

**IDENTIFICATION OF QUANTITATIVE TRAIT LOCI (QTL) FOR RESISTANCE TO  
FUSARIUM CROWN ROT IN WHEAT AND A SURVEY OF *FUSARIUM*  
*PSEUDOGRAMINEARUM* AND *F. CULMORUM* IN THE PACIFIC NORTHWEST OF  
THE U.S.**

By

GRANT J. POOLE

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

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of  
GRANT J. POOLE find it satisfactory and recommend it be accepted.

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Timothy Paulitz, Ph.D., Chair

---

Kimberly Garland-Campbell, Ph.D.

---

Richard W. Smiley, Ph.D.

---

Timothy D. Murray, Ph.D.

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THE U.S.**

Abstract

by Grant J. Poole, Ph.D.  
Washington State University  
May 2010

Chair: Timothy D. Paulitz

Fusarium crown rot (FCR), caused by a complex of *Fusarium* species, of which *F. pseudograminearum* and *F. culmorum* are the most important, reduces wheat yields in the Pacific Northwest (PNW) of the U.S. by an average of 9%. The objectives of this research were to: 1) conduct a thorough survey of *Fusarium* spp. in 2008 and 2009 throughout 105 wheat fields in Oregon and Washington; 2) correlate FCR severity ratings in wheat to DNA extracted from infected wheat stems through the use of Q-PCR techniques; 3) develop greenhouse inoculation methods for screening for FCR resistance; and 4) map resistance to FCR in two recombinant inbred line populations with a resistant Australian hard red wheat line ‘Sunco’.

Results from the FCR survey showed that four species of *Fusarium* were isolated into pure culture from 99% and 97% of the fields surveyed in 2008 and 2009, respectively, showing the widespread occurrence of the pathogen. Although previous efforts had been made to extract *Fusarium* DNA from infected stems and quantify the amount of pathogen DNA using Real Time Quantitative PCR (Q-PCR) technology, results from this study did not indicate a strong positive correlation between Fusarium crown rot severity ratings from field samples in the 2008 survey and DNA concentrations using Q-PCR.

To accurately describe Quantitative Trait Loci (QTL) for FCR resistance, it was necessary to develop a reliable inoculation method for screening mapping population lines. Optimization experiments to assess the virulence of five PNW isolates of *F. pseudograminearum* and to determine the best method of inoculation revealed that one *Fusarium* isolate was consistently the most virulent across studies. Placing millet seed colonized with *F. pseudograminearum* at the stem base of the seedling resulted in the most consistent virulence and differentiation between the parents of two mapping populations. Two recombinant inbred line mapping populations with the parents Sunco/Macon and Sunco/Otis were developed for the purpose of constructing a genetic linkage map for identifying QTL describing FCR resistance. Significant QTL were identified on chromosome 3BL with a LOD = 14 and LOD = 10 for the Sunco/Macon and Sunco/Otis mapping populations, respectively.

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# CHAPTER 1

## LITERATURE REVIEW

### I. INTRODUCTION

Wheat (*Triticum aestivum* L.) production was approximately 659.3 million tons in 2009, which was second to maize (796.3 tons) and higher than rice (433.5) according to Foreign Agricultural Service production statistics (USDA-FAS, 2009). Due to its diversity of uses, protein content, and storage qualities, wheat is one of the most common staple food crops for more than one-third of the world's population. Wheat originated in southwestern Asia over 10,000 years ago and is related to wild species that still can be found in Lebanon, Syria, northern Israel, Iraq, and eastern Turkey. Breeding of wheat for genetic improvements in crop production began in the early 1800s (Sleper and Poehlman, 2006).

Cook (2001) estimated that soil-borne root diseases cost the U.S. about 10 million tons in wheat production annually. He also stated that the development of host plant resistance is the most effective and affordable opportunity to combat soil-borne diseases. Fusarium crown rot, caused by *F. pseudograminearum* (O'Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*) and *F. culmorum* (Wm. G. Sm.) Sacc. is one of the most damaging soil-borne diseases of dryland wheat in the world, and in the Pacific Northwestern (PNW) United States (Cook, 1992; Burgess et al., 2001; Backhouse et al. 2004; Nicol et al., 2007). Crown rot is considered the most important Fusarium disease of wheat in Australia (Burgess et al., 2001) and ranked second in economic impact to Septoria leaf blotch (*Septoria tritici* (Rob ex. Desm.) = *Mycosphaerella*

*graminicola* (Fuckel) Schroeter), causing losses of AU\$56 million annually (Chakraborty et al., 2006). Infection by *Fusarium* crown rot results in rotting of the stems, crown, and root tissues resulting in a chocolate-brown lesion in the crown region of wheat (Cook and Veseth, 1991). Crown rot is typically associated with wheat production in semi-arid areas where wheat matures under hot-dry conditions because the fungus colonizes stressed plants during the grain filling period (Burgess et al., 2001).

An economic analysis conducted in 1994 from 13 fields evaluated for the impact of crown rot estimated that yield loss represented 97 million kg (3.5 million bu.) in production at a value of \$13 million for the PNW region of the USA (Smiley et al. 2005a). Yield losses from crown rot have been documented to range from 9% to 89% with an average of 9% in the PNW, with world wide losses exceeding 30% (Cook, 1992; Kane, 1987; Burgess et al. 2001; Dodman & Wildermuth, 1987; Klein et al. 1991; Hekimhan et al., 2004). Smiley et al. (2005a) documented yield loss as high as 61% following artificial inoculation with a mixture of five *F. pseudograminearum* isolates. Yield loss estimates for bread wheat from surveys in Turkey range from 24% to 42% for natural infections (Hekimhan et al. 2004; Nicol et al. 2001).

Yield loss resulting from *Fusarium* crown rot infection is difficult to assess on a regional basis as infection occurs at or near the soil line and is not clearly visible (Strausbaugh et al. 2004), with the exception of the formation of white heads for a brief period of time prior to straw ripening (Burgess et al. 2001). White heads form as a result of destruction of the vascular system by the fungus during colonization resulting in an inability of the plant to transport water and nutrients to the head during grain filling. It is also difficult to quantify the type and amount of soil-borne fungi responsible for infection

(Okubara et al. 2005). With recent advances in molecular technology, quantitative real-time polymerase chain reaction (Q-PCR) technology, the amount and species of the pathogens can be estimated. Smiley et al. (2005b) conducted extensive surveys of northern Oregon and southeastern Washington, however the central northernmost portions of Washington dryland wheat producing areas needed to be surveyed more extensively. The first objective of this study was to survey *Fusarium* crown rot distribution over a broader area throughout the northern and central Washington areas of the PNW region. The second objective of this study was to assess the use of Q-PCR for the quantification of pathogenic *Fusarium* DNA in soil and plant tissue as a potential diagnostic tool for disease management.

Management of crown rot in the past has relied on cultural practices that provide partial control and are not reliable for limiting damage (Cook, 1981; Paulitz et al. 2002; Smiley and Patterson, 1996). Host plant resistance is the most efficient and reliable approach to reducing yield losses due to *Fusarium* crown rot (Cook, 2001). Evaluation of *Fusarium* crown rot cultivar resistance dates back to the 1960s in Australia (McKnight and Hart, 1966; Purss, 1966). Evaluation of PNW germplasm by Smiley and Yan (2009) showed there is a high degree of variation in varietal response to *Fusarium* crown rot over years and across sites. As a result no cultivars in the PNW have been identified as being consistently tolerant to the disease. Several isolates of *Fusarium* spp. have been collected and cultured from the PNW. The third objective of this study was to assess wheat cultivars to identify genes responsible for host plant resistance to these local isolates. Currently there are no published results for mapping the partial genetic resistance to *Fusarium* crown rot inherent in the Australian spring wheat cultivar 'Sunco'. The fourth

objective of this study was to identify quantitative trait loci for crown rot resistance in a Sunco/Macon recombinant inbred line mapping population.

This literature review provides a synopsis of the history, surveys, biology and epidemiology of *Fusarium* crown rot, greenhouse bioassay screening techniques, identification of QTL for *Fusarium* crown rot resistance, and genetic characterization of *Fusarium* species.

## **II. CROWN ROT CAUSED BY *FUSARIUM* SPP.: BIOLOGY, LIFE CYCLE, AND HISTORY**

### **Biology**

Common names of crown rot include dryland foot rot, dryland root rot, foot rot, *Fusarium* crown rot, and *Fusarium* root rot. Crown rot is caused by a complex of *Fusarium pseudograminearum* (O'Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*), *F. culmorum* (Wm. G. Sm.) Sacc., *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus*), *F. avenaceum* (Fr.:Fr.) Sacc. (= *Gibberella avenacea*), and *Microdochium nivale* (Fr.) Samuels & I.C. Hallet (= *Monographella nivalis*) (Cook, 1968; Cook and Veseth, 1991; Smiley et al., 2005a.; Nicol et al., 2006). In Australia, and other parts of the world, 'crown rot' is the accepted common name of the disease and is understood to be caused mainly by *F. pseudograminearum* and *F. culmorum* (Burgess et al., 2001).

*B. sorokiniana* causes 'common root rot' alone or in association with *Fusarium* sp. (Cook and Veseth, 1991). Although the symptoms of common root rot and crown rot are similar, the symptoms are distinguishable (Cook, 1968). Common root rot is characterized by a dark brown to black discoloration of the sub-crown internode if caused

by *B. sorokiniana*, and chocolate to reddish brown symptoms on roots if caused by *Fusarium* species (Purss, 1970; Cook and Veseth, 1991).

Typical symptoms of crown rot include a dark brown discoloration (with an occasional pink tinge) of the crown region and lower one, two, or sometimes three internodes (Cook, 1968; Cook and Veseth, 1991). Burgess et al. (2001) reported that lesions extend to the fourth or fifth node and can be accompanied by salmon colored sporodochia. Infection of the crown region leads to destruction of the vascular system and the subsequent formation of white heads containing little to no seed (Cook, 1968; Burgess et al. 2001; Nicol et al. 2001). Although there are several other soil-borne root diseases that lead to the development of white heads in wheat, 'white heads' are often used as an indicator of crown rot injury (Burgess et al. 2001; Cook, 1968; Nicol et al. 2007).

The first visual symptoms of the disease on seedlings are a uniform browning of the stem bases (Burgess et al. 2001). Cook (1968) conducted a time of infection study and reported that initial infection occurred around 4 weeks after emergence. In that study 97% of the plants were symptomless 2 weeks after emergence. Cook (1968) concluded that the initial infections occurred through openings made during crown root emergence. He also postulated that disease development was limited during the winter, but resumed in the spring and the disease life cycle followed that of the winter wheat. Symptoms and adverse effects of crown rot are much more pronounced under drought conditions in dryland wheat producing regions throughout Australia, Europe, North America, North and South Africa, and West Asia, Turkey, and South America (Smiley et al. 2005a.; Nicol et al. 2007; Chakraborty et al. 2006).

The dominant causal pathogen and occurrence of *Fusarium* spp. within a given location varies from year to year (Smiley and Patterson, 1996). Smiley et al. (2005b) found that infection by *F. pseudograminearum* caused a greater incidence of lesions of the sub-crown internode and crowns than *B. sorokiniana* or *F. culmorum* in field trials. *F. pseudograminearum* and *F. culmorum* led to a greater abundance of white heads and reduction in seedling stand than any of the other species involved in the infection complex (i.e. *B. sorokiniana*, *F. avenaceum*, and *Microdochium nivale*) at two locations during 2003 and 2004. Yield was consistently reduced with *F. pseudograminearum* and *F. culmorum*, but not with *B. sorokiniana* (Smiley et al. 2005b).

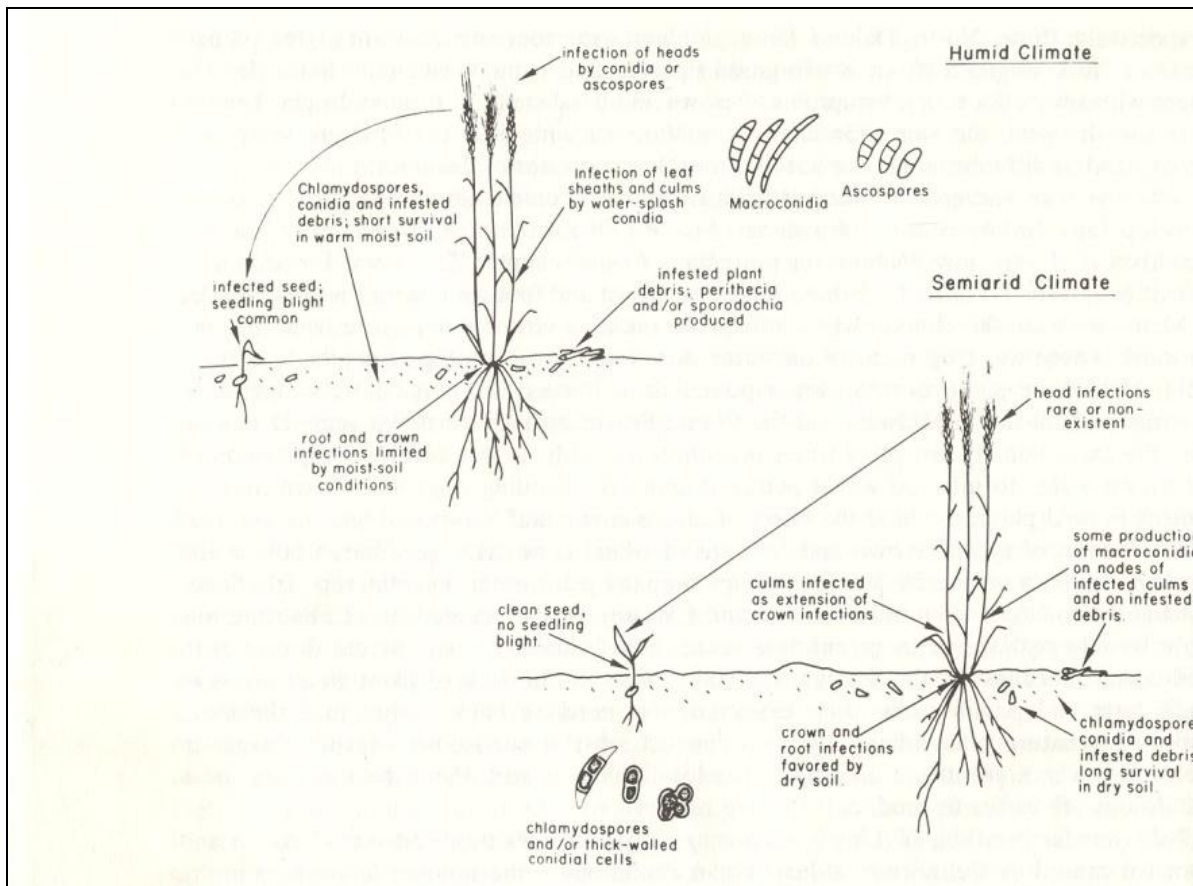
### **Life Cycle**

The *Fusarium* crown rot pathogens survive in the soil as chlamydospores in dead organic matter and plant debris (Paulitz et al. 2002). Summerell et al. (1990) found that the main inoculum source was infected residue. Cook (1968) reported that *Fusarium culmorum* survives in the soil as thick-walled chlamydospores embedded in organic matter or formed within macroconidia. Chlamydospores have the potential for long-term survival in soil and plant debris. Chlamydospores can form from macroconidia (endoconidial chlamydospores) or hyphae (mycelial chlamydospores) (Cook, 1968; Sitton and Cook, 1981; Inglis and Cook, 1986). Inglis and Cook (1986) reported that endoconidial chlamydospores are the predominant type for *F. culmorum* in eastern Washington wheat fields in Adams, Lincoln and Whitman counties. Of these endoconidial chlamydospores most occurred in chains of two, three or four adjacent intercalary macroconidial cells with little variation in shape. Sitton and Cook (1981) showed that some chlamydospores formed from macroconidia of *F. pseudograminearum*

were short-lived (5.5 years) compared to chlamydospores formed from *F. culmorum* macroconidia which survived 8.5 years.

Burgess et al. (2001) stressed that *F. pseudograminearum* persists in Australia as hyphae in stem tissues that were colonized during the parasitic phase of the life cycle. In dry and arid climates *F. pseudograminearum* dominates with infections localized to the crown region (Figure 1). Its life cycle is readily distinguished from *F. graminearum*, which produces the sexual stage (teleomorph *Gibberella zeae*) that results in formation of perithecia and ascospores and leads to head infections (Nelson et al. 1981). Head (blight) infections are rare with *F. pseudograminearum* or *F. culmorum* in an arid environment. Inglis and Maloy (1983) were the first to report head scab caused by *Gibberella zeae* on irrigated wheat in eastern Washington.

Paulitz et al. (2002) reported that both *F. culmorum* and *B. sorokiniana* survive as chlamydospores and pigmented conidia, either in plant residue or soil. Propagules infect newly sown plants leading to the colonization of crowns and lower internodes (Nelson et al. 1981). Infection of roots does not appear to be common (Burgess et al. 2001). Culms become infected as an extension of crown infections. If inoculum is severe and conditions are moist, infection prior to emergence can result in damping-off and seedling death. Macroconidia are produced in infected crowns and return to the soil in debris as a long-term inoculum source (Nelson et al. 1981).



**Figure 1.** Life cycle of *Fusarium pseudograminearum* (semi-arid climate) and *F. graminearum* (humid climate), formerly known as *Fusarium roseum* and *F. graminearum* Group 1 and Group 2, respectively (Nelson et al. 1981).

*Fusarium* head blight, caused by *F. graminearum*, occurs occasionally under irrigated conditions or in high elevation (late maturing) areas but is not considered a serious problem in the predominantly rain-fed PNW region (Paulitz et al. 2002).

Fernandez and Chen (2005) found that although *F. culmorum* and *F. graminearum* both caused head blight infections, *F. culmorum* was the most virulent species on wheat crowns. *F. avenaceum*, *F. equiseti* and *F. poae* were also evaluated and found be less virulent on wheat (Fernandez and Chen, 2005).



*Fusarium* species limit yield by rotting seed, seedlings, roots, crowns, basal stems, or heads (Smiley et al. 1996; Paulitz et al. 2002; Smiley et al. 2003). Infection of seedlings and basal stems leads to yield loss from damaged seedlings, pre-harvest lodging, and impaired grain filling (Schilling et al. 1996). Crown rot infection causes a reduction in wheat straw production, grain yield, and grain quality (i.e. mainly test weight). Crown rot tends to have a greater impact than other soil-borne root rotting diseases in dryland or rain-fed wheat producing regions (Smiley et al. 2005a). Although *F. culmorum* and *F. pseudograminearum* both cause significant yield reductions, the latter tends to occur in the greatest frequency in Oregon and Washington (Smiley et al. 2005b). Occurrence of crown rot is favored by wet conditions shortly after seeding and dry conditions between anthesis and maturity (Paulitz et al. 2002; Smiley et al. 1996).

### **History of Surveys of Fusarium Crown Rot**

Burgess et al. (2001) stated that Hynes (1923) was the first to describe an isolate of *Gibberella saubinetii*, which resembled the currently named *Gibberella zeae* (teleomorph of *F. graminearum*) as the causal pathogen of crown rot. Hynes (1923) described the fungus by its inability to produce perithecia in culture, from which others have since postulated that it could have been the earliest evidence of *F. pseudograminearum* occurring in wheat (Burgess et al., 2001). Burgess et al. (2001) also stated that the earliest evidence of resistance to crown rot in published literature was described in Australia by McKnight and Hart (1966).

According to Purss (1970), the earliest documented mention of resistance in wheat to “foot rot” or common root rot was in Australia in 1922 by Hamblin (1922). Purss (1970) further mentioned that Hynes (1938) was the first to confirm the resistance

identified by Hamblin (1922). Purss (1970) evaluated 18 cultivars from the world collection, six from Canada, and eight local cultivars for resistance to common root rot. 'Gala', a cultivar with a high level of resistance to crown rot, was not as resistant to common root rot. Purss (1970) summarized that although common root rot did not cause significant above ground symptoms, its infection had an "insidious effect on the plant." At the time of this publication, resistance to common root rot and crown rot was thought to be "multigenic" (Purss, 1970).

Economic damage from crown rot infections were first documented in the PNW region of the U.S. in 1964 with estimated yield losses up to 50% (Cook, 1968). Cook (1968) described the fungus as *F. roseum* Lk. (emend. Snyder & Hans.) f. sp. *cerealis* (Cke.) Snyder and Hans.) and delineated the fungal cultivars as 'Culmorum', 'Graminearum', or 'Avenaceum'. Greater than 90% of the isolates from diseased fields in Washington in 1964 were *F. roseum* f. sp. *cerealis* 'Culmorum' and *F. roseum* f. sp. *cerealis* 'Graminearum', with 'Culmorum' predominating. *F. roseum* f. sp. *cerealis* 'Avenaceum' was isolated occasionally. The fundamental documentation of nomenclature used to describe these species in literature is now *F. graminearum*, *F. culmorum*, and *F. avenaceum*.

Cook (1968) was the first to conduct detailed survey studies of crown rot in winter wheat in the PNW. Yield losses of 17% and 50%, respectively, were reported for two winter wheat populations with different *Fusarium* population densities. Within the last 40 years crown rot has been well documented throughout the Pacific Northwest (PNW) wheat producing states of Washington, Oregon and Idaho (Cook, 1980; Smiley and Patterson, 1996; Strausbaugh et al. 2004; Smiley et al. 2005a). More recent research

has shown yield losses in the 0 to 35% range, with an average of 9.5% in a study evaluating 13 sites that had *Fusarium* infection in Washington and Oregon (Smiley et al. 2005a).

Burgess et al. (1975) conducted surveys in Australia and found that *Fusarium roseum* 'Graminearum' was the predominant pathogen associated with crown rot in the eastern wheat belt of Australia during the 1972, 1973, and 1974 growing seasons. Surveying was conducted by taking tiller samples from plants showing symptoms 50 to 75 m inside the field border in a 25 m arc during the time of white-head formation. Isolates were identified as *F. roseum* 'Graminearum' Group 1 isolates because they did not form perithecia in culture or on sterile wheat straw. Burgess et al. (1975) confirmed work by McKnight and Hart (1966) that found the predominant causal pathogen of crown rot was *F. roseum* 'Graminearum'.

U.S. surveys for common and *Fusarium* root rots have been conducted in California, Colorado, Wyoming, and Texas (Scardaci and Webster, 1982; Hill et al. 1983; Specht and Ruch, 1988). In Colorado, Wyoming, and Texas the main pathogens associated with common root rot were *B. sorokiniana* and *F. acuminatum*. Although *F. equiseti* was frequently isolated in Texas, it was not virulent (Specht and Rush, 1988). In California *F. culmorum*, *F. graminearum*, and *B. sorokiniana* were most frequently isolated from cereals with root rot symptoms (Scardaci and Webster, 1982).

Klein et al. (1990) surveyed the incidence of white heads in wheat in the northern areas of the wheat belt in New South Wales from 1976 to 1981. Sampling was conducted by taking infected tillers from five sub-sites in a sampling arc located at least 150 m from the field border. Incidence of white heads across cultivars was generally low (less than

5%), but their presence was associated with crown rot. Results showed the predominant crown rot pathogen was *F. graminearum* Group 1 which was isolated from 97% of 1450 stems showing white head symptoms. *F. equiseti* and *B. sorokiniana* were frequently isolated from stems and sub-crown internodes as well (Klein et al. 1990).

Smiley and Patterson (1996) surveyed 288 non-irrigated winter wheat fields in the PNW during 1993 and 1994. Samples were collected from random fields by taking 15 stems and soil samples in a 10 to 15 m circle located 20 to 70 m into each field. *F. graminearum* Group 1 was the dominant and most wide spread pathogen associated with crown rot samples from 19 counties throughout the PNW region. Although *F. culmorum* was widely distributed in soil, it was detected in plants half as often as *F. pseudograminearum*. Complex interactions between cropping systems and the environment influence pathogen distribution of these species. Smiley and Patterson (1996) proposed “Fusarium foot rot” as the name used to describe the disease.

Smiley et al. (1996) evaluated crown and root rot diseases of winter wheat under different crop rotations and cultural practices over 3 years (1989 to 1991) in the PNW. Take-all (*Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver var. *tritici* J. Walker) and eyespot (*Pseudocercospora herpotrichoides* (Fron.) Deighton) were associated with high precipitation sites, while Rhizoctonia root rot (*Rhizoctonia solani* Kühn AG-8) and Fusarium crown rot were favored by locations with drought conditions. Occurrence of crown rot increased with increasing surface residue from previous crops as well as the application of inorganic N fertilizers. The occurrence of crown rot also was greater under annual cropping systems (continuous wheat). Overall root diseases were less prevalent in wheat-pea rotations compared to annual and summer-fallow dryland wheat rotations.

Backhouse and Burgess (2002) conducted a climatic analysis of the distribution of *F. pseudograminearum*, *F. graminearum*, and *F. culmorum* in Australia using the climate matching system BIOCLIM. Records for this study were obtained from a survey database at the University of Sydney Fusarium Research Laboratory. BIOCLIM aided in describing *Fusarium* distributions through quantitative climatic data. *F. graminearum* was most closely associated with warmer temperatures (greater than 18 °C) and high rainfall (greater than 195 mm). *F. pseudograminearum* did not appear to be limited by climatic conditions in this study as it was found to be very widespread. *F. culmorum* was more limited in distribution as populations were associated with lower temperatures and higher rainfall compared to *F. pseudograminearum*. Sitton and Cook (1981) and Smiley and Paterson (1996) reported *F. pseudograminearum* tended to dominate in counties where the maximum temperature in July was warmer than counties where *F. culmorum* dominated.

Backhouse et al. (2004) conducted an extensive survey of 409 wheat, barley or durum wheat fields from the eastern Australian grain belt from 1996 to 1999. *F. pseudograminearum* was nearly the only pathogen isolated from samples, but *F. culmorum*, *F. avenaceum*, *F. crookwellense*, and *F. graminearum* were also isolated. *F. culmorum* accounted for more than 70% of isolates from samples collected from the high rainfall region (Victorian > 500 mm) and the southeast region of Southern Australia. *F. culmorum* represented 18% of the isolates collected from the moderate rainfall region (350-500 mm), and 7% of the isolates from the mid-Northern low rainfall region. The proportions of *F. culmorum* were significantly correlated with climatic conditions in this study, especially November rainfall and temperature. It was concluded that *F.*

*pseudograminearum* dominated the region and *F. culmorum* was more confined to areas with cooler temperatures and higher rainfall. The authors postulated that rainfall may have a stronger influence on the occurrence of *F. culmorum* than temperature.

Surveys of root diseases were conducted in southern Idaho by Strausbaugh et al. (2004) to identify the dominant soil-borne pathogens present in 133 wheat and barley dryland (230 to 360 mm) and irrigated fields during 2001 and 2002. This region, known as the Intermountain West Region of the PNW, is unique due to its high elevation and cooler temperatures. *F. culmorum* and *B. sorokiniana* were isolated among other pathogens including other *Fusarium* spp. (*F. equiseti*, *F. reticulatum*, *F. acuminatum*, *F. semitectum*, *F. tricinctum*, and *F. crookwellense*), *Rhizoctonia solani*, and *Gaeumannomyces graminis* var. *tritici*. Ninety-six percent of the fields sampled had lesion nematodes (*Pratylenchus* spp.), 78% had stunt nematodes (*Tylenchorhynchus* spp.) and cereal cyst nematodes (*Heterodera avenae*) to a lesser extent. The authors concluded that *F. culmorum* is more prevalent in cooler climates and plant breeders should screen for resistance to *B. sorokiniana* and *F. culmorum*. This study showed the prevalence and importance of crown and common root rot pathogens in the PNW region and confirms other literature that *F. culmorum* is associated with cooler temperatures. However, this crown rot assessment was based on roots, not crowns. Burgess et al. (2001) suggested that root infections of crown rot rarely occur in Australia with *F. pseudograminearum*. Root infections are more common with *F. culmorum* infections (Dr. J. Nicol, personal comm.).

