BmJ), difenoconazole-mefenoxam seed treatment (ST), and FCR resistance on spring wheat cultivars Hank and Volt⁷ at Huntley and Manhattan, MT in 2009.

Treatment ⁸	Variable response												
	Huntley						Manhattan						
	Yield (kg/ha)			DSS ² (%)			DNA cpn ³	Yield (kg/ha)			DSS (%)		
	Hank	Volt	Average	Hank	Volt	Average	Average	Average	Hank	Volt	Average	Average	
Control	3,253	3,133	3,193	64.3	56.4	60.3 a ¹	44,673 a ⁵	4,339	53.6	54.4 a	54.0 a	86,040 a	
BmJ	3,631	3,405	3,518	65.5	56.6	61.0 a	53,970 b	4,426	48.9	46.9 b	47.9 bc	28,642 b	
Seed treatment	3,663	3,066	3,365	65.1	59.9	62.5 a	18,645 bc	4,366	50.5	43.5 b	47.0 c	33,757 b	
BmJ+ ST	3,643	3,046	3,345	62.6	49.3	55.9 b	9,149 c	4,440	58.1	45.0 b	51.6 ab	34,182 b	
Average	3,548 a ⁴	3,162 b		64.4 a	55.5 b				52.8 a	47.4 b			
Factor	P-value ⁶												
Treatment (Trt)	n/s			0.002			0.002	n/s	n/s	0.035	0.009	0.0325	
Cultivar (Cv)	<0.001			<0.001			n/s	n/s			0.001	n/s	
Trt * Cv	n/s			n/s			n/s	n/s			0.015	n/s	

¹ Means followed by the same letter in each column are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

² Disease severity scores (DSS) of FCR was calculated as: $DSS = [(Class\ 1 * N^{\circ}Plant + Class\ 2 * N^{\circ}Plant + Class\ 3 * N^{\circ}Plant + Class\ 4 * N^{\circ}Plant) / (4 * Total\ number\ of\ plants\ assessed)] * 100$, where $N^{\circ}Plant$ = number of first internodes of plants rated in one of the 4 classes of severity (1 = 0–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% of the internode discolored).

³ Average *Fusarium* DNA copy number of *tri5* gene for natural infection of populations of *F. culmorum*, *F. graminearum*, and *F. pseudograminearum* using real-time qPCR analysis observed for each treatment in samples taken from the base of first internode of 30 wheat stems.

⁴ Means followed by the same letter in the row between two cultivars are not significantly different according to Fisher's protected LSD ($\alpha=0.05$).

⁵ Means comparison was done on $\log(x + 1)$ values ($x = Fusarium$ DNA copy number of *tri5* gene).

⁶ *P*-values obtained from multifactorial ANOVA for each particular factor assessed in each variable for each field experiment, where Trt = Treatments; Cv = cultivars; Cv * Trt = Interaction between treatment and cultivar; and n/s = no significant differences associated to that factor (*P*-value higher than 0.05).

⁷ Cultivars had differential resistance to FCR with cv. Hank being susceptible and cv. Volt being moderately resistant.

⁸ Seed treatment was done with difenoconazole-mefenoxam (Dividend[®] XL RTA, Syngenta Crop Protection, Inc., Greensboro, NC) in a dose of 0.65 g / 100 g of seeds. BmJ was applied in a dose of 0.35 g / 1.0-L of the commercial product BmJ WP (Montana Microbial Products, Inc, 3×10^{10} spores per gram) at 22 and 34 days post planting (dpp) at Huntley and at 19 and 34 dpp at Manhattan. Combined

treatment (BmJ+ST) was done at the same dosages of the products already described and during dates of application described for each location.

CHAPTER 6

GENERAL CONCLUSION

This dissertation work was able to show the importance and impact of the diseases involved in the dryland root rot complex (DLRRC) in Montana. Additionally, this research project showed the importance to understand the dynamic between the pathogens involved in the DLRRC and to develop new strategies to confront these diseases. This research is the first survey where Fusarium crown rot (FCR) and common root rot (CRR) diseases were assessed using two real-time qPCR assays. These results obtained by the qPCR assays were comparable to traditional isolation methods used for other authors. In this research was determined that CRR is the most wide spread pathogen in “the Golden Triangle” area of wheat production in Montana, while FCR was more variable on the surveyed area and with FCR absent from the soil type *Gb5* located in Fergus and Judith Basin counties. Through an intensively sampling was possible to suggest that when severe both diseases are causing significant yield losses for grower in the sampled area. Our estimations suggest that approximately 12% of the surveyed field could have yield losses caused by FCR, while around 15% of the fields could be affected noticeably by CRR. Through analysis of relationship of environmental variables and pathogen distribution was possible to show that CRR and *F. pseudograminearum* were positively correlated with temperature while *F. culmorum* negatively correlated with temperature, suggesting that CRR and *F. pseudograminearum* are adapted to warmer conditions while *F. culmorum* is adapted to cooler areas in the surveyed regions and those results were in concordance with conclusions reported by other authors. Additionally, it was demonstrated clearly that FCR is more severe in spring wheat

under Montana conditions. Assessment of the competition dynamics between pathogens showed that the 1 and 3g rates of *F. pseudograminearum* inoculum were associated with reduction of *Bipolaris sorokiniana* populations in the first internode. However, reciprocal association between *B. sorokiniana* and *Fusarium* populations was not possible to determine because the high indigenous populations of *Bipolaris* present in the experimental sites. *Fusarium* and *Bipolaris* populations in the first internode of wheat stems were increased from heading until harvest, while *Fusarium* populations colonized first internode earlier than *Bipolaris* and that preemptive colonization may explain reductions of *Bipolaris*. Moreover, inoculation noticeably increased incidence of infection and coinfection relative to natural settings. At the seedling stage, both fungi alone or combined reduced the seedling counts when compared to a control non-inoculated for the three locations. Work conducted to develop an integrated management program for FCR, one of the diseases of the DLRRC, determined that difenoconazole–mefenoxam at rate of 0.65g/100g seed (Dividend RTA, Syngenta) fungicide seed treatment reduces FCR severity, while seed treatment with *Bacillus pumilis* isolate 314-16-5 and *T. harzianum* (T-22) provided statistically better control different than the untreated on the cultivar Hank in glasshouse trials. Our research was able to determine that cultivar Volt has partial resistance and has higher levels of innate chitinase and β -1, 3-glucanase activity compared to four more susceptible spring wheat cultivars. Also, induction of SAR with foliar applications of *Bacillus mycooides* isolate BmJ or acibenzolar-S-methyl (Actigard, Syngenta) reduced the severity of FCR compared to a water control. In cv. Hank and Volt, BmJ application increased specific activities of peroxidase and chitinase, while ASM increased β -1, 3-glucanase. This research project is also the first demonstration of BmJ induction of SAR in a monocot.

Integration of the management tools: difenoconazole-mefenoxam seed treatment, cultivar resistance, and SAR induction did not reduce disease severity more than use of cultivar resistance plus fungicide seed treatment or SAR induction in glasshouse trials and in an irrigated field trial conducted in Manhattan, MT. However, in a dryland field trial (Huntley, MT), integration of all three management tools reduced disease severity and FCR populations as measured using qPCR more than individual tools.

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