Smiley et al. (2005a) conducted an extensive survey of crown rot in wheat and barley in the PNW region of the U.S.. These trials were the first yield loss estimates for

the region in controlled field experiments. Yield loss was evaluated by analyzing diseased tillers sampled from commercial fields and plots of cultivars inoculated with *F. pseudograminearum* compared to non-inoculated naturally infested soils. Crown rot decreased yield in 13 commercial fields by as much as 35% with an average of 9.5%. Under inoculated conditions yield loss was as high as 61%. The incidence of crown rot was the greatest in seasons with low precipitation and above ground symptoms were not always obvious under moderate pathogen pressure (Smiley et al. 2005a). This research is commonly cited and represents reliable controlled experiments to assess yield loss in the Oregon and Eastern Washington regions of the PNW.

Smiley et al. (2005b) described the pathogenicity of fungi associated with the crown rot complex in experiments conducted during 2003 to 2004 in greenhouse tests and at two field locations. Pathogenicity was evaluated for 178 isolates in the greenhouse, of five species (cited previously) involved in the ‘crown rot complex’. Twenty-four isolates of *B. sorokiniana*, *F. culmorum*, and *F. pseudograminearum* were also evaluated in field experiments. Across experiments crown rot occurrence caused by those species significantly reduced plant height, or was negatively correlated with plant height. In field experiments with spring wheat, stand establishment and density of mature heads were reduced with increased crown rot severity. *F. culmorum* and *F. pseudograminearum* caused the greatest amount of yield loss and disease severity. Smiley et al. (2005b) recommended that future research should use a mixture of isolates to evaluate varietal resistance to be representative of native pathogen populations.

Bentley et al. (2006a) conducted surveys from wheat and other grasses in New Zealand. Although *F. oxysporum* was isolated in the greatest frequency (20%), it was

secondary in causing disease to *F. culmorum* and *F. pseudograminearum* species. *F. culmorum* was isolated from 16% of all wheat stems and *F. pseudograminearum* was isolated from 1.5% of wheat stems. The authors concluded that the cool mild climate in New Zealand compared to other regions led to the dominance of *F. culmorum* in the crown rot complex.

The dominant species associated with Fusarium foot rot and head blight differs among countries and occurrence is qualitatively related to climate (Backhouse and Burgess, 2002). Prior to Bentley et al. (2006b) there were no detailed reports of the relative distribution and importance of *Fusarium* species associated with crown and root rot of wheat in Turkey. Random wheat samples were collected from three ecological wheat producing regions in northern Turkey. Results indicated that of the 15 *Fusarium* species isolated from wheat stem bases, *F. culmorum* was isolated most frequently at 28% of the sites sampled, and *F. pseudograminearum* was isolated from only 8% of the sites.

Tunali et al. (2006) obtained 51 isolates representing 13 *Fusarium* species from 32 fields from a survey of Northern and Central regions of the Anatolian plain in Turkey. Approximately 49% of the samples were taken from a region where the annual rainfall exceeded 453 mm and 51% were taken from regions where annual precipitation was 252 to 452 mm. *F. culmorum*, *F. pseudograminearum*, and *F. graminearum* were isolated (in that order of frequency) from discolored crown regions of the winter wheat cultivar 'Pehlivan' and identified as the most virulent species associated with crown rot in the survey.



Chakraborty et al. (2006) provided an international review of literature on Fusarium crown rot and head blight population structure and epidemiology. The review reported that *F. pseudograminearum* is the dominant pathogen responsible for crown rot in Australia and other arid parts of the world. Although crown rot has received less attention than Fusarium head blight world wide, it occurs in most cereal producing regions of the world including Australia, Europe, North America, South America, West Asia, South Africa, and North Africa. The distribution of *F. pseudograminearum* and *F. culmorum* in causing crown rot infections is most likely linked to climate as several studies suggest that *F. culmorum* is the dominant pathogen in cooler / wetter climates (Backhouse and Burgess, 2002; Strausbaugh et al. 2004; Smiley et al. 2005a). Several studies have found that tillage and crop rotation have a significant effect on the occurrence of crown rot in the PNW (Smiley et al. 1996; Paulitz et al. 2002).

### **Fusarium Crown Rot in Different Cropping Systems and Cultural Control**

Much of the research documenting the impacts of cropping systems and soil management practices on these soil-borne diseases was initiated in the mid-1960s (Cook, 1968). Smiley et al. 1970 reported that anhydrous ammonia NH<sub>3</sub> fertilizer significantly reduced *Fusarium* spp. (mainly *F. culmorum*) populations in a 4 to 5 cm zone of Palouse silt loam and Ritzville silt loam soil in situ laboratory experiments. Later reports showed little or no decline in the number of *Fusarium* spp. propagules in the soil tillage layer following banding applications in field experiments due to an insufficient distribution of anhydrous ammonia throughout the layer (Smiley et al. 1972).

Papendick and Cook (1974) provided the most comprehensive report of the earliest evidence pertaining to cultural practices on the development of Fusarium foot rot

in eastern Washington. Experiments were conducted over three growing seasons (1970 to 1972) in Lincoln County to assess the effects of planting date, nitrogen fertilization and planting density on the development of Fusarium foot rot caused by *F. roseum* f. sp. 'Culmorum' (currently referred to as *F. culmorum*). The research also utilized soil and plant water potentials to assess the respective moisture content during the duration of the studies. Results showed that high nitrogen rates (>60 kg N/ha) accompanied by increased planting densities (60 kg seed/ha in rows 30 cm apart) resulted in the greatest occurrence of Fusarium foot rot with the driest conditions and lowest water potentials of -42 bars in June (Papendick and Cook, 1974).

Plant water potentials remained steady at -20 to -25 bars regardless of the treatment until May (Papendick and Cook, 1974). The authors reported that lethal attacks to plants by the disease occurred under drier plant water potentials of -35 to -42 bars during June, while at -30 to -33 bars crown rot acted as a slow-decaying disease. High nitrogen rates were more significant in determining disease severity than *F. culmorum* inoculum density (propagules/g soil). The authors concluded that early planting, high nitrogen rates, and high planting densities contributed to the development of lush vegetative growth that resulted in greater transpirational capacities that led to increased water deficit in April and early May resulting in elevated crown rot occurrence (Papendick and Cook, 1974).

As mentioned previously, crown rot is severe following dry conditions between anthesis and maturity (Nelson et al. 1981; Paulitz et al. 2002; Smiley et al. 1996). Field conditions that are thought to contribute to a greater occurrence of Fusarium crown rot and Pythium root rot include annual cropping, summer fallow cropping systems, early

planting, high nitrogen content in soil, and the presence of surface crop residue (Smiley et al. 1996). Wildermuth et al. (1997) found that crown rot was significantly higher in conservation tillage treatments where stubble was retained (32% infection) compared to where it was removed through disc tillage (4.7% infection).

No-till and reduced tillage practices (conservation tillage) are becoming more popular in Washington, Oregon, and Idaho (Paulitz et al. 2002). The total conservation tillage acreage that accounts for no-till and reduced tillage practices represents 5.7% of the acreage in the PNW region. Across the U.S., conservation tillage was practiced on over 17% of the acreage in 2000 (Paulitz et al. 2002). These reduced tillage practices can reduce operational costs and equipment depreciation as well as improve soil water holding capacity, microbial activity and lead to reduced wind and surface erosion from residue on the soil surface. However, tillage is often responsible for alleviating the reproductive structures and host material of soil-borne pathogens involving *Fusarium* spp. and *Pythium* spp. (Paulitz et al. 2002). The additional pressure from soil-borne pathogens under conservation tillage practices has made growers reluctant to adopt the technology (Paulitz et al. 2002).

Cultural methods for crown rot control include delayed planting dates and optimized nitrogen applications to reduce late season water stress (Paulitz et al., 2002). Because options for chemical control are limited and not cost effective, identifying genetic resistance in wheat remains the most important tool to control crown rot (Cook, 2001; Yahyaoui et al. 2003).

*Fusarium pseudograminearum* remains the most prominent species and subject of study for evaluating and developing genetic resistance in wheat populations. Australia

has made progress with the discovery of cultivars ‘Kukri’ and ‘Sunco’, which have adult-plant resistance to *F. pseudograminearum* (Wallwork et al. 2004; Mitter et al. 2006). The term 'adult-plant resistance' is often used to distinguish resistance evident in older plants from that observed in seedling screening assays, and will be discussed in more detail in subsequent sections.

Wildermuth et al. (2001) found that crown depth, which ranged from 17 mm to 2 mm (above ground), was correlated to the relative susceptibility of 13 cultivars to crown rot ( $r^2 = 0.57$ ,  $P \leq 0.05$ ). Results showed that as crown depth increased, relative susceptibility of cultivars to crown rot increased, with the exception of ‘Sunco’. Cultivars with greater than 55% susceptibility to crown rot (based on a proportionate scale of the highly susceptible cultivar ‘Puseas’), had crown depths between 9.5 mm and 15.8 mm. The more resistant cultivars, such as ‘2-49’, had crown depths shallower than 6.7 mm, with the exception of ‘Sunco’ (crown depth = 17 mm) (Wildermuth et al. 2001).

Crown depth in seedlings was highly related to crown rot susceptibility in the greenhouse as well ( $r^2 = 0.8$ ,  $p \leq 0.001$ ). The correlation between crown depth measured in the greenhouse and field were highly correlated ( $r^2 = 0.9$ ), as was the correlation between crown depth (field) and relative cultivar susceptibility ( $r^2 = 0.89$ ). As the planting depth of the cultivars increased from 25 mm to 75 mm, there was shallower crown depth formation in more resistant cultivars (‘2-49’ and ‘IRN497’) but significantly deeper crown depth formation for more susceptible cultivars (‘Kamilaroi’ and ‘Cunningham’) (Wildermuth et al. 2001).

It has been postulated that the depth of crown formation in wheat could explain two types of crown rot resistance; that of which can be detected in seedlings and adult

plants (common in cultivars such as ‘2-49’) and resistance that can only be detected in adult plants in the field (common in ‘Sunco’ and ‘Pelsart’) (Wildermuth et al. 2001). This research documented that the depth of crown formation is a dominant genetically controlled trait that is linked to partial resistance (Wildermuth et al. 2001). Methods of screening for resistance in controlled environments are needed in order to evaluate a range of wheat genotypes for resistance to crown rot (Wildermuth and McNamara, 1994).

### **III. BIOASSAYS FOR IDENTIFYING CROWN ROT RESISTANCE**

Field screening of cultivars for disease resistance can be time-consuming and limited by seasonal crop growing constraints and environmental variability (Wildermuth, 1994). Methods for screening plant material year around in greenhouses can be very efficient for cultivar screening. Early efforts in the mid-1960s failed to adequately mimic field inoculation of wheat with *Fusarium* to obtain crown rot symptoms at plant maturity (Liddell, 1986). However, Burgess et al. (2001) reported that Purss (1966) found no correlation between seedling blight in greenhouse tests and tolerance to mature plants in the field using a “seed inoculation” technique. Subsequent studies found significant correlations between greenhouse tests and field trials for crown rot disease ratings (Wildermuth and McNamara, 1994; Mitter et al. 2006; Li et al. 2008).

A study was conducted to evaluate the inoculation potential of *Fusarium*-colonized wheat chaff (Liddell, 1986). Results from these experiments showed that sufficient inoculum was needed to cause uniform infection; however excessive amounts skewed results by causing premature death of wheat plants (Liddell, 1986). Other recommendations from this study encouraged the use of pasteurized soil to inhibit secondary effects from soil microflora.

Wildermuth and McNamara (1994) evaluated wheat seedlings for resistance to *Fusarium* grown in a controlled greenhouse environment to speed the process of selection for *Fusarium* tolerance. Wheat and barley grain was autoclaved and colonized with *F. pseudograminearum*. Wheat leaf sheaths were rated for necrotic lesions after 22 days. Seedling evaluations were compared with mature plant ratings from wheat grown under inoculated conditions in the field with a resulting correlation coefficient of  $r=0.78$  ( $p<0.01$ ). However, at growth chamber temperatures below 25 ° C, differences among cultivars for crown rot susceptibility were not as pronounced (Wildermuth and McNamara, 1994). Furthermore, the use of this method requires a lot of greenhouse space and can be inconvenient (Mitter et al. 2006).

Adult and seedling resistance have been documented. The seedling stage of growth has been defined within the first 45 days, while a mature plant is considered an adult for this comparison (Wallwork et al. 2004; Mitter et al. 2006). It appears that the most resistant genotypes can be identified in seedling bioassays, but genotypes with adult-plant resistance (such as that of ‘Sunco’). Thus the potential for significant resistance to crown rot to remain undetected in seedling screening programs exists (Wallwork et al. 2004). Seedling resistance can be evaluated during the first 45 days of the seedling phase of growth. Adult plant resistance is documented by evaluating mature plants for crown rot symptoms and damage.

Wallwork et al. (2004) developed an improved method of screening wheat cultivars for crown rot resistance. The method used seedlings grown in open-ended tubes in galvanized baskets placed in outdoor sand-based terraces. This method was used to detect adult plant resistance to *F. pseudograminearum* and *F. culmorum*. The authors

add that screening of plants in field-sown trials often is unreliable due to uneven natural infection, contamination by other pathogens, or adverse environmental conditions that affect results.

A refinement of these methods was conducted by Mitter et al. (2006). The objective of these experiments was to find a method for screening high volumes of seedlings for *F. pseudograminearum* resistance in a greenhouse environment. Single seedlings were grown in square seedling pots, inoculated 13 to 14 days after planting with *F. pseudograminearum*, and incubated under high relative humidity (60%/ 80% (+ or – 10%) day / night) conditions for 48 hours at 25 C. Following incubation, the original growing conditions were restored and crown rot severity was assessed 35 days after inoculation. Sixteen cultivars were compared using this technique in the greenhouse and then followed by in-field evaluations where the plants were rated at maturity. The two methods were highly correlated according to Spearman's rank correlation coefficient ( $r_s = 0.987$ ), proving that the greenhouse screening technique was sufficient to appropriately screen cultivars for resistance to *F. pseudograminearum* (Mitter et al., 2006). Crown rot severity for 'Sunco' was 0.54 according to the crown rot index (CRI) calculated by the authors (CRI = length of stem discoloration/seedling height) x (number of leaf sheaths with necrosis).

Li et al. (2008) conducted the most recent research pertaining to seedling inoculation for crown rot resistance. Experiments were conducted in the greenhouse to evaluate soaking seedlings (age 1 to 4 days old) in a conidial suspension varying in concentrations ranging from  $5 \times 10^5$  to  $1 \times 10^7$  conidia per mL of solution (water + 0.1% tween (v/v)). In addition to these treatments the duration of inoculation (1 to 10 min of

soaking), incubation period (0, 24, and 48 hr at 100% relative humidity and darkness), the effect of water stress on crown rot symptom development, and comparisons to the Mitter et al. 2006 stem droplet method were conducted. Plants were rated on the basis of a 0 to 5 scale (0=no disease; 5=severe crown rot symptoms). There was not any effect of duration of inoculation or incubation period. However, increasing the conidial concentration to  $1 \times 10^6$  conidia per mL resulted in greater levels of infection. Overall disease severity was significantly greater than plants inoculated with the Mitter et al. (2006) method. The authors summarized that this was a simple reliable method for screening resistant germplasm, but did note that there was a high mortality among the most susceptible cultivars evaluated with this seedling dipping method.

Seedling and adult plant screening bioassays described previously have been useful in identifying Quantitative Trait Loci (QTL) for plant resistance (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006). Liu (1998) defined a QTL as “genes affecting quantitative traits”, such as disease symptoms or signs.

#### **IV. GENETIC RESISTANCE AND DEVELOPMENT OF MOLECULAR MARKERS IN WHEAT**

##### **Host Plant Resistance to Crown Rot in Wheat**

Purss (1970) commented that the first resistance for “foot rot” or common root rot was mentioned around 1922 (Hamblin, 1922). World wide sources of resistance have been evaluated since that time (Purss, 1966; McKnight and Hart, 1966; Purss, 1970; Nelson and Burgess, 1994; Smiley et al. 2003). Although Cook (1968) surveyed several fields, Smiley et al. (2003) was first reported quantifying cultivars for *Fusarium* susceptibility in inoculated field experiments in the PNW. Nelson and Burgess (1994)



reported that a wide range of wheat genotypes had been evaluated over the prior two decades. Nelson and Burgess (1994) evaluated nine cultivars of oats, barley, and wheat for resistance and found oats to be the most resistant, followed by wheat and barley. In fact, crown rot symptoms were not observed on any of the oat plants (Nelson and Burgess, 1994).

Cook (2001) suggested that resistance could be due to escape or tolerance mechanisms rather than true host resistance. He suggested the possibility that superior performance of soft white winter wheat cultivars, when infected with *F. culmorum* under dryland conditions in the PNW was associated with the ability to avoid or tolerate water stress. Nelson and Burgess (1994) stated that although crown rot severity differed among genotypes, complete resistance to infection had not been demonstrated in Australia. Tolerance or resistance to crown rot and common root rot has been described as 'polygenic' (Purss, 1970; Burgess et al. 2001; Collard et al. 2005; Bovill et al. 2006). Although various QTL have been identified for crown rot resistance, the analysis of trait inheritance and genetic mapping in bread wheat has proven to be difficult due to the complex genetic structure of hexaploid wheat (Sleper and Poehlman, 2006).

The *Triticum* species are grouped into three ploidy classes: diploid ( $2n = 2x = 14$ ), tetraploid ( $2n = 4x = 28$ ), and hexaploid ( $2n = 6x = 42$ ). There are currently 11 species of diploid and tetraploid wheats and six hexaploid species. *Triticum aestivum*, common hexaploid bread wheat, and *Triticum turgidum*, tetraploid durum wheat are most important for commercial production (Sleper and Poehlman, 2006). *Triticum timopheevii* is a wild tetraploid species and has been the source of several disease resistance genes (Kammholz et al. 2001; Bovill et al. 2006).

## **Genetic Mapping**

Genetic mapping is the determination of relative positions of loci along a chromosome and is essential for quantitative genetic analysis (Lynch and Walsh, 1998). According to Hartl and Jones (2006) ‘genes’ are the elements of inherited traits that are transmitted from parents to offspring in reproduction. ‘Alleles’ are the different forms of a particular gene (e.g. allele ‘A’ or allele ‘a’). The genotype is the genetic constitution, or molecular make-up of an organism or cell. The phenotype is the observable properties of a characteristic governed by the gene, which can be affected by the environment. A genetic ‘marker’ can be a variant in DNA that is a known gene, protein, or represent a fragment of DNA without a known function. A gene can be considered a marker if it has a known function and detectable variation. In order to be of use as an indirect selection tool, the gene and the marker should be tightly linked, that is they should exhibit simple inheritance that can be followed through generations (Hartl and Jones, 2006; Liu, 1998).

Genetic linkage is defined as the association or inheritance of genes located in close proximity on the same chromosome (Liu, 1998). Linkage is estimated from the degree of “recombination”, or the number of progeny phenotypes that are intermediate or different than parental phenotypes. The recombination frequency between different genetic loci is associated with the mathematical map distance between those loci. For example, if there is a recombination frequency of 1 percent in progeny from a controlled parental cross, then the genes of interest are located 1 map unit or centimorgan (cM) apart, that is 1 map unit = 1% recombination = 1 centimorgan (Hartl and Jones, 2006; Liu, 1998). Recombination results from random “crossing over”, the exchange of DNA

molecules between chromosomes during meiosis. If there is a high degree of recombination, genetic linkage will be low (Hartl and Jones, 2006).

The maximum frequency of recombination between any two genes is 50%. For a recombination frequency of 50%, two genes would be assumed to be on different chromosomes and / or assorted independently. Genes are considered to be tightly linked at recombination frequencies of 10% or less. A genetic map that includes all of the known genes in a chromosome constitutes a “linkage group” (Hartl and Jones, 2006).

Mapping information can be useful in determining parental crosses and selection strategies in a breeding program (Eagles et al. 2001). Efforts to combine the maps of several important genotypes through molecular techniques have led to a more thorough knowledge of the wheat genome (Somers et al. 2004). Molecular mapping through the use of molecular markers allows for the generation of chromosome maps. Types of DNA molecular markers include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), and simple sequence repeats (SSR) microsatellite are DNA-based markers (Eagles et al. 2001). Another common marker type utilized in molecular mapping are known as single nucleotide polymorphisms (SNP).

A private institution known as ‘Triticarte’ has used proprietary markers utilizing a micro-array platform to generate numerous genetic maps of bread wheat and barley (Akbari et al. 2006). These markers are typically known as ‘DArT’ markers (Diversity Arrays Technology<sup>®</sup>). The concept of the formation of these segments of DNA is similar to that of AFLP. A restriction enzyme is used to digest (or cut) genomic DNA into fragments. Adapters are annealed to fragments that are amplified with PCR.

Libraries of genomic representations are generated, cloned, denatured, and hybridized to microarrays. These dominant marker clones are then scored for polymorphism through the use of DARTSOFT analysis software, which utilizes ANOVA to statistically calculate the difference between allele clusters. If two allele clusters are readily distinguishable, and the between cluster variance was at least 80% of the total variance, the clone is labeled 'polymorphic' and deemed a satisfactory marker clone. DArT marker technology will be utilized in this research to generate linkage maps for two recombinant inbred line populations (Sunco/Macon and Sunco/Otis).

Detection of genes through the use of DNA markers allows for the quantification of variation in genomic DNA. The utilization of methods including 'gene pyramiding' and 'rapid backcrossing' can be implemented through the use of molecular markers (Eagles et al. 2001). In the case of gene pyramiding, several genes identified through the use of markers are crossed into a single cultivar resulting in improved plant resistance. Rapid backcrossing allows for the acceleration of the process of incorporating a trait into a genotype (Eagles et al. 2001). Recently quantitative trait loci (QTL) for resistance to *F. pseudograminearum* have been identified through DNA marker techniques in Australia (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006).

Bread wheat (*Triticum aestivum* L.) has been genetically mapped for all 7 chromosome groups using RFLP-based molecular markers (Cadalen et al., 1997). The first map of the cultivar 'Sunco' was generated from doubled-haploid lines derived from a cross of 'Sunco' x 'Tasman' (Chalmers et al., 2001). These linkage maps were constructed with RFLP, AFLP, microsatellite markers, known genes, and proteins.

## **Identification of Quantitative Trait Loci (QTL)**

Genes affecting quantitative traits are often referred to as Quantitative Trait Loci (QTL) (Liu, 1998). However, a QTL is not a gene. Paterson (1998) defines a QTL as “chromosomal locations of individual genes or groups of genes which influence complex traits”. The QTL represents a region on the chromosome that is connected to, or expresses, a quantifiable phenotypic trait of interest (e.g. disease resistance). Liu (1998) describes a QTL as a term used to describe a situation when more than one gene is responsible for a varying phenotype. Linkage between a gene, or genes of interest, and a marker is the basis for QTL identification with the ultimate goal of marker assisted selection (MAS) (William et al. 2007).

The first step in identifying a QTL is to develop a genetic map, of which requires the development of a mapping population (recombinant inbred line, doubled haploid, F<sub>2</sub> intercross etc.) from a controlled cross between parents for the trait of interest (Liu, 1998). For example, for mapping crown rot resistance a cross was made between a partially resistant parent ‘W21MMT70’ and a moderately susceptible parent ‘Mendos’ (Bovill et al. 2006). Decisions of the parents depend on the objectives of the experiment, but parents must have sufficient variation (i.e. polymorphic) for the trait of interest at both the molecular (DNA) and phenotypic levels (Liu, 1998). Variation in parents at the DNA level is essential for tracing recombination frequency (Liu, 1998).

Phenotypic polymorphism is variation in the degree of disease or trait of interest. Genetic polymorphism between the parents can be defined as the detectable heritable variation at a locus identified by a marker. Loci polymorphisms are inferred from marker segregation patterns. If a marker segregates, that is it is polymorphic among the progeny

in a pattern, then it has heritable polymorphism. Microsatellite markers are often “hyper-variable”, co-dominant in nature, and have high heterozygosity in many populations. An acceptable genetic marker is polymorphic between parents, heritable, repeatable, and can be genetically interpreted (Liu, 1998).

There are four steps to constructing a genetic map with a large set of markers; 1. Pair wise linkage analysis for all possible two-locus combinations; 2. Group the markers into different linkage groups; 3. Order markers within each linkage group; 4. Estimate multi-point recombination fractions among the adjacent loci (Liu, 1998).

Determining robust recombination frequencies for molecular map development are often conducted through the use of computer programs such as ‘Map Maker’ (Lincoln et al. 1993). MapMaker<sup>®</sup> is a computer program with the capability to calculate genetic linkages through the use of molecular marker data. For QTL identification ‘QTL Cartographer’ is a commonly used computer program to calculate statistics correlating phenotypic and genotypic recombination data to assign subsequent QTL locations to marker locations on chromosomes (N.C. State Univ., Raleigh NC, 2004).

### **QTL Identification for Crown Rot Resistance in Wheat**

Due to the difficulty in phenotypically assessing crown rot resistance, identifying markers linked to resistance would facilitate cultivar development (Wallwork et al. 2004). Prior to 2004, there were no reports of the genetic location of resistance genes for crown rot in wheat. Since Wallwork et al. (2004), three additional studies have mapped QTL for crown rot resistance in bread wheat (Collard et al. 2005; Bovill et al. 2006; Ma et al. 2009).

Wallwork et al. (2004) developed 100 doubled haploid (DH) homozygous inbred lines from a Kukri ('PR' parent)/Janz ('S' parent) cross. The progeny of this cross was screened in an outdoor terrace system inoculated with *F. pseudograminearum* and *F. culmorum* and growth of lines to maturity to quantify adult plant resistance. Plants were scored on the basis of a 0 to 5 rating system (0 = no disease; 5 = >75% disease browning). A bulked segregant analysis (BSA) was conducted by pooling DNA into resistant and susceptible groups. Wallwork et al. (2004) analyzed one hundred between-bulk AFLPs for linkage with crown rot resistance in the 12 lines (6 susceptible lines and 6 resistant lines).

Wallwork et al. (2004) discovered the first crown rot (QTL) resistance locus in wheat with polymorphic markers on chromosome 4B near the dwarfing gene *Rht1* in the cultivar 'Kukri'. The QTL was approximately 20 cM from the *Rht1* locus. Collard et al. (2005) confirmed this QTL on 4B at 19.8 cM using a seedling assay similar to Wildermuth and McNamara (1994). The terrace disease-screening method was used to evaluate lines carrying this QTL. A large proportion of the resistant lines were shorter (having the *Rht1* gene), whereas susceptible lines were taller. A correlation coefficient of -0.59 was found between height and resistance (i.e. shorter lines were more resistant). 'Kukri' was documented as having adult plant resistance.

Collard et al. (2005) constructed a population of doubled-haploid lines from the parental cross '2-49' (partially resistant) x 'Janz' (susceptible). The combined effect of the best marker associated with five crown rot resistance QTL identified by composite interval mapping explained 40.6% of the overall phenotypic variance in resistance. The QTL explaining the greatest variation were located on chromosomes 1D and 1A, with

LOD scores of 7.5 and 3.2 explaining 21% and 9% of the variation, respectively. These resistance alleles were inherited from 2-49, but additional alleles were discovered on chromosome 2B and inherited from the susceptible parent 'Janz'. Additional QTL were identified on chromosomes 2A, 2B, 4B, and 7B.

Bovill et al. (2006) evaluated a doubled haploid population derived from a cross between parental lines 'W21MMT70' (partially resistant) x 'Mendos' (moderately susceptible). Three putative QTL were identified and located on chromosomes 2B, 2D, and 5D with LOD scores of 5.6, 2.2, and 9.4, respectively. The authors postulated that the resistance locus on the short arm of 2B was possibly from an introgression from *T. timopheevii*. This introgression has been documented in 'Sunco' (Kammholz et al. 2001). These QTL are the most recent and closely associated loci to identify genetic potential resistance to *F. pseudograminearum*.

Both Collard et al. (2005) and Bovill et al. (2006) concluded that the resistance loci identified on chromosome 2B, from the respective susceptible parents ('Janz' and 'Mendos'), was due to transgressive segregation. Collard et al. (2005) mentioned that due to transgressive segregation, the 2B QTL may not show up in seedling screening assays. The QTL on 2B (introgression from *T. timopheevii*) is also in close proximity to the *Sr36* resistance gene (Bovill et al. 2006). Collard et al. (2005) reported that the QTL identified on 1DL, tightly linked by flanking markers (2.1 cM) and explaining 21% of the variation, had the greatest potential for use in marker assisted selection (MAS). Collard et al. (2006) confirmed the QTL on 1DL as being inherited from Gluyas Early and the QTL on 2B was inherited from 'Janz'. However, screening of tightly flanked SSR markers to the 1D and 1A QTL on a subset of elite wheat genotypes were not



polymorphic in all backgrounds. There is a need to identify more tightly flanking markers for backcrossing to elite pedigrees (Collard et al. 2006).

Ma et al. (2009) reported the most recent QTL for resistance to Fusarium crown rot from CSIRO in Australia. Ninety-two lines from a recombinant inbred line F8 mapping population was used in this study and derived from a cross between ‘CSCR6’ and ‘Lang’. The population was screened with *Fusarium graminearum* and *F. pseudograminearum* isolates. The seedling dipping method using a conidial concentration of  $1 \times 10^6$  as outlined by Li et al. (2008) was used for inoculation. A total of six replicated trials were carried out on the mapping population (two for the *F. pseudograminearum* isolate and four for the *F. graminearum* isolate). Correlations between one of the *F. graminearum* screens was weak ( $r = 0.50$ ), but strong for the other four (ranging from  $r = 0.82$  to  $0.99$ ).

A wheat DArT array consisting of 776 polymorphic markers were used in conjunction with 54 SSR markers to generate a linkage map with a total distance of 912.9 cM. No markers were assigned to chromosomes 4D, 5D, or 6D. A total of 144 of the markers were mapped to chromosome 3B.

QTL analysis revealed two QTL for the *F. pseudograminearum* isolate on chromosomes 3BL and 4B using interval mapping (IM) with a LOD = 10.2 and 3.1 (for trial #1), respectively. The QTL on the end of the long arm of chromosome 3B had a LOD = 10.3 for both trials for *F. pseudograminearum* and a combined LOD = 11.1 explaining 41.6% of the variation in the data. The flanking DArT markers for the major QTL on chromosome 3B were wPt10505 and wPt2277. Resistance QTL were detected in the same location (3BL) and magnitude for all four *F. graminearum* isolate screening

trials. The authors postulated that crown rot resistance was not species-specific as the two *Fusarium* isolates returned similar QTL with the same chromosomal location (3BL).

There were not any SSR markers identified in the region of the QTL on chromosome 3B.

To date a total of 11 QTL located on chromosomes 1A, 1D, 2A, 2B, 2D, 3B, 4B, 5D, and 7B have been identified and correlated to crown rot resistance. The only overlap of QTL from these three studies was on chromosomes 2B and 4B. This illustrates genetic variation in the crown rot resistance mechanism and the potential for ‘gene pyramiding’ to enhance resistance (Bovill et al., 2005).

### **Marker Assisted Selection (MAS)**

Markers for marker assisted selection are often identified through QTL analysis and are those that are conserved among genotypes with a high degree of inheritance and can be effectively used to select for the trait of interest (William et al. 2007). The use of DNA markers to screen for genetic traits in marker-assisted selection (MAS) can be used to screen large quantities of plant material in a relatively short period of time at a reduced cost, compared to traditional breeding techniques (William et al. 2007). The application of QTL analysis is the use of markers linked to a QTL in marker assisted selection (Paterson, 1998). Until recently there has been little application of markers for use in MAS in breeding programs due to a lack of reliable markers and economic feasibility associated with transgressing multiple QTL regions into desirable germplasm (Paterson, 1998; William et al. 2007).

Currently there are some barriers to MAS (William et al. 2007). One is a lack of applicable markers available for traits of interest. Many markers are associated with qualitative traits, which can be more effectively screened with traditional phenotypic

methods (William et al. 2007). Although markers exist for certain genetic traits, selection in a breeding program involves selecting for traits that involve additional factors for which markers are not available. 'Indirect selection' is a term used to describe the use of a marker to select for a trait of interest including disease resistance (William et al. 2007).

Some of the advantages of indirect selection with markers would be as follows:

1) Screening for multiple genes for gene pyramiding; 2) Selecting for recessive traits; 3) Expense in screening; 4) Use of lab or greenhouse facilities for screening (not limited by seasonal and geographic considerations); 5) Heritability of traits (QTL may be affected by the environment); 6) Early generation selection with low amounts of seed; 7) Early detection or screening of young seedlings (William et al. 2007). A lack of confidence in published results has been mentioned as an additional barrier to MAS. Another disadvantage for MAS would be for users lacking resources to utilize MAS there can be a high initial expense in establishing a lab with the proper instrumentation. Markers must be reliable, repeatable, and must have been validated (William et al. 2007).

There are advantages and disadvantages to adopting MAS (William et al. 2007). Genetic markers allow for the detection of differences at the molecular level. Thus MAS allows for gene pyramiding, as individual genes responsible for a trait that can be detected with markers may not otherwise be phenotypically expressed. This similar concept can be applied to using markers to breed for a recessive trait that would otherwise require an extra generation of selfing (William et al. 2007).

Depending on the resources of the end user, the expense of the MAS strategy can be an advantage to the alternative expenses and time for labor and materials required for field and greenhouse screening systems. However, if resources and instrumentation for

MAS are not available, one disadvantage can be the system can be substantially more expensive to adopt initially. Acceptable markers can be an advantage to field screening techniques by removing the element of environmental variation. Also seasonal limitations of screening in the field in terms of time and pathogen pressure are by-passed with MAS. The heritability can be much more accurate for these reasons as well, but only for proven reliable markers. Assay requirements for protein and other quality analysis at early generation selection stages are bypassed with MAS as well (William et al. 2007).

Several researchers have expressed the need for improving methods to phenotype plants as they can be time-consuming, laborious, and expensive (Wildermuth and McNamara, 1994; Mitter et al. 2006; Nicol et al. 2007). Collard et al. (2006) stressed the need for the development of markers closely linked to crown rot resistance for use in a MAS program. Collard et al. (2006) summarized the process of finding a marker is a process that involves several steps including but not limited to QTL identification, confirmation of accuracy of the QTL location, and ‘marker validation’ which refers to the process of confirming the effectiveness of markers linked to putative QTL in one mapping population in target backgrounds of other populations and germplasm. To date none of the crown rot QTL have been confirmed in independent populations. Once a durable marker is identified it must be polymorphic (or differ in size) in target genotype backgrounds so it can be traced and used for indirect selection (Collard et al. 2006).

Collard et al. (2005) reported a major QTL located on chromosome 1D (*QCr.usq-1D1*) with a LOD = 7.5 explaining 21% of the phenotypic variation from a 2-49 x Janz population. Collard et al. (2006) was able to confirm that QTL in another background

population (Gluyas early x Janz), but screening of tightly linked SSR markers on a small sample of elite wheat genotypes indicated that not all of the linked markers were polymorphic in all backgrounds. Collard et al. (2006) summarized that there was a need to identify more SSR markers linked to the QTL region of interest to evaluate for polymorphism in elite germplasm backgrounds. The need remains for the identification of durable SSR markers that can be used in MAS for crown rot resistance.

## **V. POPULATION DIVERSITY AND MOLECULAR CHARACTERIZATION OF *FUSARIUM* SPECIES FOR IDENTIFICATION AND QUANTIFICATION**

Symptoms of crown rot begin as small (0.5 to 4 cm) necrotic lesions on the coleoptile, subcrown internode or leaf sheaths and progress to uniform browning from the base of the stem to the fourth internode (Burgess et al. 2001). Traditional diagnostic methods of identifying and re-isolating *Fusarium* species have been based on morphological characteristics of conidia from cultures grown on nutrient weak selective media (Burgess et al. 2001). Initially *Fusarium graminearum* was placed into two groups, designated Group 1 and Group 2, distinguished within the anamorphic species on the basis of host pathogenicity and the origin of the isolate (Burgess et al. 2001). Burgess et al. (1988) reported the key diagnostic feature distinguishing the two groups was the formation of homothallic perithecia of *Gibberella zeae* in Group 2 on carnation leaf agar. Group 1 isolates did not form perithecia in culture and were presumably heterothallic. The groups appeared to have different geographic distributions, with Group 1 being found in hot and dry climates while Group 2 isolates were found in wetter climates. Thus, distinguishing species involved in the root rot complex required a certain level of mycological expertise (Burgess et al. 2001).

Root diseases caused by soil-borne pathogens are often present in the soil as a complex and produce similar symptoms making it difficult to isolate and identify the specific causal pathogen of the disease. Oftentimes symptoms of eyespot (caused by *Tapesia yullundae* and *T. acuformis* anamorph *Pseudocercospora herpotrichoides*), *Fusarium*, *Microdochium* and common root rot (*B. sorokiniana*) are confused and competition on media may favor one species over another (Nicholson et al. 1998). With the advent of molecular techniques, such as PCR, DNA can be amplified and detected in plant tissues and soil (Schilling et al. 1996). *Fusarium graminearum* Group 2 and *F. culmorum* were distinguished using randomly amplified polymorphic DNAs (RAPD) analysis (Shilling et al. 1996).

Schilling et al. (1996) were the first to develop diagnostic molecular PCR-based assays for detecting differences among *Fusarium* species. SCARs (sequence-characterized RAPD fragments) were used to generate primers in an attempt to distinguish three *Fusarium* spp. The internal transcribed spacer region (ITS) of nuclear ribosomal DNA were amplified with universal primers (OPT18 (SCAR2-35: 5'-GATGCCAGAC-3' and UBC85 (SCAR85): 5'-GTGCTCGTGC-3') and analyzed for sequence variation among *Fusarium* species.

The primer OPT18 amplified a fragment of 470 bp that was specific to *F. culmorum*, whereas primer UBC85 amplified a distinct fragment of 410 bp specific to *F. graminearum* Group 2. Although ITS sequences of *F. culmorum* and *F. graminearum* were not polymorphic, there was sufficient variation between the ITS of *F. culmorum* / *F. graminearum* and *F. avenaceum*, allowing for the development of species-specific primers only for *F. avenaceum*. RAPD fragments were identified and used to develop

primers for selective amplification of *F. culmorum* and *F. graminearum* (Shilling et al. 1996).

The primers developed for *F. culmorum* correctly detected 65 of 69 *F. culmorum* isolates (Shilling et al. 1996). The four that were undetected were confirmed to be *F. culmorum* by morphological identification. Possibilities for non-amplification of these primers were postulated to be due to alterations in annealing sites. This study also assayed plant tissue for the presence of *Fusarium* species. The amplification of SCAR2-35 (OPT18 primer) and SCAR85 (UBC85 primer) revealed the presence of both *F. culmorum* and *F. graminearum* in plant-tissue extracts Shilling et al. 1996).

Nicholson et al. (1998) used RAPD assays to distinguish *F. culmorum* from *F. graminearum*. Three primer pairs were used including OP-T01 (Fc01) (GGGCCACTCA), OP-T16 (Fg16) (GGTGGACGCT), and OP-U17 (Fcg17) (ACCTGGGGAG). The *F. culmorum* primer (Fc01) was used in the detection of inoculum load and timing of inoculation on winter wheat stem bases infected with *F. culmorum*. Fc01 detected all 21 *F. culmorum* isolates evaluated in this study. Analysis of the ITS region of ribosomal RNA (rDNA) showed no differences between *F. culmorum*, *F. graminearum*, or *F. crookwellense* (Nicholson et al. 1998). Primer Fcg17 detected both *F. culmorum* and *F. graminearum*, indicating the two species are closely related as shown by Shilling et al. (1996). The *F. graminearum* primers (Fg16 and Fg16N) were used to detect the trichothecene gene which was correlated to colonization and virulence of *F. graminearum* on grain. PCR assays from wheat tissue were positively correlated with disease severity ratings. The DNA detection limit was 1 pg for

both species. Primers specific to *F. graminearum* and *F. culmorum* were successfully used to detect the presence of each pathogen in diseased rye and wheat tissues.

Aoki and O'Donnell (1999a) observed morphological characters of 17 *F. graminearum* Group 1 strains and 15 *F. graminearum* Group 2 strains to identify differences between the groups. They also analyzed DNA sequence data from the  $\beta$ -tubulin gene introns and exons of these groups and other *Fusarium* spp. to investigate phylogenetic relationships among them. Two species-specific *F. pseudograminearum* PCR primer pairs were developed from the aligned translation elongation factor (EF-1 $\alpha$ ) gene, which were Fp1-1 (5'-CGGGGTAGTTTCACATTTTC(C/T)G – 3') and Fp1-2 (5' – GAGAATGTGATGA(C/G)GACAATA – 3').

Based on the ability to differentiate between *F. graminearum* Groups 1 and 2 through molecular techniques, Aoki and O'Donnell (1999a) described *F. graminearum* Group 1 as a distinct species, which they called "*Fusarium pseudograminearum*" with a distinct phylogeny. A thorough description of *F. graminearum* Group 1 morphology and growth habit was published by Burgess et al. (1975).

Morphological differences were reported between *F. graminearum* Groups 1 and 2 in width of 3- and 5-septate conidia (Aoki and O'Donnell, 1999a). Conidia of Group 1 were typically widest at the middle septum compared to those of Group 2. The widest position on the conidia of Group 2 strains were frequently 30% to 40% from the apex, that is between the 1<sup>st</sup> and 2<sup>nd</sup> septum for 3-septate conidia, and near the 2<sup>nd</sup> septum for 4-septate conidia, and between the 2<sup>nd</sup> and 3<sup>rd</sup> (middle) septum for 5-septate conidia (Aoki and O'Donnell, 1999a). The upper halves of the conidia of Group 2 are often thicker



than the bottom halves, whereas conidia of Group 1 (*F. pseudograminearum*) have a symmetrical decrease in width from the middle region (Aoki and O'Donnell, 1999a).

In summary, Aoki and O'Donnell (1999a) described *F. pseudograminearum* on the basis of four different characters: 1) slower growth rate on weak nutrient media (SNA); 2) symmetrical decrease in conidial width from the middle septum; 3) uniform length of 3-septate conidia formed under BLB light and complete darkness; and 4) the absence of homothallic production of perithecia. The widest portion of the conidia was described as the most reliable morphological characteristic for distinguishing *F. pseudograminearum* from *F. graminearum*.

However, Aoki and O'Donnell (1999b) found seven combinations of single-conidial isolates of *F. pseudograminearum* (out of 153 possible combinations) that yielded mature heterothallic perithecia of *Gibberella* sp. containing viable ascospores. Molecular techniques serve as the most definitive method for identifying *F. pseudograminearum* (Aoki and O'Donnell, 1999a). Phylogenically *F. graminearum* seems to be more closely related to *F. culmorum* than to *F. pseudograminearum*.

Williams et al. (2002) conducted PCR assays on 79 isolates of 12 different *Fusarium* species. Primers were developed by cloning RAPD amplicons. A universal primer combination comprised of OPB-07 (5' – GGTGACGCAG – 3') and OPD-03 (5' – GTCGCCGTCA – 3') amplified a 1020 bp segment specific to *F. pseudograminearum*. RAPD fragments were cloned and designated 'FPG' with the sequences (FPG-F – 5' – GTCGCCGTCACTATC – 3'; FPG-R – 5' – CACTTTTATCTCTGGTTGCAG – 3'). This FPG species-specific primer set amplified 40 out of 42 *F. pseudograminearum* isolates. Two isolates showed a mis-identification of *F. pseudograminearum*. AFLP

analysis of these mis-identified isolates showed they were more similar to *F. graminearum* than *F. pseudograminearum*.

PCR assays to identify *Fusarium* species were validated by extracting DNA from seedlings infected with single or multiple isolates, as well as by extracting DNA from cultures from infected mature wheat stems (Williams et al. 2002). Seedlings were inoculated with *F. pseudograminearum*, *F. graminearum*, and *F. culmorum* by placing a 3-mm PDA plug against the stem of young seedlings, covered by 2 cm of soil. DNA was also extracted from cultures of field samples from mature wheat stems. DNA was extracted from inoculated seedlings by freezing with liquid nitrogen, crushing to a fine powder and extracting with several liquid chemical compounds and re-suspended in 10mM Tris-HCL, 1 mM EDTA (pH 8.0) containing 40 µg/mL RNAase. Purified DNA was amplified with FPG (*F. pseudograminearum* species-specific primer), SCAR 2-35 (OPT-18 specific to *F. culmorum*), and SCAR 85 (UBC85 specific to *F. graminearum*) (Williams et al. 2002).

In two out of three double inoculations to plants, amplicons were generated from each primer set, indicating the primers ability to detect different species in inoculated wheat tissue (Williams et al. 2002). SCAR 85 (UBC85) failed to amplify DNA from *F. pseudograminearum* and *F. graminearum*. The SCAR 2-35 (OPT18) primer set produced non-specific amplification from one of the seedlings infected with both *F. pseudograminearum* and *F. graminearum*. In summary only the FPG and FAC primers accurately identified *F. pseudograminearum* and *F. acuminatum*, respectively (Williams et al. 2002).

Direct DNA extractions from mature wheat stems did not always produce reliable amplification results, of which the authors attributed to potential polysaccharide contamination of the extracts (Williams et al. 2002). A method was developed that allowed for mycelial growth in the extraction tube and nine out of ten stems produced enough mycelium for DNA isolation using this technique. Species-specific amplification was successful for eight out of nine of these DNA extractions. In the ninth sample amplicons were produced for both *F. pseudograminearum* and *F. culmorum*, and only the former was cultured from five stem sections. The authors postulated that the presence of *F. pseudograminearum* was most likely dominant in the stem. Although there was enough *F. culmorum* was present in tissue to be amplified, it was more difficult to subculture and isolate from stems. Genotyping isolates of *Fusarium* using AFLPs proved to be reliable in this study (Williams et al. 2002).

More recently, PCR assays have been developed for the specific identification of *F. culmorum* (Klemsdal and Elen, 2006; Leisova et al. 2006). Klemsdal and Elen (2006) used nested PCR to increase sensitivity and detect *F. culmorum* in infected cereal tissues. They wanted to accurately detect the presence of *F. culmorum* to prevent contamination of cereal products by its production of carcinogenic mycotoxins that are harmful to humans and animals. The detection limit for *F. culmorum* was 5 to 50 fg of purified target DNA and allowed the detection of 1% infected wheat seeds in a mixture of uninfected grains. The authors concluded that the detection of *F. culmorum* in cereal samples using the nested PCR method was 100 times more sensitive than previously documented PCR methods. The method was also more efficient and cost effective for commercial use.

## ***Fusarium pseudograminearum* and *F. graminearum* population distribution and diversity**

Several studies have evaluated the population dynamics of *F. pseudograminearum* and *F. graminearum* in surveys in Canada and Australia (Scott et al. 2004; Akinsanmi et al. 2006; Mishra et al. 2006; Bentley et al. 2008). Surveys of *Fusarium* species associated with crown rot and head blight have shown that *F. pseudograminearum* is more closely associated with crown rot in the Queensland, New South Wales, and Southern Australia regions, whereas *F. graminearum* is more localized to moist regions of northern New South Wales and is more closely associated with *Fusarium* head blight (Backhouse et al. 2004).

Akinsanmi et al. (2006) evaluated the genetic diversity of *F. graminearum* and *F. pseudograminearum* using AFLP analysis. Sixty three of 149 AFLP loci were distinct between the two species using  $X^2$  analysis. Results showed that 70 of 72 isolates of *F. pseudograminearum* and 56 of 59 *F. graminearum* isolates had distinct haplotypes. Phenotypic experiments were conducted between the two species for growth rate and sporulation, but no statistical differences were found. The genotypic diversity of these haplotypes was highly associated with aggressiveness for the two pathogen species. Within fields, the spatial diversity of *F. graminearum* was high, while that of *F. pseudograminearum* was low with a high degree of gene flow among isolates. The authors concluded that these differences illustrated diversity expressed with the homothallic sexual reproductive nature of *F. graminearum* versus the heterothallic asexual reproductive nature of *F. pseudograminearum*.

Mishra et al. (2006) investigated the genetic diversity of *F. pseudograminearum* populations from the Canadian provinces of Alberta and Saskatchewan. Nuclear ribosomal DNA (nrDNA) intergenic space region (IGS) and inter-simple sequence-repeat (ISSR) molecular markers were used to evaluate 26 isolates from infected wheat grains collected during 1998 and 2000. Restriction analysis of the IGS region resulted in 12 polymorphic markers of 18 screened. ISSR resulted in 118 polymorphic markers of 120 screened. Genetic diversity and gene flow estimates within populations were high and significant recombination within these populations was assumed. The authors postulated that regular gene flow and random mating between isolates from different populations could result in potentially damaging genotypes (Mishra et al. 2006). Amplified fragment length polymorphism analysis (AFLP) from the *Tef-1* gene (translation elongation factor - 1 $\alpha$ ) revealed five distinctive clusters of *Fusarium* species that corresponded to *F. pseudograminearum*, *F. culmorum*, *F. crookwellense*, and *Chionochloa rubra* ssp. *cuprea* (Bentley et al. 2006).

Bentley et al. (2008) assessed genetic diversity within and between populations of 217 isolates of *F. pseudograminearum* with AFLP analysis. These isolates were from eight populations from three key regions of Australia; northeastern, south central, and southwestern Australia. From the *F. pseudograminearum* population 176 haplotypes were identified and grouped into two main clusters characterized by their geographic origin (northeastern or southwestern Australia).

The southern populations were unique from the northeastern population by greater levels of population differentiation and genetic identity amongst the populations. South central and southwestern isolates were genetically similar with low genetic

differentiation. There were also high levels of gene flow within the northeastern population, which was in agreement with Akinsanmi et al. (2006). However, there was restricted gene flow between regions. It was hypothesized that populations from northeastern and southern Australia were independent from different founding effects or from geographic isolation. Although it has been postulated that sexual recombination is a key factor in genetic diversity observed among *F. pseudograminearum* populations, it is uncertain as the teleomorph *Gibberella coronicola* has been reported only occasionally in field and laboratory conditions (Bentley et al. 2008).

Data from Bentley et al. (2008) confirmed this theory as PCR amplification of conserved portions of mating loci (*MAT-1* and *MAT-2* loci) exhibited linkage disequilibrium indicating a low level of sexual recombination in *F. pseudograminearum* populations. These results were in contradiction with Akinsanmi et al. (2006) and are more consistent with low sexual recombination that would be expected as *F. pseudograminearum* reproduces mainly asexually via conidia. It was postulated that under field conditions sexual recombination remains unknown, but is estimated to be occasional, if at all. The differences in *F. pseudograminearum* population structure between northeastern and southern Australia were attributed to selection, genetic drift, population bottlenecks, and founder effects.

### **Quantitative (Real Time) Polymerase Chain Reaction for *Fusarium* quantification**

In contrast to the conventional PCR approach, the advent of quantitative or 'real time' PCR (referred to as Q-PCR ) allows for the monitoring and quantification of DNA during thermocycling. Q-PCR is convenient in pathological studies because it allows for the quantification of pathogenic DNA that cannot be readily extracted or cultured from

host tissue or soil samples (Okubara et al. 2005). The sensitivity of Q-PCR detection is high as symptomless plants can contain inoculum of the pathogen (Okubara et al., 2005).

The basis of Q-PCR is that the amplicon (or amplified PCR product) is monitored (quantified) as the PCR thermocycler is going through the heating / cooling cycles (Okubara et al. 2005). Through the use of a binding fluorescent dye (SYBR green 1) or complementary probe to target DNA, the amplicon, can be detected and monitored (Okubara et al., 2005). The point at which detection of the amplicon accumulates to supersede background DNA levels is called the cycle threshold ( $C_T$ ). The  $C_T$  level is important because the quantity of target DNA is inversely proportional to the  $C_T$  value (Okubara et al., 2005). The greater the amount of initial DNA in the target sample, the earlier the  $C_T$  value (Stratagene, La Jolla, CA., 2004). The  $C_T$  value and amplification figures from the software associated with the real time thermocyclers are used to make inferences about the quantity of the target DNA in the sample. These values are calibrated from known concentrations of pathogens in soil and diseased plant tissue (rated or quantified) to develop standard curves for assaying pathogen species extracted from field soil and tissue samples (Hogg et al. 2007).

The simplest, least expensive, and most universal Q-PCR detection method is the use of a universal fluorescent DNA dye called 'SYBR green I' (Molecular Probes, Eugene, Oreg.). SYBR green I binds to the minor groove of double-stranded DNA within the region amplified by two PCR primers, and its fluorescence is detected by the Q-PCR model thermocycler (Leisova et al. 2006). The fluorescence of bound SYBR green 1 dye is detectable because its fluorescence is about 20-fold greater than unbound dye.

The major disadvantage of Q-PCR systems is the relatively high cost of the equipment (thermocyclers and supporting assay equipment) (Okubara et al. 2005).

Q-PCR has been successfully used for symptom diagnosis and culture-based assessments from agricultural studies (Okubara et al. 2005). Q-PCR has been used in several studies to quantify the amount of *F. culmorum* and *F. pseudograminearum* in diseased wheat tissue (Leisova et al. 2006; Hogg et al. 2007). Leisova et al. (2006) used Q-PCR to detect *F. culmorum* in infected seeds. The detection limit was 0.9 pg of *F. culmorum* genomic DNA. Significant correlations were found ( $r^2 = 0.98$ ) between DNA concentration,  $C_T$  values, and deoxynivalenol (DON) content (a commonly used method for assaying mycotoxins such as those produced by *F. culmorum*) of seed samples inoculated with *F. culmorum*.

Strausbaugh et al. (2005) isolated *Fusarium* from wheat and barley roots common to southern Idaho and rated root lesions in four growth chamber and two field assays. They developed Q-PCR assays using TaqMan probes to quantify Fusarium crown rot in diseased wheat and barley tissues. Primers Gzgae87T forward and reverse primers and a Gzgae87T probe were developed from the trichodiene synthase (*tri5*) gene of *F. graminearum*. However, the *tri5* gene based primers were not species-specific and amplified *F. culmorum* and *F. pseudograminearum* in addition to *F. graminearum*. The primer set did not amplify pure cultures of *F. avenaceum*, *F. acuminatum*, *F. semitectum*, *F. equiseti*, *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Pythium* spp. and 10 of the most common fungal saprophytes isolated from wheat and barley roots from southern Idaho. *F. culmorum* infections resulted in the largest infected root area.



Field assays resulted in 245 and 307 root isolates at two locations, respectively, and resulted in the following isolates in order of decreasing amplitude; *F. culmorum*, *B. sorokiniana*, *F. equiseti*, *F. acuminatum*, *F. reticulatum*, *F. semitectum*, *Gaeumannomyces graminis* var. *tritici*, *F. chlamydosporum*, *F. crookwellense*, other *Fusarium* sp., and *Rhizoctonia* spp. (Strausbaugh et al. 2005). These assays were able to detect *F. culmorum* in root tissue down to 61 pg. In greenhouse assays with *F. culmorum* inoculated plants were found between the percent infected root area and *Fusarium* DNA quantities in tissue. However, most *Fusarium* crown rot measures use a crown / basal internode / lower stem based rating system (Burgess et al. 2001). Thus there was a need to evaluate species-specific primers and compare them to crown rot severity ratings of wheat stem bases (Hogg et al. 2007).

Hogg et al. (2007) used the (*tri5*) gene based primers and probes developed by Strausbaugh et al. (2005) to relate 22 *Fusarium* species (including *F. pseudograminearum* and *F. culmorum*), and additional isolates from 12 fungal species, to field inoculated experiments during 2004 and 2005. The primer used in the study was from the cloned fragment of the *tri5* gene from a *F. culmorum* isolate and was designated 'Fusclone' with the forward sequence (5' – CGGGTCCAGATGTTTGCCAT – 3') and the reverse sequence (5' – ACTGCTCAATCCAGCATCCC – 3'). The Q-PCR assay detected all of the *Fusarium* crown rot isolates, but did not amplify any of the other species evaluated. The sensitivity of the assay was the greatest with *F. graminearum* with step-wise diminishment from *F. pseudograminearum* to *F. culmorum*.

Field trials were inoculated with *F. pseudograminearum*. Disease severity scores (DSS) and *Fusarium* DNA quantities were negatively correlated to yield and comparable

in predicting yield reductions with r values = -0.64 and -0.77, respectively. The location or length of lesion up to the fourth internode did not appear to be correlated to DNA quantities. The authors summarized that the Q-PCR assay used was able to accurately estimate disease severity and subsequent yield loss from *Fusarium* crown rot infection under inoculum pressure and favorable climatic conditions for disease development (Hogg et al. 2007).

From these studies it appears that Q-PCR is an adequate tool for assessing *Fusarium* populations in an epidemiological context. Traditional methods of plating and identification are cumbersome and require mycological expertise. Furthermore, some *Fusarium* spp. can be extracted from soil using dilution plating, but Q-PCR has been used to more readily identify quantities of soil-borne pathogens from soil samples (Schroeder et al. 2006).

## VI. SUMMARY

Crown rot has been known to cause significant yield losses in wheat throughout the PNW region of the U.S. and the world. Previously, there were no effective methods of isolating and quantifying *Fusarium* sp. from soil samples. With the advent of Q-PCR thermocyclers for amplifying pathogenic DNA, and published primers for *F.*

*pseudograminearum* and *F. culmorum*, levels of *Fusarium* sp. can be rapidly and accurately quantified in plants and soil for use in epidemiological survey studies.

Although genetic resistance in cultivars has been identified, its genetic location would facilitate breeding efforts. QTL, identified chromosomal regions of potential resistance genes, have been identified for crown rot resistance through the utilization of high-throughput greenhouse assays. Identification of QTL would allow for the potential use of marker assisted selection (MAS) for applications of gene pyramiding and rapid backcrossing of crown rot resistance genes into locally adapted PNW wheat cultivars.

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## **CHAPTER 2**

## CHAPTER 2

### A SURVEY OF FUSARIUM CROWN ROT IN DRYLAND WHEAT IN THE PACIFIC NORTHWEST OF THE U.S.

Grant Poole<sup>1</sup>, Richard W. Smiley<sup>2</sup>, Timothy D. Murray<sup>1</sup>, Julie M. Nicol<sup>3</sup>, Kimberly Garland-Campbell<sup>4</sup>, and Timothy C. Paulitz<sup>4</sup>. <sup>1</sup>*Washington State University, Dept. of Plant Pathology, Pullman, WA, 99164-6430; E-mail: [gpoole@wsu.edu](mailto:gpoole@wsu.edu); FAX: 509-335-9581;* <sup>2</sup>*Oregon State University, Pendleton, OR.;* <sup>3</sup>*CIMMYT International Maize and Wheat Improvement Centre, Ankara, Turkey;* <sup>4</sup>*USDA-Agriculture Research Service, Pullman, WA.;*

#### ABSTRACT

Fusarium crown rot is one of the most widespread root and crown diseases of wheat in the Pacific Northwest of the U.S. but its distribution in specific areas of the Pacific Northwest remains unknown. Accurate surveys of pathogen and disease presence are needed to determine the extent and damage due to crown rot. Surveys were previously conducted in northern Oregon and south central Washington in 1993 and 1994, representing a more arid region within the Pacific Northwest. Our objectives were to conduct a survey covering the diverse dryland wheat-producing areas of Washington and Oregon and to determine the geographic species distribution of causal agents of Fusarium crown rot. In this study 105 fields were surveyed during 2008 and 2009. One-thousand and fifty tillers were cultured of the study to obtain 768 isolates. Crown rot symptoms were reported in every field in both years with a crown rot severity rating ranging from

0.18 to 5.12 in 2008 and 0.48 to 5.42 in 2009. Isolates of *Fusarium* spp. were obtained from 99% of fields in 2008 and 97% of fields in 2009. *Fusarium culmorum* was isolated from 31% of the symptomatic stems surveyed, closely followed by *F. pseudograminearum* isolated at a frequency of 30% (symptomatic stems) averaged over both survey years. The 2 year mean field proportions where only *F. culmorum* or *F. pseudograminearum* were isolated was 33% and 36%, respectively, while both species were isolated from 26% of the fields, while 5% of the fields contained isolates of minor species or no isolations. Overall isolation frequency means for other minor species included *F. crookwellense*, *F. acuminatum*, *F. equiseti*, and *Bipolaris sorokiniana* at 13%, 1%, 1%, and 2%, respectively. Species composition and disease severity varied significantly among climatic regions. *F. pseudograminearum* occurred in significantly greater frequency in areas of the PNW with hotter and drier weather patterns (Region 5), whereas *F. culmorum* occurred in greater frequency from regions with moderate to high moisture and cooler temperatures (Regions 1 and 4). Isolates of *F. pseudograminearum* and *F. culmorum* were capable of infecting and causing Fusarium crown rot symptoms on winter wheat cultivars.

## **INTRODUCTION**

Crown rot is one of the most widespread root and crown rot diseases of dryland wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) in the Pacific Northwest (PNW) region of the United States (Smiley et al. 2005a). This disease causes average annual yield losses of 9.5% at a value of U.S. \$13 million in the PNW (Smiley et al. 2005a). Crown rot infections limit yield by rotting seed, seedlings, roots, crowns, and

basal stems resulting in seedling mortality, premature lodging, and impaired grain filling (Smiley et al. 1996; Schilling et al. 1996; Paulitz et al. 2002).

Smiley et al. (2005a) conducted a survey of crown rot in wheat and barley in the PNW accompanied by yield loss estimates. Crown rot decreased yield in 13 commercial fields by as much as 35%, with an average of 9.5%. Under inoculated conditions yield loss was as high as 61%. The incidence of crown rot was the greatest in seasons with low precipitation and aboveground symptoms were not always obvious even under moderate disease pressure (Smiley et al. 2005a).

Crown rot is caused by a complex of *Fusarium pseudograminearum* (O'Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*), *F. culmorum* (Wm. G. Sm.) Sacc., and *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus*), (Cook, 1968; Cook and Veseth, 1991; Smiley et al., 2005a; Nicol et al., 2006). Other pathogens of lesser importance capable of causing crown infection include *F. avenaceum* (Fr.:Fr.) Sacc. (= *Gibberella avenacea*) and *Microdochium nivale* (Fr.) Samuels & I.C. Hallett (= *Monographella nivalis*) (Smiley et al. 2005a). *F. pseudograminearum* and *F. culmorum* are the most dominant pathogens causing crown rot in the PNW. It is widely accepted that *F. pseudograminearum* is the dominant species responsible for causing crown rot in arid regions of the PNW and Australia (Burgess et al. 1975; Backhouse and Burgess, 2002; Chakraborty et al., 2006; Smiley et al. 2005b). 'Crown rot' is generally the accepted name when *Fusarium* spp. are the dominant causal pathogens, and 'common root rot' is the term that describes the disease when *B. sorokiniana* is the causal pathogen (Purss 1970; Burgess et al. 2001; Cook and Veseth, 1991; Smiley et al. 2005a).

Although the symptoms of ‘common root rot’ and ‘crown rot’ are similar, they are distinguishable (Cook, 1968). The disease caused by *B. sorokiniana* is characterized by a dark brown to black discoloration of the sub-crown internode, whereas *Fusarium* species cause chocolate to reddish brown symptoms on roots or shoot internodes (Purss, 1970; Cook and Veseth, 1991). Burgess et al. (2001) suggested that lesions extending to the fourth or fifth node were accompanied by salmon-colored sporodochia, which are masses of macroconidia. The infection of the crown region leads to a constriction of the vascular system and the subsequent formation of whiteheads containing little to no seed (Cook, 1968; Burgess et al. 2001; Nicol et al. 2001). Whiteheads are often used as an indicator of crown rot injury and as a measure to quantify the amount of disease present (Burgess et al. 2001; Cook, 1968; Nicol et al. 2007).

Several *Fusarium* surveys have been conducted throughout the world (Cook, 1968; Smiley and Patterson, 1996; Smiley et al. 2002; Backhouse and Burgess, 2002; Backhouse et al. 2004; Smiley et al. 2005a). The earliest documentation of *Fusarium* crown rot in the PNW was reported by Cook (1968). Yield losses of 17% and 50%, respectively, were reported for two winter wheat fields with different densities of *Fusarium* populations. Cook (1968) described the fungus as *F. roseum* Lk. (emend. Syd. & Hans.) f. sp. *cerealis* (Cke.) Syd. and Hans. and delineated the fungal cultivars as being 'Culmorum', 'Graminearum', or 'Avenaceum'. Greater than 90% of the isolates from diseased fields in Washington in 1964 were *F. roseum* f. sp. *cerealis* 'Culmorum' and *F. roseum* f. sp. *cerealis* 'Graminearum', with 'Culmorum' being predominantly present. *F. roseum* f. sp. *cerealis* 'Avenaceum' was isolated occasionally from those samples. Studies conducted in the U.S. states of California, Colorado, Wyoming, and

Texas found that *F. culmorum*, *F. graminearum*, and *B. sorokiniana* were most commonly associated with root and crown rot symptoms (Scardaci and Webster, 1982; Hill et al. 1983; Specht and Rush, 1988). Results from surveys were conducted in the PNW in 1993 and 1994 from 288 dryland fields showed *F. pseudograminearum* was the dominant pathogen, with *F. culmorum* detected less often (Smiley and Patterson, 1996). Complex interactions between cropping systems and the environment influence the distribution of these species (Smiley et al. 1996). Trends have been reported where *F. pseudograminearum* dominated in areas where the maximum temperature in July was warmer (31° C) than counties where *F. culmorum* dominated (28° C) (Sitton and Cook, 1981).

Smiley and Patterson (1996) conducted an extensive survey of the PNW region and reported the occurrence of *F. pseudograminearum* was more frequent than *F. culmorum* in nine counties and the opposite was true in three counties. *F. culmorum* was more prevalent than *F. pseudograminearum* in Lincoln and Adams counties in 1993 and only Lincoln county in 1994. The percentages of fields yielding isolates of *F. pseudograminearum* were higher (40% in 1993 and 23% in 1994) than *F. culmorum* (20% in 1993 and 10% in 1994) in both survey years. There was a significant correlation between precipitation and elevation with the occurrence of *F. pseudograminearum* ( $r^2 = 0.46$ ,  $P = 0.05$ ) suggesting that its occurrence may be less prevalent as both elevation and temperature increase. There also was a weak but consistent correlation of *F. pseudograminearum* occurrence with the mean monthly temperature during July ( $r^2 = 0.42$ ,  $P = 0.06$ ). Smiley and Patterson (1996) concluded that there were no relations between the occurrence or frequency of Fusarium foot rot pathogens with six agronomic

zones defined by Douglas et al. (1990). The authors also concluded that *F. pseudograminearum* was the dominant pathogen responsible for causing Fusarium crown rot throughout the region.

In Australia, Burgess et al. (1975) found that *F. graminearum* Group 1, now referred to as *F. pseudograminearum*, was the dominant pathogen responsible for crown rot infection. Klein et al. (1990) surveyed fields in Australia and found that whitehead occurrence was correlated to crown rot occurrence with *F. graminearum* Group 1 as the dominant pathogen isolated. Backhouse and Burgess (2002) conducted a climatic analysis of the distribution of *Fusarium* spp. and found *F. pseudograminearum* did not appear to be limited by climatic conditions as it was found to be very wide-spread. *F. culmorum* was more limited in distribution and populations were associated with lower temperatures and higher rainfall compared to *F. pseudograminearum*. The authors also utilized 16 climatic parameters to conduct a Principle Component Analysis (PCA) where the first two principle components explained 52% and 21%, respectively, of the variation in the survey data set. Survey sites were clustered in accordance with the first principle component in two clusters which corresponded to winter and summer rainfall. The authors concluded that the distribution of *F. culmorum* was less restricted in the predominantly winter rainfall cluster. The sites from which *F. culmorum* was collected tended to be clustered in areas with low temperature (<22 °C) and higher levels of rainfall (>350 mm).

Backhouse et al. (2004) surveyed wheat, barley or durum wheat fields from the low rainfall (250 to 500 mm) areas near Queensland and New South Wales eastern Australian grain belt finding *F. pseudograminearum* as nearly the only pathogen

(approximately 99%) isolated from samples. However, *F. culmorum* was isolated from 70% of samples from the Victorian high rainfall region (>500 mm). Other species isolated included *F. avenaceum*, *F. crookwellense*, and *F. graminearum*.

Strausbaugh et al. (2004) found that *F. culmorum* and *B. sorokiniana* were the soil-borne pathogens most frequently isolated from 81 wheat fields and 52 barley fields from 13 counties in Southeastern Idaho during 2001 and 2002, among other pathogens including other *Fusarium* spp. They documented the prevalence and importance of crown and common root rot pathogens throughout Idaho and confirmed other literature associating *F. culmorum* with cooler temperatures. Smiley and Patterson (1996) and Smiley et al. (2005a) conducted extensive surveys of *Fusarium* spp. throughout northern Oregon and southeastern Washington, but there remained an extensive area in north central and eastern Washington that needed to be surveyed for *Fusarium* crown rot. Many of these areas have different climatic conditions than regions previously surveyed.

Douglas et al. (1990) published agronomic zones developed on the basis of growing degree days, precipitation, and soil depth (Table 1; Figure 1). Average annual precipitation and growing degree days were calculated based on 30 year National Weather Service data. The information on soil depth was obtained from soil surveys published by the Soil Conservation Service. Cropping system information was summarized for each zone by the authors to further characterize and define each agronomic zone. The zones were suggested for use as a geographical base for extrapolating research results (Figure 1).

The objective of this work was to; 1. determine crown rot occurrence and *Fusarium* species distributions covering the broad geographic region comprising the



major wheat producing areas of Washington and Oregon; 2. analyze survey results according to the agronomic zones developed by Douglas et al. (1990).

**Table 1.** Pacific Northwest Agronomic zones, the number of field collection sites within each zone, and parameters used to develop the respective zones.

Agronomic zone <sup>1</sup>	Number of fields sampled per zone <sup>2</sup>	Characteristics of climatic region <sup>3</sup>	Precipitation (mm) <sup>4</sup>	GDD <sup>5</sup>	Soil Depth (m) <sup>6</sup>
1. Annual Crop	5	Wet Cold	>400	<700	---
2. Annual Crop	18	Wet Cool	>400	700-1000	---
3. Annual Crop	7	Fallow-transition	350-400	700-1000	> 1m
4. Annual Crop	20	Dry	250-400	<1000	< 1m
5. Grain fallow	41	Dry	<350	NA	> 1m
6. Irrigated	9	Hot dry summers Irrigation required	NA	NA	

<sup>1</sup> Agronomic zones were developed by Douglas et al. (1990) and Douglas et al. (1992) on the basis of precipitation, growing degree days, and soil depth.

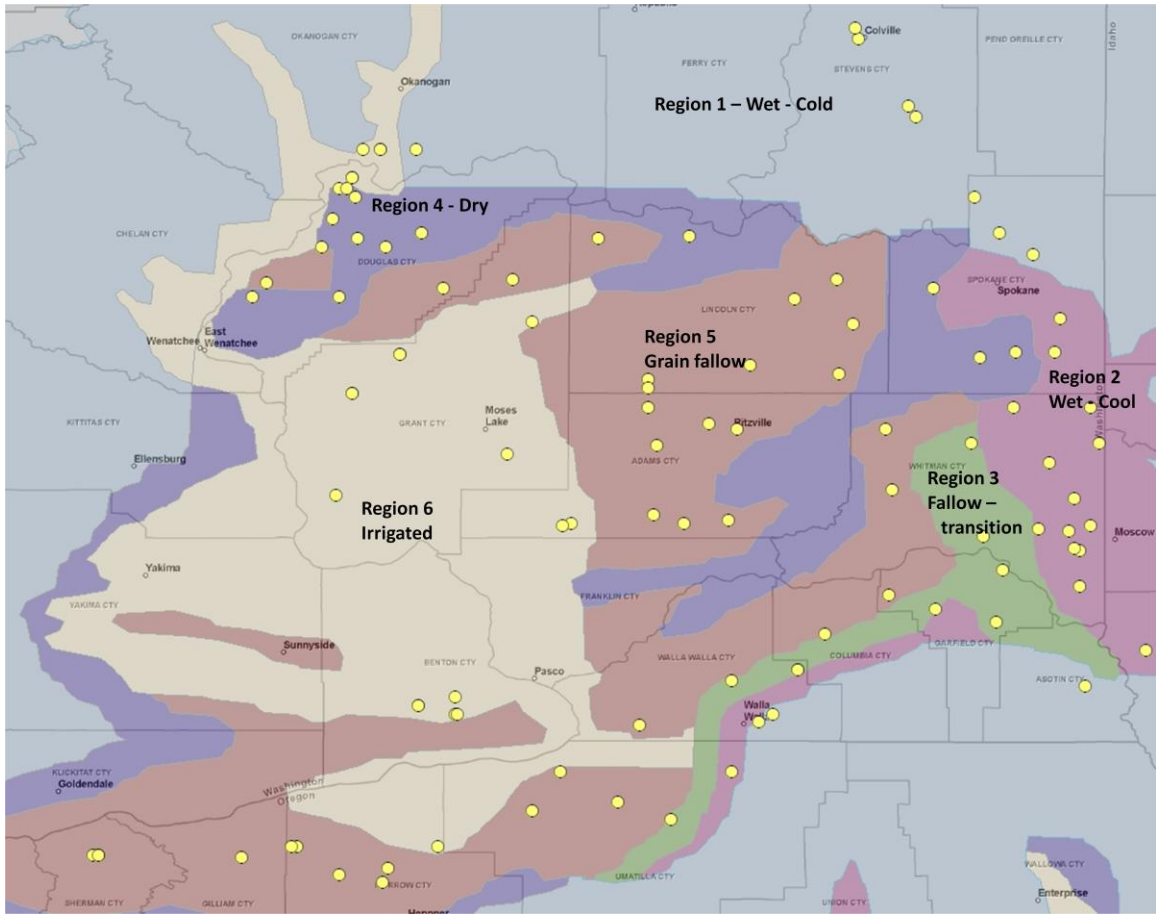
<sup>2</sup> Number represents the number of fields that were sampled within each agronomic zone in the Fusarium crown rot survey conducted in 2008 and 2009.

<sup>3</sup> Characteristics of each climatic region were developed on the basis of precipitation, temperature and cropping system wherever each was appropriate to characterize the respective region. ‘Wet’ areas received over 400 mm, and ‘Dry’ areas were placed into two subclasses (250 to 400 mm and 350 to 400 mm).

<sup>4</sup> Precipitation values were obtained for the National Weather Service 30 year precipitation records.

<sup>5</sup> GDD = Growing degree days were calculated by adding daily maximum and minimum air temperatures (°C) and dividing by two, discarding values less than zero and summing GDD values accumulated from 1 Jan – 31 May.

<sup>6</sup> Soil depth was estimated on the basis of Soil Conservation Service County soil survey maps and was included in zone delineation as an indicator of water storage capacity.



**Figure 1.** Map of six Agronomic zones for the dryland Pacific Northwest developed by Douglas et al. (1990) on the basis of precipitation, growing degree days, and soil-type. Agronomic regional zones are depicted by color polygons: Region 1 (light blue), Region 2 (dark red), Region 3 (green), Region 4 (dark blue), Region 5 (red), Region 6 (tan). Yellow points represent the 105 fields sampled in 2008 and 2009 for the Fusarium survey.

## **MATERIALS AND METHODS**

### **Sampling of field sites**

Selected survey sites included 210 randomly selected commercial fields located across the major wheat producing regions of Washington (17 counties) and Oregon (5 counties), including 21 Washington State University Cooperative Extension Uniform Cereal Cultivar Testing sites for winter wheat. Locations were surveyed during the summers of 2008 and 2009.

Fields were selected on the basis of their location within the major dryland wheat producing areas of Washington and Oregon. The approximate sampling distance between fields ranged from 17 to 80 km. Field sampling frequency was greater where *Fusarium* crown rot was known to persist in northern Oregon and north central Washington areas that had not been sampled previously. Fields were sampled randomly over a broad geographic area covering the summer fallow winter wheat region from 13 Washington counties and 5 Oregon counties. Given the crop rotation was predominantly grain-fallow, fields were sampled in 2009 in the closest proximity of those sampled in 2008, within 3.2 km. Sampling began when wheat was fully mature after harvest began in the Pendleton region around July 7<sup>th</sup> in 2008 and July 10<sup>th</sup> in 2009. Sampling followed the harvest pattern throughout the region through approximately September 15<sup>th</sup> in 2008 and September 25<sup>th</sup> in 2009 in the Pullman and Spokane areas of eastern Washington.

Five samples were randomly taken from each field. A sample was defined by a 1-m row of wheat stubble and roots taken with a shovel. A single sample was taken after walking 100 m into a field from the edge. Four additional samples were taken from that point by walking 25 m between each sample in a zig-zag pattern. Samples were placed in

a labeled paper bag and taken back to the Plant Pathology Farm near Pullman, WA to be scored at a later time. At the time of scoring, 10 stems were randomly chosen from plants within the sample. A total of 10 stems per sample were scored for crown rot severity based on the 0 to 10 scale for crown score according to the methods of Nicol et al. (2004). The number of nodes with disease symptoms was also recorded. At each sample a 2.5-cm diameter soil core was taken to a depth of 4-cm and stored in a -80 °C freezer in the lab in Pullman, WA to be used for soil DNA extraction. GPS coordinates were recorded using a 'Magellan'<sup>®</sup> device (Triton model, Magellan mfg., San Dimas, CA) at each 1-m row sample in 2008, but only at each field in 2009. A total of 5,100 tillers were selected and scored each year (2008 and 2009) (10,200 tillers rated total) for crown rot severity.

### **Isolation and culturing techniques**

A stem was randomly selected from a group of symptomatic stems from each sample, 5 stem samples per field (500 isolates total), from diseased crown and sub-crown internode tissue. A 2-cm diseased stem section was cut from each of the randomly selected symptomatic tillers. Stems were surface sterilized in 0.05% NaOCl for 30 s followed by a 6 minute rinse in ddH<sub>2</sub>O and placed on Synthetischer Nährstoffärmer agar (SNA) (Nirenberg, 1976) (1 g KH<sub>2</sub>PO<sub>4</sub>, 1 g KNO<sub>3</sub>, 0.5 g MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.5 g KCl, 0.2 g glucose, 0.2 g saccharose, and 20 g of agar per 1 L of media). Cultures were placed at room temperature in natural light for 3 days.

After 3 days, 1-cm pieces were cut from the SNA agar and fungal growth surrounding each cut stem piece was transferred to a fresh SNA plate. Plates were incubated for 3 weeks in the dark. Fungal species present were identified on the basis of

morphological characters of macroconidia observed through a light microscope at 40 X and 100 X (Nelson et al. 1981; Lesley et al. 2006).

When different species were isolated from a single field, single-spore cultures were made. Each SNA plate was viewed under the dissecting scope and a 1-cm piece of agar containing sporodochia was cut out and placed in 1 mL of water in a 5 mL tube. The tube was vortexed for 5 s and 90µL was pipetted onto a ½ strength PDA plate containing chloroamphenicol. Plates were incubated for 24 hrs at 22 C. Plates were viewed under the dissecting microscope and single germinated spores were cut out and transferred to a new SNA plate to serve as monospored isolations.

### **Data Analysis**

Means representing overall frequency of isolation of various fungi associated with crown rot were calculated. Species occurrence represented a mean across samples of *F. pseudograminearum*, *F. culmorum*, *F. crookwellense*, *F. acuminatum*, *F. equisiti*, and *Bipolaris sorokiniana* from a single randomly sampled cultured stem within each sample. No other descriptive statistics were used to analyze this qualitative data set.

The dependent variables from each survey sample were analyzed using Proc GLM ANOVA performed with SAS v.6.12 (SAS Institute Inc., Cary, NC.). Experimental units were individual stems randomly selected from 10 plants within a sample. The dependent variables were crown rot severity score and the number of diseased nodes from 10 stems per sample. The independent variables were field (random effect) and sample (random effect). The experimental design was a nested design where samples were nested within fields and stems were nested within transects. The random

field effect was tested with the sample (field) mean square error term and the random sample effect was tested with the stem (sample) mean square error term.

To analyze year differences for the dependent variables crown rot severity and the number of nodes with symptoms, 10 individual stems within a sample served as the experimental unit. Fields were nested within years. The independent variables were year, field, and sample. The mean square error of the field (year) term was used to test for the year effects and the sample (field) mean square error term was used to test for significance for the field effect. Least squares means were used to calculate means of dependent variables (crown rot score and number nodes with symptoms) and Proc GLM ANOVA was performed with SAS.

#### Agronomic zone ANOVA model

In another analysis field survey locations for each individual year were placed within agronomic zones developed by Douglas et al. (1990). The experimental units were means of 10 stems averaged within a sample for each field (sample or replications = 5 per field). Sample means served as replications within each field. The independent variables were agronomic zones (fixed effect) and fields within agronomic zones (random effect). Since agronomic zones were assigned to specific areas they were designated as fixed effects. The model error mean square field (agronomic zone) was used to test agronomic zones for significance. The dependent variables analyzed according to this model were crown rot severity and the number of nodes with symptoms over years.

In another analysis field survey locations were again placed within agronomic zones developed by Douglas et al. (1990). Independent variables were agronomic zones

(fixed effect) and fields (random effect). To test the dependent variables of *F. pseudograminearum* and *F. culmorum* isolation frequency, the mean number of isolations per field of each respective species was averaged across the 5 samples within a field (i.e. numbers represented a value between 1 and 5). The isolation number of each species per field served as the experimental unit. Duncan's least significant difference (LSD) multiple range test was used to calculate mean separation values and Proc GLM ANOVA was performed with SAS. Following statistical analysis values were converted from numbers to a frequency of isolation proportion (%) per five samples within each field within each agronomic zone.

Given that the two main *Fusarium* spp. (*F. pseudograminearum* and *F. culmorum*) seemed to dominate in certain agronomic zones of the Pacific Northwest, a t-test analysis was conducted to test the differences between mean isolation frequencies of *F. pseudograminearum* and *F. culmorum* within each zone over the survey years 2008 and 2009. Sampling locations between years were aligned and means for *F. pseudograminearum* and *F. culmorum* isolation frequencies were calculated within fields across the years 2008 and 2009. An F-test was conducted for the frequency of isolation within each field for each species (*F. pseudograminearum* and *F. culmorum*) to test for equality of variance. Wherever F-tests were not significant ( $p > 0.05$ ) a t-test for equal variance between *Fusarium* spp. means for each region was conducted. Wherever F-tests were significant ( $p < 0.05$ ) a t-test for unequal variance between *Fusarium* spp. was conducted. Regression analysis was also conducted on the mean isolation frequency for *F. pseudograminearum* and *F. culmorum* averaged within fields and across survey years 2008 and 2009.

## RESULTS

The mean frequency of isolation from randomly selected symptomatic stems of *F. culmorum* and *F. pseudograminearum* was very similar at 31% and 30%, respectively, over both survey years (Table 2). Within years, the frequency of isolation for *F. pseudograminearum* (26%) and *F. culmorum* (23%) were slightly lower in 2009 than in 2008. Other species were also isolated from infected stems, including *F. crookwellense*, *F. acuminatum*, *F. equisiti*, and *B. sorokiniana* at frequencies of 13%, 1.3%, 0.3%, and 1.7%, respectively (Table 2).

**Table 2.** Frequency of isolation of each respective species isolated from the total (525) sampled wheat stems in the 2008 and 2009 PNW Fusarium crown rot field survey.

Fungal Species <sup>1</sup>	Frequency of isolation <sup>2</sup>		Mean <sup>3</sup>
	2008	2009	
<i>F. pseudograminearum</i>	34%	26%	30%
<i>F. culmorum</i>	38%	23%	31%
<i>F. crookwellense</i>	12%	14%	13%
<i>F. acuminatum</i>	1.6%	1%	1.3%
<i>F. equisiti</i>	0.6%	0%	0.3%
<i>Bipolaris sorokiniana</i>	1.4%	2%	1.7%

<sup>1</sup>Fungal species were identified according to Lesley et al. 2006. Stem isolates were cultured from SNA media and placed in slides for morphological identification.

<sup>2</sup>Frequency of isolations represent the proportion of samples (per 525) isolated from the entire survey for 2008 and 2009 survey years, respectively.

<sup>3</sup>Mean represents the average for each species isolated across the 2008 and 2009 survey years.



Significant differences in the field sample ANOVA model in crown rot severity ( $p < 0.0001$ ) and number of nodes with symptoms ( $p < 0.0001$ ) were detected among fields for both the 2008 and 2009 survey years. The mean crown rot severity rating among fields was 2.2 in 2008 and 2.9 in 2009, with a coefficient of variation (CV) of 60 and 57 for each year, respectively. Field ratings for crown rot severity (rated 0 to 10) ranged from 0.2 to 5.1 in 2008 and from 0.5 to 5.4 in 2009. The  $R^2$  of the model was 0.54 and 0.48, for 2008 and 2009, respectively showing a reasonable fit of the data to the field sample ANOVA model for both years. There was a significant difference between years in crown rot severity ( $p < 0.0001$ ) and the number of nodes with symptoms ( $p = 0.0001$ ) for the Year ANOVA model (Tables 3 and 4). Most of the variation was accounted for between the survey years 2008 and 2009, followed by between field variation.

There were significant statistical differences between crown rot severity ( $p < 0.0001$ ), nodes with symptoms ( $p < 0.0001$ ), and isolation frequencies for *F. pseudograminearum* ( $p < 0.0001$ ) and *F. culmorum* ( $p < 0.0001$ ) during 2008 and 2009 for the Agronomic zone ANOVA model. In both 2008 and 2009 crown rot severity was the greatest in Region 3, the fallow transition zone extending from St. John to Walla Walla in Washington State. Region 3 had significantly greater number of nodes with crown rot symptoms in both years than Regions 1 and 4 (Table 5).

In 2009 Region 4 had the least amount of crown rot severity and significantly greater frequency of *F. culmorum* isolation than Regions 2, 3 and 5. Region 1 had the least crown rot severity and lowest frequency of *F. pseudograminearum* in 2008 and a significantly greater frequency of *F. culmorum* isolation over both survey years compared to Regions 2, 3 and 5 (Table 5).

**Table 3.** Results for crown rot severity (rated 0 to 10) from the Year ANOVA model analyzed over years 2008 and 2009 from the Fusarium survey.

Source	DF <sup>1</sup>	Sum of Squares	Mean Square	F-Value	P-value <sup>2</sup>
Model <sup>3</sup>	636	20110.92	31.62	15.03	0.0001
Error	9735	20485.85	2.10		
Total	10371	40596.78			
<hr/>					
Year <sup>4</sup>	1	1219.56	1219.56	579.55	0.0001
Field (Year) <sup>5</sup>	102	6097.36	59.77	28.41	0.0001
Sample (Field) <sup>6</sup>	429	3199.45	7.45	3.54	0.0001

<sup>1</sup> DF = degrees of freedom (DF = (n) -1, where n=number of samples).

<sup>2</sup> P-values were considered significant at P=0.05.

<sup>3</sup> The ANOVA model according to year and field where the dependent variable, crown rot severity, was rated from 10 individual stems within a sample which served as the experimental unit. The independent variables were year, field, and sample.

<sup>4</sup> Year effect was analysed for the 2008 and 2009 Fusarium survey years.

<sup>5</sup> Fields were nested within years 2008 and 2009.

<sup>6</sup> Five samples were taken within each field randomly in a zig-zag pattern.

**Table 4.** Results for the number of symptomatic nodes from the Year ANOVA model analyzed over years 2008 and 2009 from the Fusarium survey.

Source	DF <sup>1</sup>	Sum of Squares	Mean Square	F-Value	P-value <sup>2</sup>
Model <sup>3</sup>	636	4395.29	6.91	12.74	0.0001
Error	9735	5279.86	0.54		
Total	10371	9675.16			
<hr/>					
Year <sup>4</sup>	1	179.45	179.45	330.88	0.0001
Field (Year) <sup>5</sup>	102	1504.88	14.75	27.20	0.0001
Sample (Field) <sup>6</sup>	429	699.00	1.62	3.00	0.0001

<sup>1</sup> DF = degrees of freedom (DF = (n) -1, where n=number of samples).

<sup>2</sup> P-values were considered significant at P=0.05.

<sup>3</sup> The ANOVA model according to year and field where the dependent variable, the number of nodes with symptoms, was rated from 10 individual stems within a sample which served as the experimental unit. The independent variables were year, field, and sample within a field.

<sup>4</sup> Year effect was analysed for the 2008 and 2009 Fusarium survey years.

<sup>5</sup> Fields were nested within years 2008 and 2009.

<sup>6</sup> Five samples were taken within each field randomly in a zig-zag pattern.

**Table 5.** Crown rot severity, average number of nodes with symptoms, and isolation frequency per field for *F. pseudograminearum* (*Fp*) and *F. culmorum* (*Fc*) within six PNW Agronomic zones.

Agronomic zones <sup>1</sup>	Crown rot severity <sup>3</sup>		Nodes with symptoms <sup>4</sup>		Isolation frequency (%) <sup>5</sup>			
					<i>Fp</i>		<i>Fc</i>	
	2008	2009	2008	2009	2008	2009	2008	2009
1. Wet/ Cold	0.8 d <sup>2</sup>	3.2 b	0.6 d	2.0 b	0 b	20 bc	76 a	38 a
2. Wet/ Cool	2.2 b	3.4 b	1.4 b	1.8 b	28 a	22 abc	30 b	26 bc
3. Fallow- transition	3.1 a	4.2 a	1.7 a	2.4 a	36 a	32 ab	26 b	4 d
4. Dry	1.5 c	2.1 d	0.8 c	1.2 c	28 a	4 c	52 ab	50 a
5. Grain fallow	2.3 b	2.7 c	1.4 b	1.3 c	44 a	38 a	32 b	12 cd
6. Irrigated	2.8 a	3.1 b	1.5 b	1.8 b	16 ab	26 ab	48 ab	16 bcd
Mean <sup>6</sup>	2.2	2.9	1.3	1.5	34	26	38	24
LSD	0.3	0.3	0.1	0.2	27	17	27	19.8

<sup>1</sup> Agronomic zones were developed by Douglas et al. (1990) and Douglas et al. (1992) on the basis of precipitation, growing degree days, and soil depth.

<sup>2</sup> Numbers within a column followed by the same letter are not significantly different according to Duncans LSD multiple range test at P=0.05.

<sup>3</sup> Crown rot severity was rated on a 0 to 10 scale (0 = no disease; 10 = significant disease/death) from each of 10 stems per sample from 5 samples within each field (50 samples per field).

<sup>4</sup> The number of nodes showing symptoms were recorded for each stem rated for crown rot severity.

<sup>5</sup> Isolation frequencies represent the number of total proportion (%) of samples where each respective species (*Fp* = *F. pseudograminearum* and *Fc* = *F. culmorum*) was isolated and morphologically identified according to Lesley et al. (2006).

<sup>6</sup> Means and Fischer LSD values represent the numbers within a column.

Region 5, the grain fallow region making up most of the eastern Washington and Oregon dryland wheat production region, was moderate in crown rot severity compared to all other regions, but had statistically lower crown rot severity compared to Region 3 in both years. Regions 2 and 6 also had moderate levels of crown rot severity over both years. Isolation of *F. pseudograminearum* seemed to be prevalent across all the regions, with the exception of Region 1 in 2008, with the greatest isolation frequency in Region 5 (Table 5).

The mean proportion of isolations of *F. pseudograminearum* and *F. culmorum* were further analyzed for their distribution across fields in six agronomic zones (Table 6). Fields within regions were separated into categories according to fields with only one of each species isolated within a field, or both species occurring within each field and averaged over the 2008 and 2009 survey years. *F. culmorum* alone was most frequently isolated from fields within Regions 1 and 4 at an equal proportion of 65%, respectively. Fields were dominated with *F. pseudograminearum* within Regions 3 and 5 at proportions of 67% and 54%, respectively. There were a high proportion of both species isolated within the same field for Regions 2 and 6, which are characterized by high rainfall and irrigation (Table 6).

**Table 6.** Distribution of *Fusarium* spp. identified in culture. Proportions of fields that contained only *F. pseudograminearum*, only *F. culmorum*, or both species within the same field for each agronomic zone.

Agronomic zone <sup>1</sup>	Mean (2-year) proportion of fields <sup>2</sup>		
	<i>F. pseudograminearum</i> <sup>3</sup>	<i>F. culmorum</i> <sup>4</sup>	Both <sup>5</sup>
1. Wet / Cold	12%	65%	19%
2. Wet / Cool	33%	30%	34%
3. Fallow- transition	67%	13%	11%
4. Dry	3%	65%	30%
5. Grain fallow	54%	14%	29%
6. Irrigated	30%	30%	33%
Mean	33%	36%	26%

<sup>1</sup> Agronomic zones were developed by Douglas et al. (1990) and Douglas et al. (1992) on the basis of precipitation, growing degree days, and soil depth.

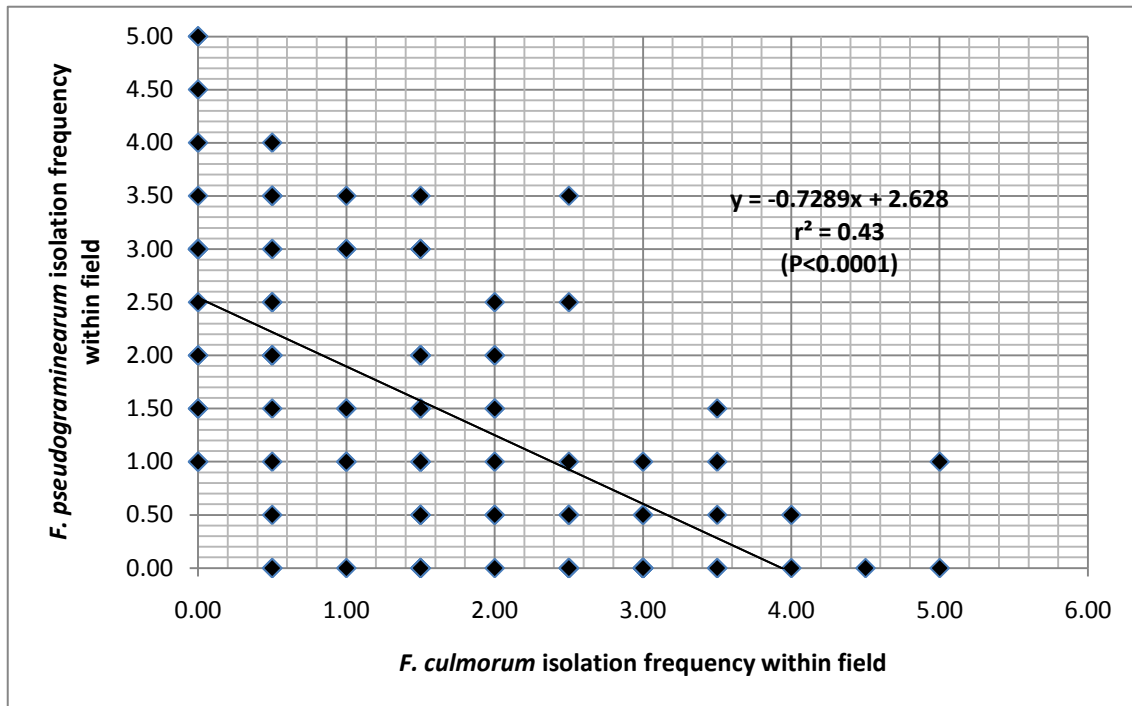
<sup>2</sup> Numbers represent 2 year mean (2008 and 2009) proportions (%) of fields where only one species or the other or both were isolated.

<sup>3</sup> Proportion of fields where only *F. pseudograminearum* was isolated averaged over the 2008 and 2009 survey years.

<sup>4</sup> Proportion of fields where only *F. culmorum* was isolated averaged over the 2008 and 2009 survey years.

<sup>5</sup> Proportion of fields where both *F. pseudograminearum* and *F. culmorum* were isolated within the same field averaged over the 2008 and 2009 survey years.

Regression analysis of isolation frequency showed a significant relationship between *F. pseudograminearum* and *F. culmorum* across the 105 fields and six agronomic zones ( $r^2=0.43$ ;  $p<0.0001$ ) (Figure 2). These regression results suggest a trend that the isolation of one species may dominate within a given field over the other (in comparing the two most frequently isolated *Fusarium* spp.). T-tests of mean isolation frequencies across the years for *F. pseudograminearum* and *F. culmorum* within each region revealed significantly higher isolations of *F. culmorum* from Regions 1 ( $p=0.02$ ) and 4 ( $p<0.0001$ ). In Region 5 there were significantly higher isolations of *F. pseudograminearum* ( $p=0.0003$ ). There were no significant differences between the isolation frequencies between *F. pseudograminearum* and *F. culmorum* for Regions 2, 3, and 6 (Table 7).



**Figure 2.** Regression analysis of isolation frequency within 105 fields (averaged across five samples) of *F. pseudograminearum* (independent variable =y) and *F. culmorum* (dependent variable =x) ( $r^2=0.43$ ;  $p<0.0001$ ).

**Table 7.** Results from the t-test analysis between population means for *F. pseudograminearum* and *F. culmorum* isolation frequencies within each Agronomic zone.

Agronomic zone <sup>1</sup>	Mean (2-year) isolation frequencies (%) <sup>2</sup>		P-Value <sup>5</sup>
	<i>F. pseudograminearum</i> <sup>4</sup>	<i>F. culmorum</i> <sup>4</sup>	
1. Wet / Cold	14 a <sup>3</sup>	2.80 b	0.0280
2. Wet / Cool	1.31	1.36	0.8740
3. Fallow -transition	1.50	1.00	0.4810
4. Dry	0.60 a	2.65 b	<0.0001
5. Grain fallow	2.06 a	1.09 b	0.0003
6. Irrigated	1.11	1.67	0.2520
Mean	1.21	1.76	-----

<sup>1</sup> Agronomic zones were developed by Douglas et al. (1990) and Douglas et al. (1992) on the basis of precipitation, growing degree days, and soil depth.

<sup>2</sup> Numbers represent 2 year mean (2008 and 2009) isolation frequencies within each field which served as replications within agronomic zones.

<sup>3</sup> Numbers followed by different letters within a row represent significant differences according to pairwise t-tests.

<sup>4</sup> Numbers represent the number of isolations per field (out of 5 samples within a field) within each respective agronomic zone for respective *F. culmorum* and *F. pseudograminearum* averaged over both survey years.

<sup>5</sup> P-values were considered significantly different at the 0.05 level according to a pairwise t-test.

## DISCUSSION

Crown rot surveys conducted in the PNW have established that *F. culmorum* and *F. pseudograminearum* are the two dominant causal agents of Fusarium crown rot (Cook, 1968; Smiley and Patterson, 1996; Smiley et al. 2005a). In this study, we conducted a survey of 210 fields in the dryland wheat producing regions of Washington and Oregon during 2008 and 2009 to determine the distribution of fungi associated with crown rot of wheat. Results confirmed previous reports that *F. pseudograminearum* and *F. culmorum* are the dominant species associated with crown rot symptoms, and were isolated in the greatest frequencies of 30% and 31%, respectively, over the two year survey. Isolates of *Fusarium* spp. were obtained from 99% and 97% of all fields surveyed in 2008 and 2009, respectively, confirming its widespread distribution in the dryland wheat producing regions of the PNW.

Other species have been associated with the crown rot and common root rot disease complex including *F. crookwellense*, *F. acuminatum*, *F. equiseti*, and *B. sorokiniana* in the U.S. (Scardaci and Webster, 1982; Specht and Rush, 1988; Hill et al. 1983). Specht and Rush (1988) reported that *F. acuminatum* was associated with root and foot rot of wheat in the Texas pan-handle. Others have reported the isolation of *F. equiseti* from stems with crown rot symptoms, but it was not considered to be pathogenic (Burgess et al. 1975; Specht and Rush, 1988; Klein et al. 1990; Strausbaugh et al. 2004). Our results showed that the isolation of *F. crookwellense*, *F. acuminatum*, *F. equiseti*, and *B. sorokiniana* at frequencies of 13%, 1.3%, 0.3%, and 1.7%, respectively, from symptomatic tissue agree with that of others (Bentley et al. 2006a; Smikey and Patterson, 1996; Specht and Rush, 1988; Strausbaugh et al. 2004).



In addition to these two main species of *Fusarium*, *F. crookwellense* was isolated from 12% and 14% of the total isolated stem samples in 2008 and 2009, respectively, according to conidial morphology outlined by Lesley et al. (2006). The mean isolation frequency was 13% averaged over both survey years. Others have reported isolation of *F. crookwellense* from diseased wheat crown tissue in surveys conducted in Australia and Turkey (Backhouse et al. 2004; Bentley et al. 2006b).

Bentley et al. (2006a) isolated *F. crookwellense* from wheat and other grassy plant crowns and verified that three isolates had been incorrectly morphologically identified as *F. pseudograminearum* and were molecularly confirmed as *F. crookwellense*. Mis-identifications are common as conidia morphology of *F. crookwellense* closely resembles that of *F. culmorum* and their geographic ranges overlap (Lesley et al. 2006). Lesley et al. (2006) reported that *F. crookwellense* can cause mild root rot of wheat in greenhouse trials. Although *F. crookwellense* has been reported in the northern U.S. (Lesley et al. 2006), to our knowledge it has not been reported in Oregon or Washington. The morphological identifications made in the current study await confirmation with conventional PCR primers (CRO-C) published by Williams et al. (2002).

Cook (1968) conducted the first surveys in the PNW, providing some of the first descriptions of the symptoms and reported that 90% of the species isolated from those areas were *F. culmorum*. Cook (1968) reported that *F. pseudograminearum* (*F. roseum* f. sp. *cerealis* ‘Graminearum’) dominated in Franklin, Walla Walla and Benton Counties. These counties would fall within agronomic zones 3, 5, and 6 according to Douglas et al. (1990), which contained significant levels of *F. pseudograminearum*. Smiley and Patterson (1996) surveyed areas of Adams and Lincoln counties that had been previously

surveyed by Cook (1968) and confirmed that *F. culmorum* was much more prevalent in Lincoln county where Cook (1968) had done previous research.

Climate and soils are highly variable in the PNW (Smiley and Patterson, 1996; Douglas et al. 1990). Smiley and Patterson (1996) reported that the occurrence of Fusarium foot rot pathogens was not related to the agronomic zones outlined by Douglas et al. (1990). However, the authors did report that the detection of *F. pseudograminearum* was weakly correlated to mean monthly temperature ( $r^2 = 0.42$ ,  $P=0.06$ ) and elevation ( $r^2 = 0.49$ ,  $P=0.03$ ). But when elevation and precipitation was regressed together with *F. pseudograminearum* there was a strong relationship ( $r^2 = 0.46$ ,  $P=0.05$ ), suggesting the species occurrence may decrease as elevation and mean annual precipitation increase.

In the current study, when survey sites were placed into six climatic regions defined by precipitation, growing degree days, and soil type (Douglas et al. 1990), there were statistical differences in crown rot severity, number of nodes with disease symptoms, and species isolation frequencies (*F. pseudograminearum* and *F. culmorum*) among the zones. *F. culmorum* was more frequently isolated from Regions 1 (cool and wet) and 4 (dry), while *F. pseudograminearum* occurred in significantly greater frequency in Region 5 (grain-fallow) according to t-test results. Region 1 is characterized by wetter and cooler climates, whereas region 4 is characterized by a dry climate and shallow soil according to Douglas et al. (1990).

Samples from Region 4 were collected from high elevation locations (2,200 feet) that experience significant amounts of snowfall. In these areas of Douglas county, snow-mold (*M. nivale* and *Typhula* spp.) has been reported to be a significant problem (Bruehl,

1982). Most of the moisture in this region comes in the fall and winter. The parameters used to define the agronomic zones described by Douglas et al. (1990) are broad and lack detail for information pertaining to snowfall, specific temperature, and moisture patterns within each zone that could potentially affect species distribution.

Backhouse et al. (2001) reported that many factors can influence *Fusarium* spp. distribution including soil type, microenvironment, and land use and vegetation type, but that on a broader regional scale climate has an overriding effect. Many species of *Fusarium* seem to be controlled by climate (Backhouse et al. 2001). Backhouse et al. (2001) stressed that many factors must be taken into consideration when evaluating species distributions including latitude, longitude, elevation, temperature, rainfall, and should be as specific so as to include the seasonality of temperature and rainfall.

Cook (1981) reported that the basic difference among populations of *F. pseudograminearum* (*F. graminearum* Group 1) and *F. culmorum* are their temperature preferences. *F. pseudograminearum* occurs in warmer areas whereas *F. culmorum* is “intermediate in temperature preference” (Cook, 1981). Others have reported climatic correlations of the occurrence of *F. culmorum* in cooler and wetter climates (Sitton and Cook, 1981; Strausbaugh et al. 2004; Backhouse and Burgess, 2002). In a more detailed survey from zones associated with climate, Backhouse et al. (2004) isolated *F. culmorum* from 70% of the diseased wheat stems collected from the Victorian high rainfall region (>500 mm). There is the potential that climate may have an effect on the distribution of *Fusarium* spp. in the PNW.

Although *F. pseudograminearum* seemed to dominate in Region 5 in this survey, characterized by grain-fallow crop rotation typical of eastern Washington and warmer

and drier weather patterns, the species was not limited to these areas of distribution. The occurrence of the two dominant *Fusarium* species was highly variable between 2008 and 2009 with significant year effects in this study showing the high degree of field variability from year to year. These results agree with that of others (Smiley and Patterson, 1996).

Other factors that could affect species distribution include crop rotational patterns and cultural practices (Burgess et al. 2001; Papendick and Cook, 1974; Nelson et al. 1981). The main rotational crops in the PNW dryland wheat producing region are peas and lentils, and to a lesser extent canola and mustard (*Brassica* sp.). To date there have been no reports of *Fusarium* spp. capable of causing crown rot on these rotational crops (Shaw, 1973; Richardson, 1990).

The other factor that could affect species distribution is the inoculum source and overwintering structure. *F. pseudograminearum* and *F. culmorum* survive as chlamydospores in the soil and host residue (Sitton and Cook, 1981). Sitton and Cook (1981) suggested that the survival of *F. culmorum* chlamydospores (8.5 years) is much longer than those of *F. pseudograminearum* (5.5 years). Smiley and Patterson (1996) reported that *F. culmorum* was detected in more soil samples than plant samples in one year (1993) but more in plant samples than the soil in the other year (1994). Inglis and Cook (1986) reported a greater decrease in *F. culmorum* chlamydospores over time at Lind (survived 215 days) compared to Pullman (survived 330 days), but field surveys still indicated greater disease severity at Lind than Pullman. Inglis and Cook (1986) concluded that environmental conditions had a greater influence than chlamydospore survival on the ability of the fungus to develop and persist as a wheat pathogen. Year to

year variation between species occurrence exists as well and survey have indicated that the dominance of species has changed over time (Cook, 1968; Burgess et al. 1975; Smiley and Patterson, 1996). There were significant differences between years for crown rot severity and the number of nodes with symptoms in the current study. Most of the variation was accounted for between years in this study, followed by the variation between fields, while the variation within field was the least for crown rot severity and the number of nodes with symptoms (Tables 3 and 4).

Results of the current study agree with others that *F. pseudograminearum* and *F. culmorum* are associated with crown rot and dominate the rain-fed dryland wheat production region of the PNW. *F. crookwellense* were identified according to conidia morphology in both survey years, however these identifications must be confirmed with genetic analysis through conventional PCR. Regional differences were found in *Fusarium* spp. distribution where *F. pseudograminearum* dominated in Region 5 the grain-fallow area, while *F. culmorum* dominated in Region 1 characterized by a wet cold climate and Region 4 characterized by a dry climate according to Douglas et al. (1990). The validity of these climatic relationships requires further research.

The objective of this research was to simply conduct a survey of *Fusarium* species distribution causing crown rot in the PNW. In this study the greatest amount of variation in crown rot severity and the number of nodes with symptoms occurred over years compared to variation between and within fields. Others have documented the high degree of variation in crown rot occurrence over years (Inglis and Cook, 1986; Smiley and Patterson, 1996).

Further research needs to continue over the course of several years to evaluate the distribution of *Fusarium* spp. causing crown rot and its potential association with climate in the PNW, as well as species population changes over time. There is a need to develop appropriately designed experiments to conduct *Fusarium* spp. distribution studies that coincide with climatic parameters. A more detailed analysis with Principal Components Analysis (PCA) would further refine the relationship between *Fusarium* spp. distribution and climate and will be conducted in the future similar to the methods described by Backhouse and Burgess (2002). PCA analysis may lend more information as to the role of temperature and precipitation in *Fusarium* spp. distribution and crown rot occurrence in dryland wheat production of the Pacific Northwest. Further documentation of crop production cultural practices and cropping history should be made to better explain regional differences in species distribution.

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## **CHAPTER 3**

## CHAPTER 3

### OPTIMIZATION OF REAL TIME QUANTITATIVE PCR (Q-PCR) FOR *FUSARIUM PSEUDOGRAMINEARUM* AND *F. CULMORUM* ON WHEAT

Grant Poole<sup>1</sup>, Fatih Ozdemir<sup>2</sup>, Seth D. Nydam<sup>3</sup>, Kurtis L. Schroeder<sup>4</sup>, Julie M. Nicol<sup>5</sup>,  
Richard W. Smiley<sup>6</sup>, Kimberly Garland-Campbell<sup>4</sup>, and Timothy C. Paulitz<sup>4</sup>.

<sup>1</sup>Washington State University, Dept. of Plant Pathology, Pullman, WA, 99164-6430; E-mail: [gpoole@wsu.edu](mailto:gpoole@wsu.edu); FAX: 509-335-9581; <sup>2</sup>Bahri Dagdas International Agricultural Research Institute, Konya, Turkey; <sup>3</sup>Washington State University, Dept. of Veterinary Microbiology and Pathology, Pullman, WA, 99164; <sup>4</sup>USDA-Agricultural Research Service, Pullman, WA; <sup>5</sup>CIMMYT (International Maize and Wheat Improvement Centre) P.O. Box. 39 Emek 06511 Ankara, Turkey; <sup>6</sup>Oregon State University, Pendleton, OR.

#### ABSTRACT

Current identification of *Fusarium* spp. causing crown rot relies on traditional culturing methods that require mycological expertise and extensive time and materials. The advent of Q-PCR (Quantitative Real-Time PCR) techniques and the development of species-specific primers for *F. pseudograminearum* and *F. culmorum* has allowed for more accurate species identification and fungal DNA quantification from wheat stems infected with crown rot pathogens. The objective of this study was to develop a Q-PCR-based assay by evaluating and optimizing DNA extraction methods to accurately assess DNA concentrations of *Fusarium* spp. in wheat stem tissue from field samples. *Fusarium* species-specific primers developed in previous studies were evaluated for use in Q-PCR

to identify and quantify *Fusarium* spp. from wheat stem tissue. Fungal DNA was extracted from local Pacific Northwest (PNW) of *F. pseudograminearum* and *F. culmorum* mycelium grown on potato-dextrose broth (PDB) and used for developing standard curves and positive controls. The use of the ‘OPT’ primers for the amplification of *F. culmorum* and the ‘FPG’ primers for the amplification of *F. pseudograminearum* yielded the most consistent Q-PCR results. The MO-BIO<sup>®</sup> Ultra Clean Soil Kit for DNA extraction produced the most consistent Q-PCR from infected wheat tissue when compared to the FastDNA kit (MP Biomedicals<sup>®</sup>). Bead beating utilizing the MO-BIO kit (using the ceramic beads from the FastDNA kit) was superior to grinding with liquid nitrogen alone for Q-PCR and DNA quantification. Optimal results were obtained by grinding with liquid nitrogen, soaking prior to bead beating and a Fast Prep speed of 5 for 45s. A Fast Prep speed of 6 resulted in lower DNA quantification possibly due to over-maceration of fungal and plant cells and subsequent fragmentation of DNA. The addition of polyvinylpyrrolidone (PVPP) was necessary for adequate DNA extraction. The analysis of 144 field samples from a crown rot field survey showed that DNA quantification from Q-PCR was not positively correlated to field crown rot ratings. However, there was a significant correlation ( $r^2 = 0.61$ ) between DNA concentration and crown rot severity in seven amplified Q-PCR samples that were extracted from individual symptomatic wheat stems.

## INTRODUCTION

Fusarium crown rot of wheat (*Triticum aestivum* L.) is caused by a complex of *Fusarium* spp. and other fungal species, of which *F. pseudograminearum* and *F. culmorum* are the most dominant. Crown rot reduces wheat yields by an average of 9.5% in the Pacific Northwestern United States, with losses as high as 30% reported (Burgess et al. 2001; Cook, 1968; Cook and Veseth, 1991; Cook, 1992; Dodman and Wildermuth, 1987; Hekimham et al. 2004; Kane, 1987; Klein et al. 1991; Nicol et al. 2001; Smiley et al. 2005a). Smiley et al. (2005a) documented yield loss in the Pacific Northwest (PNW) as high as 61% resulting from inoculation with a mixture of five *F. pseudograminearum* isolates. Yield loss resulting from Fusarium crown rot infection is difficult to assess on a regional basis as infection occurs at or near the soil line and is not clearly visible (Strausbaugh et al. 2004), with the exception of the formation of white heads for a brief period of time prior to straw ripening (Burgess et al. 2001).

Common names of crown rot include dryland foot rot, dryland root rot, foot rot, Fusarium crown rot, and Fusarium root rot. Crown rot is caused by a complex of *Fusarium pseudograminearum* (O'Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*), *F. culmorum* (Wm. G. Sm.) Sacc., *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Cochliobolus sativus*), *F. avenaceum* (Fr.:Fr.) Sacc. (= *Gibberella avenacea*), and *Microdochium nivale* (Fr.) Samuels & I.C. Hallet (= *Monographella nivalis*) (Cook, 1968; Cook and Veseth, 1991; Smiley et al. 2005a.; Nicol et al. 2001). In Australia, and other parts of the world, crown rot is the accepted common name of the disease and is understood to be caused mainly by *F. pseudograminearum* and *F. culmorum* (Burgess et al. 2001). It is widely accepted that *F. pseudograminearum* is the



dominant species responsible for causing crown rot in arid regions of the PNW region of the U.S. and in Australia (Burgess et al. 1975; Backhouse and Burgess, 2002; Chakraborty, 2006; Smiley et al. 2005b).

Traditional methods of measuring crown rot severity typically use visual rating systems (based on the proportion of symptoms) and index scales (Mitter et al. 2006; Nicol et al. 2001; Smiley et al. 2005a; Wildermuth and McNamara, 1994). Klein et al. (1990) isolated crown rot pathogens in accordance with white head symptoms and found the two were closely related. Smiley et al. (2005a) utilized crown ratings and disease severity classes to correlate disease severity to yield loss. Traditional techniques for determining crown rot severity are not only dependent on tedious rating systems, but on culturing fungal isolates and obtaining appropriate morphological characters for proper identification (Aoki and O'Donnell, 1999).

With recent advances in molecular technology such as Q-PCR technology, the amount and species identity of a pathogen can be more easily estimated by the amount of fungal DNA present (Okubara et al. 2005; Schroeder et al. 2006; Hogg et al. 2007). The development of species-specific primers for use in Q-PCR have allowed for more efficient and accurate quantification of fungal DNA at the species level. Schroeder et al. (2006) designed and utilized species-specific primers for the detection and quantification of nine *Pythium* spp. from soils collected in eastern Washington.

Q-PCR has been used in several studies to quantify the amount of *F. culmorum* and *F. pseudograminearum* in diseased wheat tissue (Leisova et al. 2006; Hogg et al. 2007; Hogg et al. 2010). Waalwijk et al. (2004) developed species-specific TaqMan based primers (PE Applied Biosystems, Foster City, CA) for *F. avenaceum*, *F.*

*culmorum*, *F. graminearum*, *F. poae*, and *Microdochium nivale* to detect fungal DNA concentrations in leaves, heads, and seed for detecting Fusarium head blight occurrence and deoxynivalenol (DON) content in grain. Fungal DNA concentrations determined by Q-PCR with primers specific to *F. graminearum* and *F. culmorum* were correlated to both the incidence of Fusarium head blight and DON content of mycotoxin in the grain. TaqMan based primers for *F. graminearum* and *F. culmorum* used in the study were developed by Nicholson et al. (1996; 1998).

Strausbaugh et al. (2005) isolated *Fusarium* from wheat and barley roots common to southern Idaho and rated root lesions in four growth chamber and two field assays. Strausbaugh et al. (2005) developed Q-PCR assays using TaqMan probes to quantify Fusarium crown rot in diseased wheat and barley root tissues. Forward and reverse primers named ‘Gzgae87T’ and a ‘Gzgae87T’ probe were developed from the trichodiene synthase (*tri5*) gene of *F. graminearum* with nucleotide sequences obtained from GenBank corresponding to the *tri5* encoding trichodiene synthase from *Fusarium* spp. (*F. poae* and *Gibberella zae* accessions). A sequence alignment was performed on the most conserved region of the *tri5* gene within the *Gibberella zae* sequence from a specific collection accession.

The *tri5* gene-based primers were not species-specific and amplified *F. culmorum* and *F. pseudograminearum* in addition to *F. graminearum*. The primer set did not amplify pure cultures of *F. avenaceum*, *F. acuminatum*, *F. semitectum*, *F. equiseti*, *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Pythium* spp. and 10 of the most common fungal saprophytes isolated from wheat and barley roots from southern Idaho. *F. culmorum* infections resulted in the largest infected root area in inoculated

greenhouse assays. These assays were sensitive enough to detect *F. culmorum* extracted from pure cultures down to 61 pg (Strausbaugh et al. 2005).

Strausbaugh et al. (2005) evaluated *Fusarium* spp. populations at two field sites in Idaho under natural inoculum pressure. The isolation frequency of *F. culmorum* was 1% at the Arbon Valley and 67% at the Ririe location, however DNA was quantifiable only at the Ririe location. Although DNA was quantifiable where *F. culmorum* was persistent, there was no correlation with field disease severity ratings under naturally inoculated field conditions. However, they did report significant correlations between DNA quantities and the percentage of infected roots in growth chamber studies where plants were inoculated with *F. culmorum* and other *Fusarium* spp.

Hogg et al. (2007) used the *tri5* gene based ‘TaqMan’ primers and probes developed by Strausbaugh et al. (2005) to relate 22 *Fusarium* species (including *F. pseudograminearum* and *F. culmorum*), and additional isolates from 12 fungal species, to inoculated field experiments during 2004 and 2005. The primers used in the study were from the cloned fragment of the *tri5* gene from a *F. culmorum* isolate collected near Loma, MT. The Q-PCR assay detected all of the *Fusarium* crown rot isolates, but did not amplify any of the other species evaluated. The sensitivity of the assay was greatest with *F. graminearum* with step-wise diminishment from *F. pseudograminearum* (51% of *F. graminearum*) to *F. culmorum* (15% of *F. graminearum*).

Field trials were inoculated with a *F. pseudograminearum* isolate (Hogg et al. 2007). Disease severity scores (DSS) and *Fusarium* DNA quantities were negatively correlated to grain yield and yield reductions with r values = -0.64 and -0.77, respectively. The location or length of lesion up to the fourth internode did not appear to

be correlated to DNA quantities. The authors concluded that the Q-PCR assay used had the ability to accurately estimate disease severity and subsequent yield loss from *Fusarium* crown rot infection under inoculum pressure and favorable climatic conditions for disease development.

Kohl et al. (2007) was able to use species-specific primers for various *Fusarium* spp. from Waalwijk et al. (2004) to detect DNA concentrations in wheat stubble residue in a crown rot epidemiological study extending 10 months after harvest. They reported that DNA concentrations in the residue decreased in node and internode tissue but not in stem base tissue over the 10 month duration. Hogg et al. (2010) followed this work with an epidemiological study of wheat residues from inoculated and non-inoculated plots in Montana. They used the TaqMan assay based on the trichodiene synthase gene (*tri-5*) to non-specifically detect populations of *F. culmorum*, *F. graminearum*, and *F. pseudograminearum*. They reported that *Fusarium* populations in non-inoculated residues were approximately 0.4% or 100 times less than that of inoculated residues. The authors found that *Fusarium* populations declined rapidly in the first few months following harvest.

The current practical application of Q-PCR technology would be to quantify disease from infected field samples with crown rot symptoms. If crown rot symptoms were correlated to DNA quantities, one could rapidly and accurately assess the degree of disease and causal *Fusarium* species from a Q-PCR assay. However, as previously mentioned, other crown rot Q-PCR assays utilized primers from the *tri5* gene region from Strausbaugh et al. (2005) that are not species-specific for *F. pseudograminearum* and *F. culmorum*, unlike primers developed by Williams et al. (2002) and Schilling et al. (1996).

Also, the assay developed by Hogg et al. (2007) was from field inoculated samples, not from naturally occurring populations in the field. From these studies it appears that Q-PCR is an adequate tool for assessing *Fusarium* populations. Traditional methods of plating and identification are cumbersome and require mycological expertise.

Furthermore, *Fusarium* spp. cannot be easily extracted and cultured from soil, but Q-PCR has been used to identify quantities of other soil-borne pathogens from soil samples (Okubara et al. 2005; Schroeder et al. 2006).

The DNA extraction techniques described by Strausbaugh et al. (2005) were based on fungal mycelia isolations from a kit extraction protocol dating to the early 1990s. More DNA extraction kits have been released by several manufacturers. A DNA extraction technique from a recently manufactured FastDNA<sup>®</sup> kit (MP Biomedicals, Solon, OH) was applied to DNA extraction from wheat stems (Hogg et al. 2007). However, the extraction protocol was modified significantly from the manufacturer protocol and was not successful in producing extractions in preliminary results performed with the current study. Thus other DNA extraction kits needed to be evaluated for use in extracting and amplifying DNA from wheat stems infected with crown rot.

Hogg et al. (2007) commented on the tough, dry nature of mature wheat stems. They subsequently modified the manufacturer protocol by adding a ceramic bead and soaking the tissue samples in the extraction buffer for 1 hour to better ensure tissue maceration and subsequent DNA quantities. Other plant extraction protocols use liquid nitrogen to aid in tissue maceration. These additional methods needed to be evaluated with another Mo-Bio<sup>®</sup> DNA extraction kit (Mo BIO Laboratories llc., Carlsbad, CA) to better optimize the DNA extraction process.

Hogg et al. (2007) diluted (1:10) the fungal DNA samples following extractions with the FastDNA kit. Inhibitors in the form of polysaccharides, humic and fulvic acids and colloidal substances in soils exist that could potentially interfere with Q-PCR and create false-negatives (Okubara et al. 2005). Diluting fungal DNA samples are meant to alleviate these effects. The Mo-Bio DNA extraction kit is designed for soil analysis and contains an inhibitor removal solution. As a result, samples processed with the Mo-Bio kit do not need dilution. During the DNA extraction-optimization process with the FastDNA kits, samples were diluted with water following failed Q-PCR. Hogg et al. (2007) diluted sample DNA extracted from mycelium using the FastDNA kit 1:5 and from infected wheat stems 1:10 (1 part extracted DNA: 10 parts water).

Strausbaugh et al. (2005) extracted fungal DNA from mycelium whereas Hogg et al. (2007) was the first to report fungal DNA extracted from crown rot infected wheat stems. It is accepted that extracting DNA from fungal mycelium will produce the most reliable DNA samples for amplification with PCR (Aoki and O'Donnell, 1999). However this process can be tedious. It requires materials that need to be placed on shakers for several days to grow mycelium in PDB for DNA extraction. The entire process can take a week or more for a large number of samples. Since macroconidia remain in culture from identified field isolates in SNA (Synthetischer Nährstoffärmer agar) (Nirenberg, 1976), there is a potential that they could be used as an easier medium for DNA extraction and species verification. To date there has been no attempt to extract DNA from macroconidia with the FastDNA kit from cultures of *Fusarium* spp. causing crown rot.

The objectives of this research were to: 1 - Optimize the DNA extraction process for accurate Q-PCR using two DNA extraction kits (FastDNA<sup>®</sup> kit (MP Biomedicals) and MO-BIO<sup>®</sup> kit (Ultra Clean Soil Kit); 2 - Optimize the grinding and extraction process using the preferred kit; 3 - Compare Q-PCR results with morphological results for appropriate species identification; and 4 - Correlate crown rot severity of ratings from field survey to DNA quantity amplified for *F. pseudograminearum* and *F. culmorum*.

## **MATERIALS AND METHODS**

### **Fungal DNA extraction from mycelium for positive controls**

*Fusarium pseudograminearum* and *F. culmorum* isolates collected from wheat fields in Washington state between 2000 and 2008 and previously identified using morphological characters (seven *F. culmorum* isolates and 13 *F. pseudograminearum* isolates) according to Lesley et al. (2006) were used as positive controls in all PCR and Q-PCR reactions. DNA of 20 *Fusarium* spp. isolates was extracted from mycelium. Fungi were grown in potato dextrose broth (PDB) for approximately 5 days and the mycelium extracted from the PDB by passing it through a vacuum funnel with filter paper (Whatman #4, 12.5 cm diameter). The mycelium that remained on the filter paper was placed into a 2-mL centrifuge bead beating tube containing a ceramic bead and extracted using the FastDNA extraction kit (Catalog # 6540-400 MP Biomedicals) (details below).

### **2008 Fusarium survey field sampling technique**

A field survey evaluating crown rot occurrence, severity, and *Fusarium* spp. distribution from PNW dryland wheat fields was conducted during 2008. The sampling protocol involved taking five samples each 1 m in length from five locations within a

field that were 25 m apart in a diagonal (i.e. zig zag) pattern throughout the field.

Sampling was conducted by walking 100 m into a field and taking a 1 m row of stubble at one of five locations, which defined a sample. Five samples (1 m stubble rows) were sampled per field and scored for crown rot severity. One stem from each sample was cultured on SNA media the *Fusarium* spp. were identified morphologically (Lesley et al. 2006). The application of Q-PCR as a diagnostic tool was applied to stem samples from samples of this survey to function as replications within a field.

### **DNA Extraction Experiments (EE-1 through EE-10)**

Two kits were used to extract DNA from infected plant tissue: the FastDNA Kit – Catalog # 6540-400 (MP Biomedicals<sup>®</sup>, Solon, OH) and the Ultra Clean Soil DNA Kit Catalog # 12800-100 (MO-BIO<sup>®</sup>, Carlsbad, CA). The 2008 *Fusarium* survey samples were used as the basis for sampling and analyzing DNA extraction techniques and methods from different sources of pathogen material (i.e. conidia and mature plant stems). Each DNA extraction experiment was assigned an ‘EE’ number delineating “Extraction Experiment” followed by the experiment number. There were a total of 10 extraction experiments labeled EE-1 through EE-10.

The objective of EE-1 was to evaluate the efficiency and efficacy of extracting DNA from concentrated macroconidia collected from SNA cultures with the FastDNA extraction kit. Macroconidia of each species was harvested by flushing plates with 5 mL of deionized distilled water into 50 mL test tubes (Falcon, Franklin Lakes, NJ.). Test tubes were centrifuged at 3,200 rpm for 4 minutes. Supernatant was removed with the exception of 1 mL. The 50mL tubes were vortexed for 5 s and the 1mL solution transferred to a FastDNA kit lysing tube and the extraction process was conducted as



outlined below. Homogenization of samples was done at a Fast Prep FP120 (QBiogene Inc., Carlsbad, CA.) speed = 5. All samples were amplified using conventional PCR as outlined below.

The objective of EE-2 was to evaluate the effectiveness of grinding stem tissue with liquid nitrogen prior to DNA extraction with the FastDNA kit according to Hogg et al. (2007) to enhance Q-PCR. Fields were selected that had isolates with morphological identifications from the 3 main *Fusarium* spp. identified in the survey (*F. pseudograminearum*, *F. culmorum*, and *F. crookwellense*). Cross sections 2 cm in length were cut from symptomatic wheat stems (approximately 20 mg tissue). Samples were either ground with liquid nitrogen or untreated and placed in the FastDNA kit lysing tubes to be ground in the 'Fast Prep' homogenizer. Homogenization of samples was done at a Fast Prep speed = 5. Samples were not diluted and were amplified using conventional PCR with the FPG and OPT primer sets.

Samples in EE-2 extracted with the FastDNA kit did not successfully amplify with PCR. Therefore the objective of EE-3 was to extract DNA using the Mo-Bio kit from a broader range of seven fields with the morphological occurrence of the 3 main *Fusarium* spp. (*F. pseudograminearum*, *F. culmorum*, and *F. crookwellense*). The extracted samples were amplified with Q-PCR. A 6 cm wheat stem cross section was cut into 1 cm pieces from each respective sample stem and ground finely in liquid nitrogen (15 to 21 mg tissue) then placed in the Mo-Bio kit lysing tube and carried through the DNA extraction process as outlined below. Homogenization of samples was done at a Fast Prep speed = 5. Samples were amplified with Q-PCR for the FPG and OPT primer sets.

Since Mo-Bio kit extractions had been more successful than FastDNA kit extractions, the objective of EE-4 was to compare the two kits under similar survey sample conditions with various morphological identifications of the three main *Fusarium* spp. isolated in the survey. A 5 cm cross section of stem base crown tissue was cut into 1 cm sections. Liquid nitrogen was used to grind the samples for the Mo-Bio kit extractions while bead beating by itself was used with the FastDNA kit. All samples were processed at a speed of 5 for 45s on the Fast Prep. The DNA extraction protocols for each respective kit were carried out as outlined below. Samples were amplified with Q-PCR for the FPG and OPT primer sets.

Given the only samples that amplified in EE-4 were extracted with the Mo-Bio kit, the objective of EE-5 was to evaluate the need for grinding stems with liquid nitrogen or bead beating with the Mo-Bio kit. A 9 cm symptomatic wheat stem / crown cross section was cut into 1 cm sections. For liquid nitrogen grinding treatments, stems were placed into 2 mL Eppendorf micro-centrifuge tubes and ground to a fine powder before being transferred to a Mo-Bio bead beat lysing tube containing one 1.4 mm ceramic bead from the FastDNA kit. 'Bead beat' samples were placed directly into Mo-Bio lysing tubes with one 1.4 mm ceramic bead for homogenization and extraction. For the 'soak' treatment the stems were placed into the bead beat lysing tubes with 60  $\mu$ L of S1 solution and 200  $\mu$ L of Inhibitor removal solution (IRS) for 1 hour on ice. All samples were homogenized at a Fast Prep speed = 5. Samples were analyzed with Q-PCR for the FPG and OPT primer sets.

The Mo-Bio kit produced the most consistent PCR amplification results to this point. The objective of EE-6 was to correlate extracted DNA concentrations to field

crown rot severity ratings (low, medium, and high) from eight stems from the 2008 Fusarium survey using the Mo-Bio kit. A 1 cm cross section of symptomatic wheat stem / crown tissue was cut from each wheat stem. Extractions were conducted with the Mo-Bio kit. Samples were soaked on ice in the S1 / IRS extraction buffer solution for 1 hour then homogenized in the Fast Prep machine at a speed = 6. Samples were amplified with Q-PCR with the FPG, OPT, and CRO-C (*F. crookwellense*) primer sets.

The objective of EE-7 was to further optimize the DNA extraction procedure with the Mo-Bio extraction kit to confirm optimal conditions for the addition of a 1.4 mm ceramic bead from the FastDNA kit to the Mo-Bio kit, the 1 hour soaking treatment in the S1 / IRS extraction buffer solutions on ice, and the Fast Prep speed = 5 or 6. A 15 cm cross section of symptomatic wheat stem / crown tissue was cut up into 1 cm sections and pooled into one sample from each sample. Samples were amplified with Q-PCR for the FPG and OPT primer sets.

The objective of EE-8 was to further evaluate all of the parameters involved in the extraction process with the Mo-Bio kit to confirm previous results and finalize DNA extraction optimization. Grinding and extraction treatments were the addition of a 1.4 mm ceramic bead from the FastDNA kit to the Mo-Bio kit, the 1 hour soaking treatment in the S1 / IRS extraction buffer solutions on ice, a Fast Prep speed = 5 or 6, and the addition or lack of PVPP. A total of 10 – 1 cm cross sections of symptomatic wheat stem tissue were pooled together for each extraction treatment. Samples were taken from four samples with crown rot severities ranging from low (1-3) to medium (4-6) to high (8-10) ratings. Samples were amplified with Q-PCR for the FPG and OPT primer sets.

The objective of EE-9 was to correlate the crown rot severity ratings from 24 field samples ranging in crown rot severity scores from sample ratings of 1 to 8 (with three replications in each rating category) to the DNA concentration results from Q-PCR. The extraction methods used were to grind plant tissue in liquid nitrogen, add a 1.4 mm ceramic bead, use PVPP, and homogenize in the Fast Prep at speed = 6 without soaking. DNA was extracted from 24 single stems that were 3 cm long cut into 0.5 to 1 cm cross sections. Samples were amplified with Q-PCR for the FPG and OPT primer sets.

The objective of EE-10 was to correlate crown rot severity with DNA concentration from samples extracted with the Mo-Bio kit by pooling stems within a sample, similar to the methods outlined by Hogg et al. (2007). Fields were grouped into 39 groups in a frequency histogram based on their crown rot severity. One field was selected at random from each of these 39 groups to represent 39 fields with varying degrees of crown rot severity ranging from 0.2 to 5.1. Nine additional fields were selected from a range of crown rot severity ratings according to geographic origin and morphological species identification to make 48 fields total. Three samples were randomly selected from each field and five symptomatic stems were selected from each sample for extraction. Two 3-mm sections were cut from each of the 5 stems (6 mm total) to make 30 mm per sample as outlined in Hogg et al. (2007). DNA extractions were made using the Mo-Bio extraction kit from each 30 mm of stem tissue per sample, similar to Hogg et al. (2007). There were a total of 144 DNA extractions that were conducted for the experiment. Each sample represented a replication of sorts within each field.

### **FastDNA extraction kit protocol**

The protocol for the FastDNA kit for both fungal mycelium and plant tissue was as follows. The extraction buffer consisted of 800  $\mu\text{L}$  of CLS-VF buffer and 200  $\mu\text{L}$  of PPS containing 25:24:1 phenol:chloroform:isoamyl-alcohol (pH=8.0) solutions from the kit. These were added to a 2.0 mL micro-centrifuge tube containing variable amounts of plant tissue ranging from 20 to 150 mg depending on the sample size and optimization experiment objective. A ceramic bead (1.4 mm) that was included in the kit was added to the tube and tissue maceration was conducted with the use of a Fast Prep FP120 instrument at speed 6 for 45 s. An alternative method of tissue maceration was evaluated where plant tissue was first ground in liquid nitrogen. The ground tissue (ranging from 20 to 150 mg) was added to a lysing tube containing cell lysing solution with a ceramic bead, and further ground using the Fast Prep instrument. In some optimization experiments two ceramic beads were added to the tube. Samples were centrifuged after maceration at 14,000 g for 12 minutes. A volume of 600 to 700 (approximately 650)  $\mu\text{L}$  of the supernatant was placed into a new 2 mL micro-centrifuge tube along with an equal volume (600-700  $\mu\text{L}$ ) of 'binding matrix' (provided in the kit). Polyvinylpyrrolidone (PVPP) was added to the each micro-centrifuge tube at a rate of 25 mg (50  $\mu\text{L}$ ) and inverted to mix. Samples were incubated with gentle agitation (low-speed vortex) for 5 min at room temperature. Samples were centrifuged at 14,000 g for 10 s to pellet the binding matrix, and the supernatant was discarded. A volume of 500  $\mu\text{L}$  of prepared SEWS-M wash solution (provided in the kit) was added to the micro-centrifuge tubes. SEWS-M is an ethanol based wash solution meant to further purify and release DNA bound in the silica membrane of the filter. Samples were then centrifuged at 14,000 g for

1 min and the supernatant was discarded. Samples were centrifuged again at 14,000 g for 10 s and residual liquid was removed with a pipette. DNA was eluted by gently resuspending the binding matrix in 100  $\mu$ L of DES solution and incubated for 5 min at 55  $^{\circ}$ C in a heat block or water bath. Samples were centrifuged at 14,000 g for 1 minute. The supernatant containing eluted DNA was transferred (avoiding the binding matrix) to a clean micro-centrifuge tube and stored at  $-20^{\circ}$ C. Samples extracted from wheat stem tissue were diluted 1:10 (1 part extracted DNA solution:10 parts water).

### **MO-BIO DNA extraction kit protocol**

Approximately 20 to 80 mg of plant tissue was added to a 2 mL micro-centrifuge tube (included in the kit), which contained small silicon pellets to aid in grinding. Plant tissue, which had been ground with liquid nitrogen or that was left un-ground (depending on the experiment objective), was added to the tubes. In some cases, a 1.4 mm ceramic bead from the FastDNA kit was also added to the tube. Micro-centrifuge tubes were gently vortexed to mix the tissue, grinding pellets and ceramic bead if it was added. However, this step did not fully grind tissue, but simply mixed contents for better homogenization in the Fast Prep unit. Sixty microliters of Solution S1 was added to each tube and the tube was inverted several times to mix. S1 solution was provided in the kit and contained SDS, a detergent that aids in cell lyses to break down fatty acids and lipids associated with the cell membrane of organisms. Two hundred microliters of solution IRS (Inhibitor Removal Solution) was added to the tubes, which were then secured in the Fast Prep instrument and operated at a speed of either 5 or 6 for 45 s. The (IRS) inhibitor removal solution is a proprietary agent that was designed by Mo-Bio to precipitate humic acids and other inhibitors of PCR reactions. Extraction tubes were centrifuged at 10,000

g for 30 s. A volume of 400 to 450 (425)  $\mu\text{L}$  of supernatant was transferred to a new 2 mL micro-centrifuge tube. A volume of 250  $\mu\text{L}$  of Solution S2 was added to the supernatant in the new tube and vortexed for 5 sec. Solution S2 contains a protein precipitation agent to remove contaminant proteins that reduce DNA purity and potentially inhibit PCR applications. Micro-centrifuge tubes were incubated at 4  $^{\circ}\text{C}$  for 5 minutes then centrifuged for 1 minute at 10,400 g. Supernatant ( $\sim 650\mu\text{L}$ ) was carefully transferred (avoiding the pellet) to a new 1.5 mL micro-centrifuge tube. Solution S3 was added at a volume of 1.2 mL to the supernatant and vortexed for 5 s. S3 is a DNA binding salt solution that contains silica, as DNA will bind to silica in the presence of high salt concentrations. Approximately 670  $\mu\text{L}$  of the supernatant was loaded into the spin filter micro-centrifuge tube (from the kit) and centrifuged at 10,000 g for 1 minute. The flow through was discarded and the remaining supernatant was added to the spin filter (two more cycles of 670  $\mu\text{L}$ ) and centrifuged for 1 minute at 10,000 g. This process was repeated three times. 300  $\mu\text{L}$  of Solution S4 was added to the spin filter centrifuge tube and centrifuged for 30 s at 10,000 g, then the remaining flow through was discarded. Solution S4 is an ethanol based solution used to wash DNA that is bound to the silica membrane in the filter and also removes residual salt, humic acid, and other contaminants. The tube was centrifuged again at 10,000 g for 1 minute. The spin filters were taken out of the centrifuge tubes and placed into tubes containing 25 mg of PVPP that had been prepared prior to extraction. A volume of 50  $\mu\text{L}$  of Solution S5 was added to the center of the spin filter and centrifuged at 10,000 x g for 1 minute. Solution S5 is a sterile elution buffer containing 10 mM Tris (pH = 8.0) designed to release the eluted DNA from the silica membrane and into the micro-centrifuge tube. Eluate containing

DNA and PVPP were mixed by tapping. The mixed solution was allowed to sit at room temperature for several minutes, then centrifuged at 10,000 g for 3 minutes and 50  $\mu$ L was transferred to a new sterile 0.5 mL Eppendorf micro-centrifuge tube to be used for PCR. Samples were stored at -20 C.

### **Conventional PCR amplification**

Conventional PCR was carried out in EE-1 and EE-2 samples using 15.5  $\mu$ L of molecular water, 6.0  $\mu$ L of DNA loading buffer (New England Biolabs, Ipswich, MA), 1.8  $\mu$ L of  $MgCl_2$  (New England Biolabs, Ipswich, MA), 2.0  $\mu$ L of oligonucleotides (dNTPs), 1.2  $\mu$ L of forward primer, 1.2  $\mu$ L of reverse primer, and 0.3  $\mu$ L of Taq polymerase (New England Biolabs, Ipswich, MA). Two microliters of template DNA was added to the reaction. The PCR (MJ Research, Watertown, MA) program included an initial denaturing temperature of 94 °C for 3 minutes. Each cycle included a denaturing temperature of 92 °C for 45 seconds, an annealing temperature of 60 °C for 45 seconds, and an extension temperature of 72 °C for 1 minute. The PCR product was visualized on a 1% agarose gel, stained with Ethidium bromide (EtBr).

### **Real Time Q-PCR amplification**

Fungal DNA from crown and sub-crown tissues were assayed using the FastStart DNA Master SYBR Green I fluorescence (Roche Applied Science, Indianapolis, Ind.) and a Light Cycler thermocycler (Roche Applied Science, Indianapolis, Ind.) according to Okubara et al. (2005). Published PCR primers (Schilling et al. 1996; Nicholson et al. 1998; Aoki and O'Donnell, 1999; Williams et al. 2002) were evaluated for species-specificity using 19 PNW isolates of *F. culmorum*, *F. pseudograminearum*, and *F. crookwellense*. The following primers were used: OPT18 (SCAR2-35: 5'-



GATGCCAGAC-3' (Shilling et al. 1996); OP-T01 (Fc01) (F – ATGGTGAAGTTCGTCGTGGC and R - CCCTTCTTACGCCAATCTCG) for *F. culmorum* (Nicholson et al. 1998); Fp1-1 (5'-CGGGGTAGTTTCACATTTC(C/T)G – 3') and Fp1-2 (5' – GAGAATGTGATGA(C/G)GACAATA – 3') for *F. pseudograminearum* (Aoki and O'Donnell, 1999); FPG-F (5' – GTCGCCGTCCTATC – 3') and FPG-R (5' – CACTTTTATCTCTGGTTGCAG – 3') for *F. pseudograminearum* ; CRO-C-F (5' – TATTGGGATCTATCCAAGTCTTGT – 3') and CRO-C-R (5' – AAGCAGGAAACAGAAACCCTTTCC – 3') for *F. crookwellense* (Williams et al. 2002).

PCR reactions were conducted according to Okubara et al. (2007) and consisted of 2.0 µL of DNA extract, 0.8 µL of each primer, and 4 mmol/L MgCl<sub>2</sub> in 20 µL. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s for annealing, and 72 °C for 10 s for extension. Annealing temperatures were 65 °C. Melting profiles of the amplicons were generated by increasing the reaction temperature from 70 °C to 96 °C in 0.1 °C increments. Fluorescence was monitored after each annealing step and every 0.4 °C to 0.5 °C during melting. Genomic DNA extracted from *F. culmorum* (F-070373) and *F. pseudograminearum* (F-100413) isolates served as positive controls. Negative controls consisted of the same reaction ingredients but included nanopure water instead of template DNA.

### **Generation of standard curves**

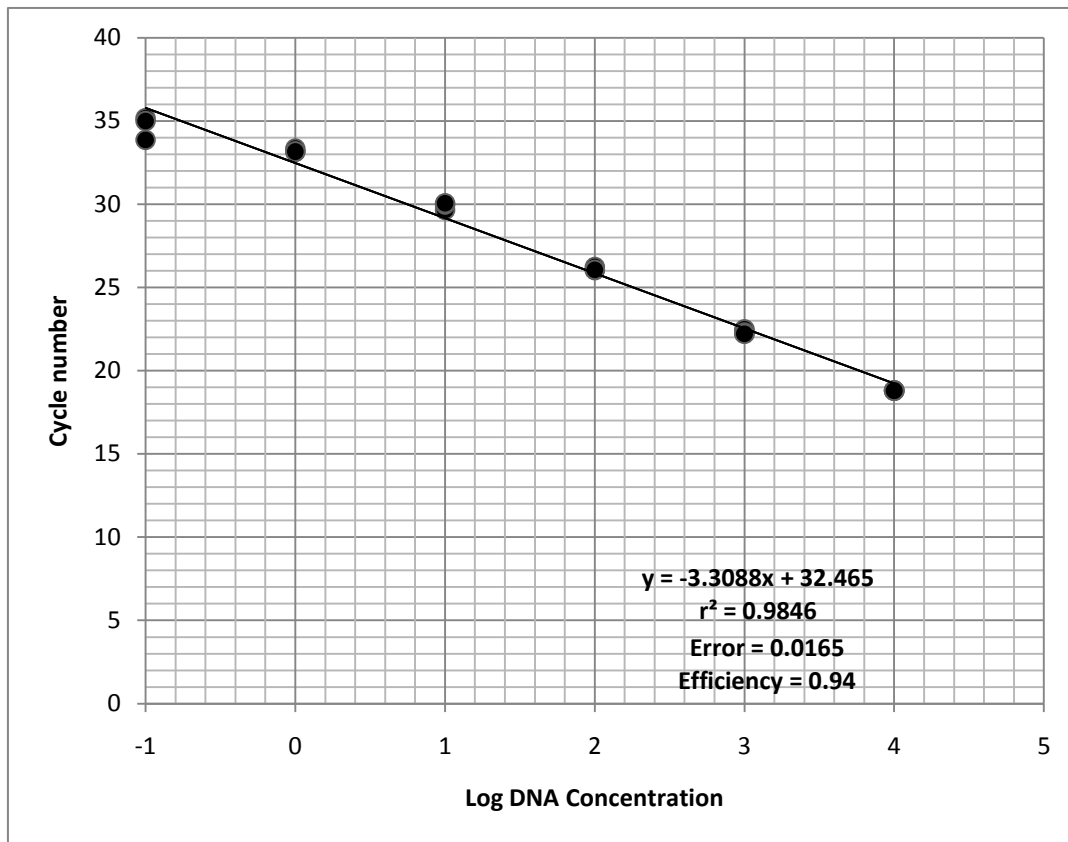
DNA was quantified according to standard curves developed on the basis of known concentrations of DNA extracted from isolates from the PNW *Fusarium* spp. collection. DNA standards were quantified prior to Q-PCR runs using fluorometry in a 200- $\mu$ L volume in 96-well plates using Hoescht 33258 dye and a Safire fluorescence plate reader (TECAN Research, Triangle Park, NC). DNA concentrations were standardized to  $\sim 5$  ng/  $\mu$ L. Standard curves were developed for FPG and OPT primers sets using positive controls for the *F. pseudograminearum* and *F. culmorum* isolates, respectively, five 10-fold dilution DNA standards ranging from 500 fg/  $\mu$ L to 10 ng/  $\mu$ L. Data was analysed with LightCycler Software version 3. The data was used to generate standard curves and standard errors and efficiencies of each DNA concentration was calculated. Results for the range of DNA quantities were plotted.

### **Data Analysis**

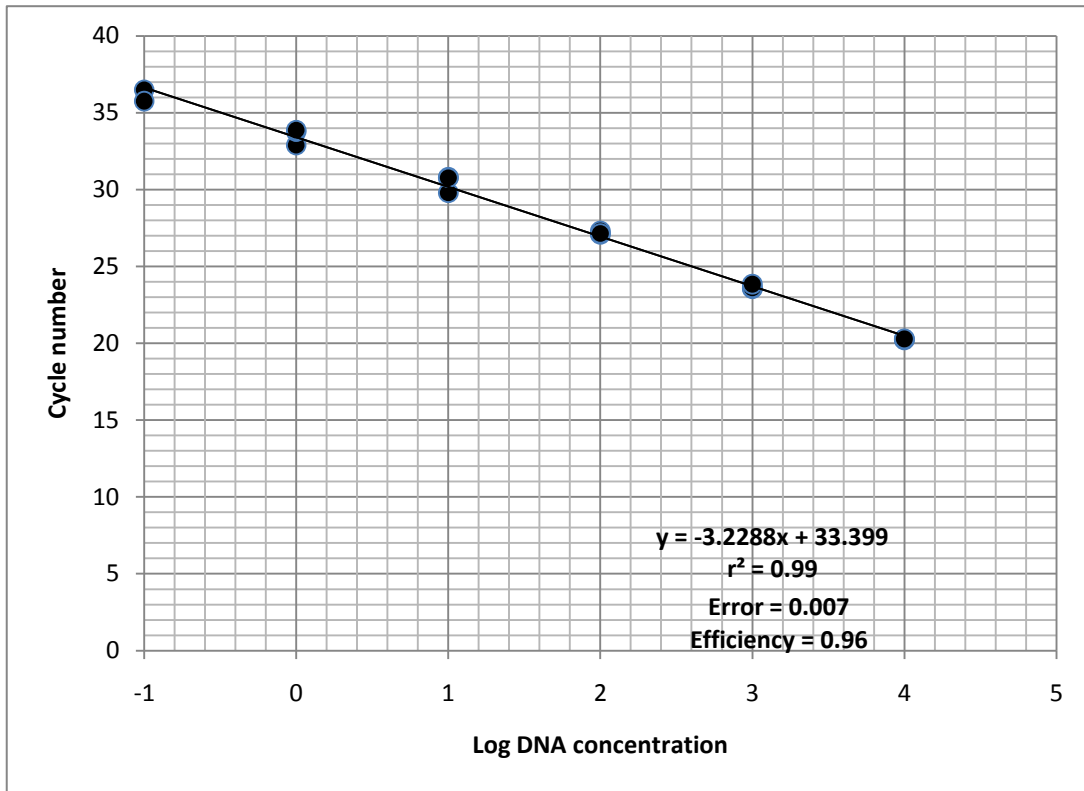
Treatments were arranged with fields as a fixed effect and independent variables were fields (48) and samples treated as replications (3). The dependent variables were crown rot severity score and DNA concentration. Three samples were randomly selected within each field and treated as the replication effect. Duncan multiple range test least significant difference (LSD) mean separation and ANOVA was performed with SAS statistical software (Version 6.12; SAS Institute Inc., Cary NC).

## RESULTS

A single primer set, 'FPG' and 'OPT', were selected and standard curves were generated for *F. pseudograminearum* and *F. culmorum* with each respective primer set. Standard curves were successfully generated using positive controls of each respective species with reproducible amplification and correlation efficiency values (Figures 1 and 2). The slopes for each species were similar with amplification efficiencies of 94% for the *F. pseudograminearum* positive control amplified with the 'FPG' primer set and 96% for the *F. culmorum* positive control amplified with the 'OPT' primer set.



**Figure 1.** Standard curves generated using Q-PCR with the 'FPG' primers developed by Williams et al. (2002) for identification and quantification.



**Figure 2.** Standard curves generated using Q-PCR with the ‘OPT’ primers developed by Schilling et al. (1996) for identification and quantification.

When the conidial samples were extracted using the FastDNA kit in the first experiment, there was positive amplification with four of the eight samples with the ‘OPT’ primers. Three of the four ‘OPT’ amplifications for *F. culmorum* agreed with morphological *F. culmorum* identifications, whereas one was morphologically identified as *F. pseudograminearum* in culture (Table 1). None of the conidial extraction samples that were identified as *F. pseudograminearum* amplified with the ‘FPG’ primer set. The overall amplification frequency from the conidial extraction was 50%.

Preliminary experiments showed that DNA extractions and subsequent Q-PCR from crown rot infected wheat stems were more effective using the Mo-Bio extraction kit rather than the FastDNA kit (data not shown). DNA extraction for EE-2 from infected

plant stems was performed using the FastDNA kit (Table 2). Grinding plant tissue with liquid nitrogen was compared to bead beating treatments using the addition of a second ceramic bead according to Hogg et al. (2007). Two of eight samples from sample 170 amplified with the 'FPG' primer set using conventional PCR for both grinding treatments when DNA was not diluted. When these samples were diluted (1:10), six of the eight samples amplified with conventional PCR; however, none of these samples amplified with Q-PCR, even following dilution (data not shown).

When DNA extraction was performed using the Mo-Bio kit in EE-3 (utilizing the liquid nitrogen grinding technique prior to homogenization), 6 of 8 samples amplified with Q-PCR, 3 with the 'OPT', and 3 with the 'FPG' primer sets, respectively (Table 3). It is important to note that morphological identifications and DNA extractions were carried out on separate stems within a given sample. Two separate stems within samples 215 and 170 that were identified as *F. pseudograminearum* amplified with the 'OPT' primer set. Likewise, two of the stems within samples that were identified as *F. culmorum* amplified with the 'FPG' primer set. One sample identified as *F. crookwellense* amplified with the 'FPG' primer set and one sample identified as *F. pseudograminearum* amplified with the 'FPG' primer set.

**Table 1.** Results from the *Fusarium* spp. conidial DNA extraction experiment (EE-1) using the FastDNA extraction kit.

Extract Sample <sup>1</sup>	Sample <sup>2</sup>	Field <sup>3</sup>	Location <sup>4</sup>	Morphological species ID in culture <sup>5</sup>	Conventional PCR Product <sup>6</sup>
1	11	3	Pendleton, OR	<i>F. pseudograminearum</i>	-----
2	41	9	Moro, OR	<i>F. culmorum</i>	OPT <sup>7</sup>
3	115	23	Pomeroy, WA	<i>F. pseudograminearum</i>	-----
4	125	25	Waitsburg, WA	<i>F. culmorum</i>	OPT
5	194	39	Odessa, WA	<i>F. pseudograminearum</i>	OPT
6	241	49	Coulee City, WA	<i>F. culmorum</i>	OPT
7	245	49	Coulee City, WA	<i>F. pseudograminearum</i>	-----
8	261	53	Douglas Co. WA	<i>F. culmorum</i>	-----

<sup>1</sup> Extract sample represents the chronological order of samples for experiment EE-1.

<sup>2</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field.

<sup>3</sup> Field number represents the field number that was surveyed in 2008.

<sup>4</sup> The location represents the nearest town location of the field surveyed during the 2008 Fusarium survey.

<sup>5</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>6</sup> Samples were analyzed with conventional PCR and products. Each cycle included a denaturing temperature of 92 °C for 45 seconds, an annealing temperature of 60 °C for 45 seconds, and an extension temperature of 72 °C for 1 minute. The PCR product was visualized on a 1% agarose gel, stained with Ethidium bromide (EtBr).

<sup>7</sup> Primers analysed for EE-1 were FPG for *F. pseudograminearum* and OPT for *F. culmorum*.

**Table 2.** Results from the DNA extraction experiment (EE-2) using the FastDNA kit comparing grinding techniques for wheat stem/crown tissue extractions.

Extract Sample <sup>1</sup>	Sample <sup>2</sup>	Field <sup>3</sup>	Tissue weight (mg) <sup>4</sup>	Grinding Method <sup>5</sup>	Morphological species ID in culture <sup>6</sup>	Conv. PCR Product <sup>7</sup>
1	169	34	21	Liquid nitrogen	<i>F. pseudograminearum</i>	----
2	169	34	21	Bead beat w/out LN	<i>F. pseudograminearum</i>	----
3	170	34	16	Liquid nitrogen	<i>F. pseudograminearum</i>	<sup>8</sup> FPG
4	170	34	22	Bead beat w/out LN	<i>F. pseudograminearum</i>	FPG
5	172	35	15	Liquid nitrogen	<i>F. culmorum</i>	-----
6	172	35	10	Bead beat w/out LN	<i>F. culmorum</i>	-----
7	465	93	20	Liquid nitrogen	<i>F. crookwellense</i>	-----
8	465	93	16	Bead beat w/out LN	<i>F. crookwellense</i>	<sup>8</sup> OPT

<sup>1</sup> Extract sample represents the chronological order of samples for experiment EE-2.

<sup>2</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field.

<sup>3</sup> Field number represents the field number that was surveyed in 2008. Samples were taken from fields 34 and 35 near Connell, WA, and field 93 in Douglas Co., WA.

<sup>4</sup> Tissue was weighed after grinding for liquid nitrogen treatments and prior to placement in the lysing tubes without grinding for bead beat treatments. Numbers represent milligrams of tissue.

<sup>5</sup> Treatments were imposed on wheat stems by grinding into a fine powder with liquid nitrogen prior to placement into lysing tubes prior to grinding with the Fast Prep. Bead beat samples were not ground with liquid nitrogen but placed into the lysing tubes with an extra ceramic bead prior to grinding in the Fast Prep.

<sup>6</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>7</sup> Samples were analyzed with conventional PCR and products. Each cycle included a denaturing temperature of 92 °C for 45 seconds, an annealing temperature of 60 °C for 45 seconds, and an extension temperature of 72 °C for 1 minute. The PCR product was visualized on a 1% agarose gel, stained with Ethidium bromide (EtBr).

<sup>8</sup> Primers analysed for EE-2 were FPG to identify *F. pseudograminearum* and OPT to identify *F. culmorum*.

**Table 3.** DNA extraction experiment (EE-3) from various fields and samples showing significant levels of crown rot where different species were isolated using the Mo-Bio Ultra Clean Soil kit and Q-PCR analysis of DNA.

Extract Sample <sup>1</sup>	Sample <sup>2</sup>	Field <sup>3</sup>	Morphological species ID in culture <sup>4</sup>	Quantitative PCR Product <sup>5</sup>
1	169	34	<i>F. pseudograminearum</i>	-----
2	170	34	<i>F. pseudograminearum</i>	<sup>6</sup> OPT
3	172	35	<i>F. culmorum</i>	<sup>6</sup> FPG
4	465	93	<i>F. crookwellense</i>	FPG
5	23	5	<i>F. pseudograminearum</i>	-----
6	115	23	<i>F. pseudograminearum</i>	FPG
7	215	43	<i>F. pseudograminearum</i>	OPT
8	234	47	<i>F. culmorum</i>	FPG

<sup>1</sup> Extract sample represents the chronological order of samples for experiment EE-3.

<sup>2</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field.

<sup>3</sup> Field number represents the field number that was surveyed in 2008. Samples were taken from field 5 in Moro Co. northern OR, field 23 near Pomeroy, WA, fields 34 and 35 near Connell, WA, field 43 near St. John WA, field 47 near Creston WA, and field 93 in Douglas Co., WA.

<sup>4</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>5</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension.

<sup>6</sup> ‘OPT’ primers correspond to the identification of *F. culmorum*; ‘FPG’ primers correspond to the presence of *F. pseudograminearum*.



In EE-4, the experiment comparing the FastDNA and Mo-Bio extraction kits, all of the Mo-Bio kit extractions amplified, but none of the extractions from the FastDNA kit amplified by Q-PCR (Table 4). All of the samples that amplified did so with the ‘FPG’ primer set. The morphological fungal identifications did not coincide with the Q-PCR amplifications in this experiment. The FastDNA extractions were later diluted 1:10 and still did not result in positive Q-PCR amplifications. Results showed that the Mo-Bio kits allowed for more efficient extraction of DNA with more consistent Q-PCR amplification over several experiments.

To further evaluate the Mo-Bio kit for extracting DNA from infected wheat stems for Q-PCR amplification, DNA extraction efficiency treatments with bead beating, liquid nitrogen grinding, and soaking treatments were compared as stand-alone treatments in EE-5. Generally, bead beating and liquid nitrogen grinding combined resulted in the greatest amount of DNA (Table 5), with the exception of the bead beat treatment for sample 115, which had the greatest DNA concentration. The two grinding treatments were not significantly different from each other across treatments. All of the treatments amplified with the ‘FPG’ primer set regardless of the morphological identification of *F. pseudograminearum* or *F. culmorum* from other stems within those samples. The exception was that of sample 115 control treatment without any soaking or bead beating, which had been morphologically identified as *F. pseudograminearum* and amplified with the ‘OPT’ primer set (amplifying *F. culmorum* according to Shilling et al. 1996).

**Table 4.** Comparison of DNA extraction with the Mo-Bio and FastDNA extraction kits (EE-4) for 12 field samples identified morphologically with various *Fusarium* spp.

Extract Sample <sup>1</sup>	Sample <sup>2</sup>	Extraction Kit <sup>3</sup>	Weight (mg) tissue <sup>4</sup>	Morphological identification in culture <sup>5</sup>	Q-PCR product <sup>6</sup>	CP value <sup>7</sup>
1	172	Mo-Bio	77	<i>F. culmorum</i>	FPG	33.7
2	115	Mo-Bio	89	<i>F. pseudograminearum</i>	FPG	26.3
3	215	Mo-Bio	107	<i>F. pseudograminearum</i>	FPG	34.3
4	234	Mo-Bio	123	<i>F. culmorum</i>	FPG	39.7
5	169	Mo-Bio	67	<i>F. pseudograminearum</i>	FPG	34.1
6	465	Mo-Bio	144	<i>F. crookwellense</i>	-----	
7	172	FastDNA	74	<i>F. culmorum</i>	-----	
8	115	FastDNA	89	<i>F. pseudograminearum</i>	-----	
9	215	FastDNA	108	<i>F. pseudograminearum</i>	-----	
10	234	FastDNA	133	<i>F. culmorum</i>	-----	
11	169	FastDNA	69	<i>F. pseudograminearum</i>	-----	
12	465	FastDNA	133	<i>F. crookwellense</i>	-----	
13	Positive	Control	-	<i>F. pseudograminearum</i>	FPG	22.9

<sup>1</sup> Extract sample number represents the chronological order of extraction samples.

<sup>2</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field.

<sup>3</sup> Extractions were made with the Mo-Bio kit or FastDNA kit.

<sup>4</sup> Tissue was weighed after grinding for liquid nitrogen treatments and prior to placement in the lysing tubes without grinding for bead beat treatments. Numbers represent milligrams of tissue.

<sup>5</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>6</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension. ‘FPG’ primers correspond to the presence of *F. pseudograminearum*.

<sup>7</sup> CP Value = crossing point value and represents the amplification cycle when DNA quantities increase above a threshold.

**Table 5.** Evaluation of bead beating and liquid nitrogen grinding of mature wheat stems to extract DNA using the Mo-Bio extraction kit (EE-5).

Extract Sample <sup>1</sup>	Sample <sup>2</sup>	Grinding Method <sup>3</sup>	Morphological identification in culture <sup>4</sup>	Q-PCR product <sup>5</sup>	DNA Conc. <sup>6</sup>
1	115	Bead beat	<i>F. pseudograminearum</i>	FPG	25
2	115	Liquid N	<i>F. pseudograminearum</i>	FPG	1.6
3	115	Soak	<i>F. pseudograminearum</i>	FPG	2.1
4	115	-	<i>F. pseudograminearum</i>	OPT	4.8
5	172	Bead beat	<i>F. culmorum</i>	FPG	4.5
6	172	Liquid N	<i>F. culmorum</i>	FPG	7.0
7	172	Soak	<i>F. culmorum</i>	FPG	3.2
8	172	-	<i>F. culmorum</i>	-----	-----
9	215	Bead beat	<i>F. pseudograminearum</i>	FPG	12.3
10	215	Liquid N	<i>F. pseudograminearum</i>	FPG	12.3
11	215	Soak	<i>F. pseudograminearum</i>	FPG	24.6
12	215	-	<i>F. pseudograminearum</i>	FPG	8.3

<sup>1</sup> Extract sample number represents the chronological order of extraction samples.

<sup>2</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field. Sample 115 came from field 23 near Pomeroy WA, sample 172 came from field 35 near Connell, WA, and sample 215 came from field a field near St. John, WA.

<sup>3</sup> Grinding methods included grinding with liquid nitrogen prior to bead beating with the Fast Prep homogenizer, bead beating alone, and soaking with bead beating.

<sup>4</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>5</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension. 'OPT' primers correspond to the identification of *F. culmorum*; 'FPG' primers correspond to the presence of *F. pseudograminearum*.

<sup>6</sup> Positive control contained 1 ng template DNA. Data units are in pg.

A small preliminary experiment (EE-6) was conducted in an attempt to correlate crown rot severity ratings and DNA concentrations from infected stems. Unfortunately there was not any direct correlation that could be drawn between crown rot severity ratings and DNA concentrations (Table 6). Those with the lowest crown rot severity ratings and identified as *F. pseudograminearum* had the greatest DNA concentrations with the 'FPG' primer set. Q-PCR was not completely correlated to the morphological species identification. Although two samples (treatments 1 and 3) morphologically identified as *F. pseudograminearum* amplified with the FPG primer set, those that were morphologically identified as *F. culmorum* (treatment 6) and *F. crookwellense* (treatment 8) also amplified with the FPG primer set (Table 6).

In the experiment EE-7, the Fast Prep grinding speed and sample preparation (soaking and grinding treatments with an extra ceramic bead) were compared to further optimize DNA extractions. Soaking combined with bead beating with the addition of a ceramic bead (from the FastDNA kit) to the Mo-Bio kit enhanced the DNA extraction. However, when bead beating and soaking treatments were combined with a Fast Prep speed of 6, the DNA concentrations dropped off substantially (Table 7). It was postulated that DNA concentrations declined at a Fast Prep speed of 6 due to over-maceration of plant tissue and shearing of fungal DNA. DNA quality was not evaluated on a gel.

EE-8 was conducted to evaluate sample preparation with liquid nitrogen grinding, soaking in the extraction buffer, the addition of a ceramic bead, the need for the addition of PVPP to the extraction process, and the Fast Prep speed with the Mo-Bio kit. The optimal treatments were grinding with liquid nitrogen followed by soaking for 1 hour in

the extraction buffer solutions (60 µL of S1 and 200 µL of IRS), the addition of a ceramic bead and a Fast Prep speed of 5 (Table 7).

**Table 6.** DNA extractions from various samples within fields from the 2008 Fusarium survey samples with the Mo-Bio DNA extraction kit to correlate DNA quantity to crown rot severity ratings (EE-6).

Extract Sample <sup>1</sup>	Field <sup>2</sup>	Sample <sup>3</sup>	(0 to 10) Rating category <sup>4</sup>	Morphological species identification from culture <sup>5</sup>	Q-PCR Results (DNA conc.) <sup>6</sup>
1	39	192	Low (1-3)	<i>F. pseudograminearum</i>	(FPG) 24.8
2	51	254	Low (1-3)	<i>F. culmorum</i>	-----
3	13	65	Medium (3-6)	<i>F. pseudograminearum</i>	(FPG) 22.4
4	35	173	Medium (3-6)	<i>F. culmorum</i>	-----
5	38	190	High (6-10)	<i>F. pseudograminearum</i>	-----
6	62	310	High (6-10)	<i>F. culmorum</i>	(FPG) 18.7
7	93	465	Medium (3-6)	<i>F. crookwellense</i>	-----
8	95	474	High (6-10)	<i>F. crookwellense</i>	(FPG) 4.09

<sup>1</sup> Extract sample represents the chronological order of samples for experiment EE-3.

<sup>2</sup> Field number represents the field number that was surveyed in 2008. Samples were taken from field 13 in Moro Co. northern OR, field 35 near Connell, WA, fields 38 and 39 near Odessa, WA, fields 51 and 93 near Mansfield, WA, field 62 near Quincy (irrigated field), and field 95 near Pullman, WA.

<sup>3</sup> Sample represents a stem sample taken from one of five 1-m samples taken from within a field.

<sup>4</sup> Samples were visually rated for crown rot severity on 0 to 10 scale (10 = heavy disease) and placed into three rating categories: Low (rating = 1 to 3), Medium (rating = 3 to 6), and High (rating = 6 to 10).

<sup>5</sup> Morphological species identifications were conducted by culturing on SNA media and identifying the respective species based on conidial morphology according to Lesley et al. (2006).

<sup>6</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension. 'OPT' primers correspond to the identification of *F. culmorum*; 'FPG' primers correspond to the presence of *F. pseudograminearum*. Data units are in pg.

**Table 7.** Comparison of sample preparation (EE-8) with liquid nitrogen grinding, soaking, bead beating, the addition of PVPP, and the Fast Prep speed to optimize DNA extractions from 2008 Fusarium Survey wheat stem samples infected with crown rot.

Field <sup>1</sup>	Grinding method <sup>2</sup>	Tissue Wt. (mg) <sup>3</sup>	CP Value <sup>4</sup>	DNA conc. per mg tissue <sup>5</sup>
40	Liquid nitrogen + Soaking + Bead Beating	22	28.53	5.045
40	Liquid nitrogen + Bead Beating	20	31.31	0.960
40	Bead beating	20	31.55	0.825
40	Liquid nitrogen (with PVPP standard)	14	30.83	1.843
40	Liquid nitrogen + Bead beating (without PVPP)	20	33.87	0.200
23	Liquid nitrogen + Bead beating (Fast Prep Speed 5)	19	26.89	16.36
23	Liquid nitrogen + Bead beating (Fast Prep Speed 6)	18	28.16	7.778
---	Positive Control	---	24.94	-----

<sup>1</sup> Field represents the field number that was surveyed in 2008. Samples were taken from field 40 near Lind, WA and field 23 near St. John, WA.

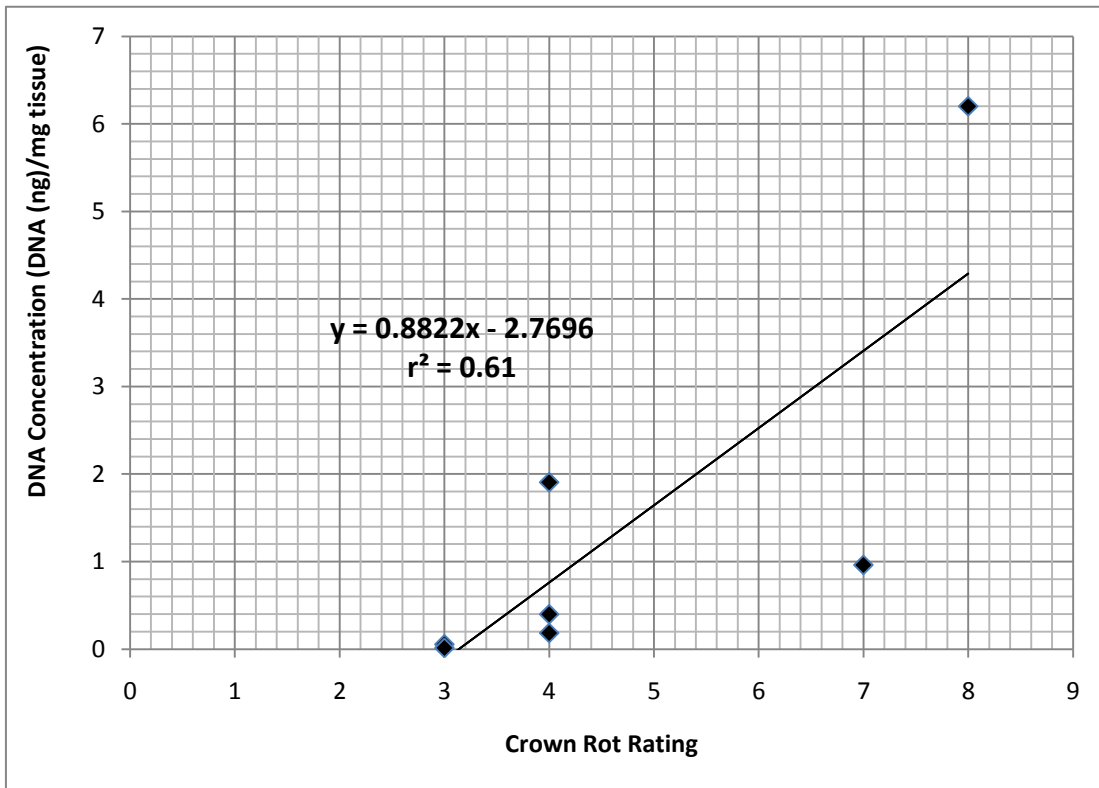
<sup>2</sup> Grinding methods included grinding with liquid nitrogen prior to bead beating with the Fast Prep homogenizer, bead beating alone, and soaking with bead beating. The Fast Prep homogenization speed was set at 5 unless otherwise indicated (field 23 samples).

<sup>3</sup> Tissue was weighed after grinding for liquid nitrogen treatments and prior to placement in the lysing tubes without grinding for bead beat treatments. Numbers represent milligrams of tissue.

<sup>4</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension. ‘FPG’ primers were used to analyse these samples for the presence of *F. pseudograminearum*. The CP value represents the crossing point value or the number of cycles where DNA was detected above a threshold.

<sup>5</sup> DNA concentration is expressed in pg DNA per mg tissue.

In an experiment to further evaluate the correlation of crown rot severity ratings to DNA concentrations (EE-9), 24 survey field sample samples were taken from single stems 3 cm in length. There were seven positive amplifications for the 'FPG' primer set from the 24 samples. Crown rot severity ratings for the seven amplifications were positively correlated to DNA concentrations with an  $r^2$  value of 0.61 for the seven samples that showed amplification (Figure 3).



**Figure 3.** Regression ( $r^2=0.61$ ;  $p<0.0001$ ) relationship between DNA concentration and crown rot rating, using single wheat stem extractions with the Mo-Bio kit and the 'FPG' primer set (EE-9).

Results from EE-10 evaluating the correlation of DNA concentration to visual crown rot severity ratings showed 74 (51%) amplifications with the ‘FPG’ primers and 30 (20%) amplifications with the ‘OPT’ primers (Table 8), while 16 (11%) sample stem samples were amplified with both primer sets (FPG and OPT). There were 16 sample DNA samples that amplified for both the FPG and OPT primer sets. Although regression analysis indicated a negative correlation between the DNA concentrations of the species within a stem ( $r^2=-0.14$ ;  $P=0.60$ ), it was not significant. There was a trend for one species to dominate in the DNA concentrations in these extracted samples that amplified with both primer sets. There were significant differences between fields for the 74 FPG amplifications ( $P<0.0001$ ) and crown rot ratings ( $P<0.0001$ ) but not for the 30 OPT amplifications ( $P=0.457$ ).

**Table 8.** Results of amplification with ‘FPG’ and ‘OPT’ primers (EE-10) from 144 DNA extractions made from 48 fields and the respective morphological identifications (*Fusarium* spp.) of the amplified samples.

Primer <sup>1</sup>	Number Amplified <sup>2</sup>	Percent of each amplified sample morphologically identified as:	
		<i>F. pseudograminearum</i> <sup>3</sup>	<i>F. culmorum</i> <sup>3</sup>
FPG	74	50%	28%
OPT	30	43%	40%

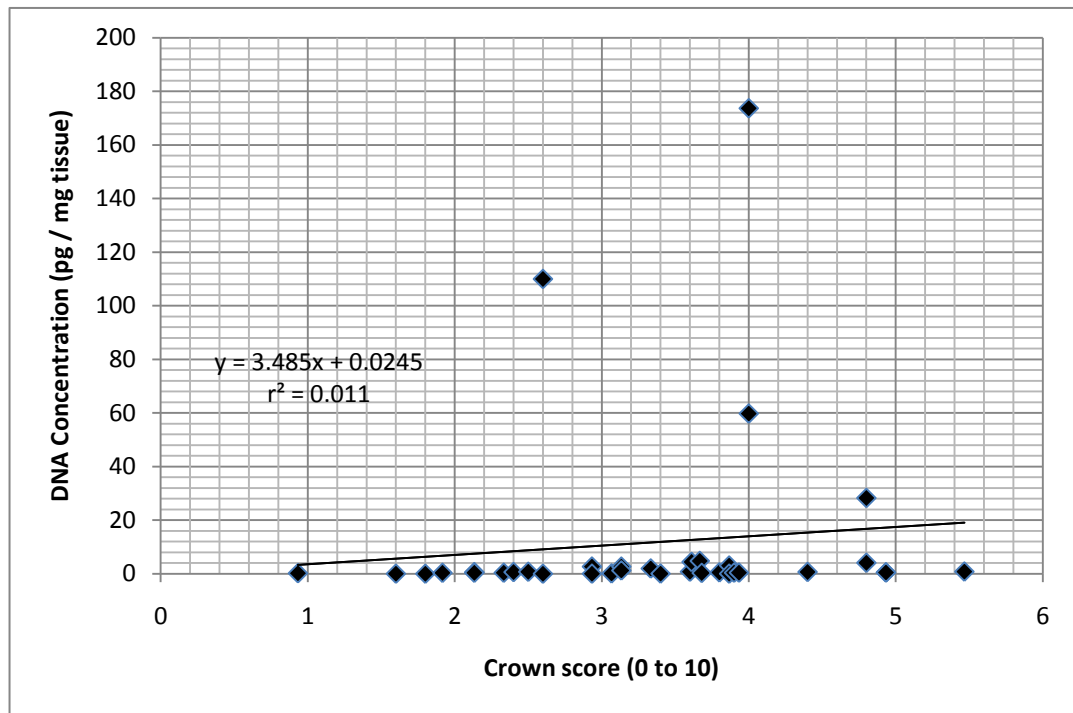
<sup>1</sup> Samples were analyzed with real-time Q-PCR. Amplification was performed at 95 °C for 10 minutes, up to 50 cycles at 95 °C for 10 s for denaturing, 5 s at 65 °C for annealing, and 72 °C for 10 s for extension. ‘OPT’ primers correspond to the identification of *F. culmorum*; ‘FPG’ primers correspond to the presence of *F. pseudograminearum*.

<sup>2</sup> Number amplified represents the number of samples out of 144 total extractions that amplified with each respective primer set.

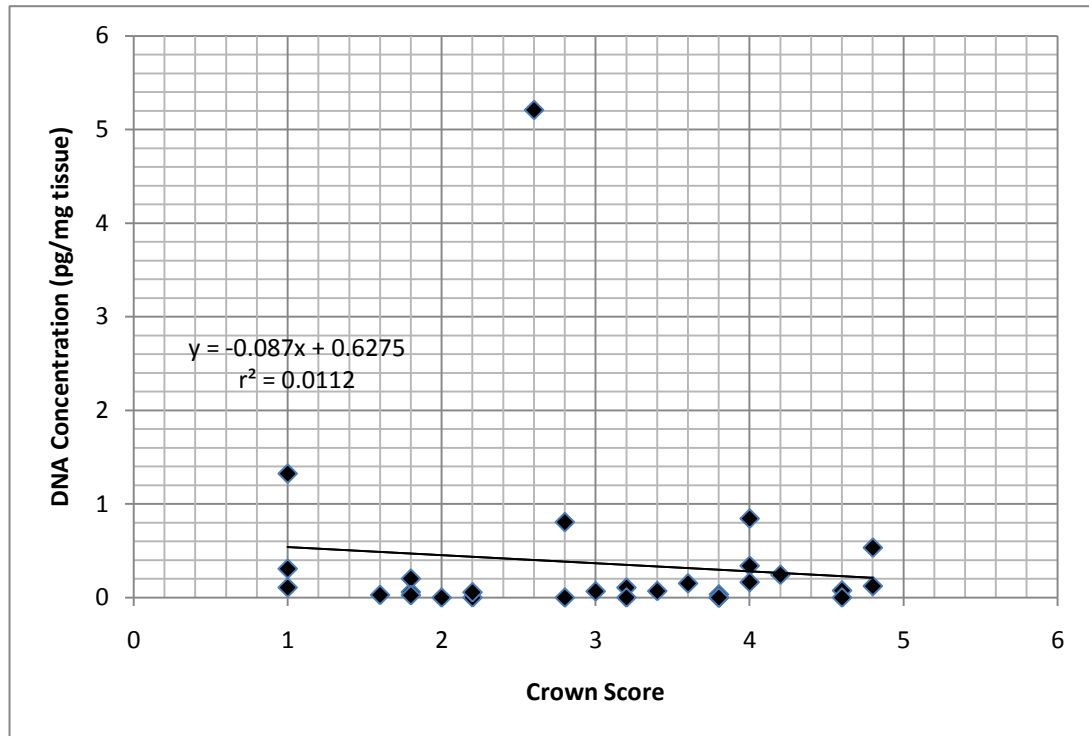
<sup>3</sup> Numbers represent the proportions of morphological identifications cultured on SNA and identified according to Lesley et al. (2006) for each respective species. Each morphologically identified stem was taken from the same field sample but from a different stem than that amplified with Q-PCR.



Of the 74 amplifications with the ‘FPG’ primers, 50% of the isolations of separate stems within those samples were identified as *F. pseudograminearum* and 28% were identified as *F. culmorum*. Of the 30 amplifications with the ‘OPT’ primers, 43% of the isolations from separate stems within those samples were identified as *F. pseudograminearum*, whereas 40% had been identified as *F. culmorum* (Table 8). In this experiment there was no significant correlation between crown rot severity and Q-PCR DNA concentrations for the amplifications of either the FPG or OPT primers (Figures 4 and 5).



**Figure 4.** Relationship between DNA concentration and crown rot severity score, from 144 DNA extractions (EE-10) made with the Mo-Bio Ultra Clean Soil extraction kit and Q-PCR with the ‘FPG’ primers.



**Figure 5.** Relationship between DNA concentration and crown rot severity score, from 144 DNA extractions (EE-10) made with the Mo-Bio Ultra Clean Soil extraction kit and Q-PCR with the ‘OPT’ primers.

## DISCUSSION

Our results are the first to use species-specific Q-PCR assays to quantify DNA in wheat stems infected with crown rot pathogens under natural conditions from a region-wide PNW crown rot survey. Hogg et al. (2007) and Strausbaugh et al. (2005) used non-species-specific ‘TaqMan’ based primers from the tri-5 genetic region to amplify *F. graminearum*, *F. pseudograminearum*, and *F. culmorum*. Although DNA was successfully quantified from naturally occurring field residues, there has not been any report of Q-PCR DNA quantities from naturally occurring fields being correlated to disease severity ratings or indexes (Strausbaugh et al. 2005; Hogg et al. 2007). The only reports of correlations between crown rot severity scores and DNA concentrations have been in inoculated field and greenhouse experiments (Strausbaugh et al. 2005; Hogg et al. 2007).

Others have reported difficulty in finding associations between crown rot severity and DNA quantities (Nicholson et al. 1998). Nicholson et al. (1998) reported that although plants receiving more inoculum tended to have more disease, visual assessments were not always an accurate indicator of inoculum levels. The authors concluded that competitive PCR revealed a clearer measure of inoculum concentration.

Strausbaugh et al. (2005) detected DNA through Q-PCR in roots highly infected with *F. culmorum* (67% isolation frequency) under natural levels of inoculum in field conditions. However, DNA concentrations were not correlated to a disease severity index. Strausbaugh et al. (2005) did report a correlation between percentage of infected roots and Q-PCR DNA concentrations in inoculated greenhouse experiments.

Hogg et al. (2007) found correlations between a calculated field disease severity score (DSS) from inoculated field plots and Q-PCR DNA concentrations. The authors attributed that the success of this correlation was due to the highly virulent *F. pseudograminearum* inoculum source in inoculated field experiments. Furthermore they reported that the relationship between DNA quantities of *Fusarium* spp. and traditional FCR disease measures was unstable over the 2 years of the study. The instability across years was attributed to low disease intensity in 2005 (the second year) from cooler and wetter climatic conditions and over-inflated disease severity ratings. The authors concluded that Q-PCR can be an effective method for assessing crown rot disease severity in mature wheat stems, but only under certain conditions that increase disease severity and symptoms. The authors noted that under conditions of low environmental stress, low pathogen populations, and less disease development the assay was not an improvement over traditional methods of culturing and isolation (Hogg et al. 2007). Hogg et al. (2010) found Q-PCR quantities to be significantly less, approximately 0.4%, from natural field inoculation compared to artificial inoculation with *F. pseudograminearum*.

In the current study we used Q-PCR and species-specific primers to quantify DNA from infected wheat stems collected from a range of crown rot severity ratings under natural field inoculum conditions. Our assays were able to successfully detect both *F. pseudograminearum* and *F. culmorum* at a rate of 49% and 20%, respectively for each species, using the Mo-Bio DNA extraction kit. DNA quantifications were not directly related to morphological identifications for each species from each respective field collection location across experiments. In this study, DNA concentration was not

correlated to crown rot severity ratings in the evaluation of 48 fields involving 144 DNA extractions. This could potentially be due to the nature of infection under natural inoculum pressure, as others have had difficulty correlating disease severity to DNA concentrations under natural conditions (Strausbaugh et al. 2005).

It is likely that the morphological identifications were not correlated to species-specific primer DNA amplifications from infected wheat stems for several reasons. Firstly, the morphological identifications and DNA extractions were made from separate stems within the same field or sample in these extraction experiments. There were fields in the survey where more than one *Fusarium* spp. occurred within the same field. It is likely that more than one species occurred in separate stems within a given field or sample. The fact that 20% of the amplified samples in EE-10 from within 48 fields amplified with both primer sets (OPT and FPG) coming from the same DNA extraction sample suggests there is the potential that both species may co-exist within a given stem. Secondly, there is a possibility that both species could potentially reside within the same stem in a field situation where both species occurred within a given field. Thirdly the species-specific primers used in this study were developed on the basis of *Fusarium* spp. in Australia (FPG) and Germany (OPT). The potential exists that amplification of PNW *Fusarium* spp. isolates may not all completely amplify using the species-specific primers used in this study (FPG and OPT).

Results from the current study showed that one of the conidial extraction samples which had been morphologically identified as *F. pseudograminearum* amplified with the OPT primer set, whereas all the other amplifications were accurate (i.e. *F. culmorum* cultures amplified with the OPT primer set). Williams et al. (2002) reported

amplification of *F. crookwellense* fungal DNA samples with both the OPT and FPG primer sets. It is possible that this *F. pseudograminearum* isolate could have been misidentified and actually was *F. crookwellense* and subsequently amplified with the OPT primer set.

Preliminary evaluations from our research with 24 DNA extractions from single stems from the survey with natural inoculum field levels showed a significant correlation between crown rot severity ratings and DNA concentration ( $r^2 = 0.61$ ;  $p < 0.0001$ ). However, in the larger experiment involving 50 fields (150 DNA extractions), samples were pooled from each survey sample similar in methodology to Hogg et al. (2007), and there were not correlations between crown rot severity ratings and DNA concentrations. Pooling the samples may have introduced variation and reduced overall DNA concentrations to the point where disease severity rating could not be accurately correlated to DNA concentrations.

The reason for this lack of correlation between crown rot severity scores and DNA concentration could have occurred for a number of reasons. First of all, there has been no report of a correlation between crown rot severity score and DNA concentration in naturally infected field samples. The only correlations between these parameters have been with inoculated experiments in the greenhouse and field (Strausbaugh et al. 2005; Hogg et al. 2007). One reason could be the lack of inoculation pressure in naturally occurring inoculum in fields. Hogg et al. (2010) showed DNA concentration in naturally occurring fields to be substantially less (0.4%) than that of inoculated field plots.

Secondly, the crown rot score seems to be normally distributed and linear between fungal occurrence and symptoms, whereas DNA concentrations are more

logarithmic in nature (Liddell et al. 1986; Nicholson et al. 1998). For example a rating of 6.0 could have 100 times more DNA than that at 5.5, whereas a rating of 3.0 may have 100 times more DNA than a rating of 1.0. Also with the pooling of symptomatic stems as was conducted by Hogg et al. (2007), there is greater likelihood that a single stem with high DNA concentration could exist that would disrupt the linear relationship between symptoms and DNA concentrations. Results show that inoculation may create more of a linear relationship between symptoms and DNA concentration (Strausbaugh et al. 2005; Hogg et al. 2007; Hogg et al. 2010).

This research demonstrated the ability to detect naturally occurring levels of *Fusarium* species in the field using species-specific primers in a Q-PCR assay. At the current stage of crown rot diagnostics research it may be more sensible, economical, and efficient to use conventional PCR to diagnose diseased stems to identify causal species. Other applications could potentially be explored for the use of Q-PCR as a tool for quantifying disease. Currently QTL identification relies on tedious rating systems to appropriately find chromosomal locations of disease resistance. If Q-PCR were validated as an accurate tool to relate visual disease severity ratings, especially under inoculated conditions, there may be an application of the technology for QTL identification. Currently the literature supports the ability to correlate disease ratings and DNA concentrations. This research should be explored with various isolates from the PNW *Fusarium* survey.

If further research were to be conducted for the use of Q-PCR as a diagnostic tool, it would have the most efficient application in accurately quantifying DNA concentrations in relation to visual disease ratings. If this research were pursued, there is

a need to evaluate the tri-5 'TaqMan' primer set on naturally inoculated survey samples used by Strausbaugh et al. (2005) and Hogg et al. (2007). Further research also needs to be conducted to evaluate the effectiveness of correlating the extraction of DNA from single stems to crown rot severity ratings using a pooling of stems within survey samples over two consecutive years. Further research comparing natural and inoculated field conditions may shed more light on the relationship between inoculum levels, disease development, and DNA concentrations from Q-PCR quantification with species-specific primer sets. Others have shown that cultural factors (nitrogen levels and planting dates) as well as water availability at the time of disease colonization during grain filling affect the rate of disease development (Papendick and Cook, 1974). It would be of interest to investigate cultural effects on the quantity of DNA under natural versus artificial inoculation to better explain the role of environmental conditions in plant stress and disease development.



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## **CHAPTER 4**

## CHAPTER 4

### EVALUATION OF INOCULATION METHODS TO ASSAY SPRING WHEAT FOR RESISTANCE TO FUSARIUM CROWN ROT IN A CONTROLLED GROWTH ENVIRONMENT

Poole, G.<sup>1</sup>, Ozdemir, F.<sup>2</sup>, Nicol, J.<sup>3</sup>, Erginbas, G.<sup>3</sup>, Murray, T.<sup>1</sup>., Smiley, R.<sup>4</sup>, Campbell, K.<sup>5</sup>, and Paulitz, T.<sup>5</sup>. <sup>1</sup>*Washington State University, Dept. of Plant Pathology Pullman, WA, 99164 USA; E-mail: [gpoole@wsu.edu](mailto:gpoole@wsu.edu); FAX: 509-335-9581;* <sup>2</sup>*Bahri Dagdas International Agricultural Research Institute, Konya, Turkey;* <sup>3</sup>*CIMMYT (International Maize and Wheat Improvement Centre) P.O. Box. 39 Emek 06511 Ankara, Turkey;* <sup>4</sup>*Oregon State University, Pendleton, OR. 97331;* <sup>5</sup>*USDA-Agricultural Research Service, Pullman, WA.*

#### ABSTRACT

Crown rot is major biotic constraint on rain fed wheat production systems throughout the world and in the Pacific Northwest (PNW) region of the U.S. Caused by a complex of *Fusarium* species, of which *F. pseudograminearum* and *F. culmorum* are the most important, crown rot reduces wheat yields by an average of 9% in the PNW. Adequate *Fusarium* screening systems must be established to appropriately phenotype mapping populations for accurate QTL identification. The objective of this research was to find an inoculation method with the greatest consistency and least variation. Two *Fusarium pseudograminearum* isolates from the PNW were used in a study to evaluate the effect of inoculation method on the performance of four spring wheat cultivars varying in



susceptibility to *Fusarium* crown rot. Three different rating systems were compared and variance was estimated for each method of inoculation. Methods of inoculation were; growing *Fusarium* on millet seed and placing near the germinated seedling; soaking germinated seedlings in a liquid conidial suspension ( $10^6$  conidia per ml) reported as ‘Nicol method’; placing a 10  $\mu$ l droplet of a liquid conidial suspension ( $10^6$  conidia per ml) in water or methylcellulose on the stem base (10 days post-germination) reported as the ‘Mitter method’; and placing an agar-based slurry containing conidia ( $10^6$  conidia per ml) in short 4-cm drinking straws at the base of the stem (10 days post germination). Rating systems were; Nicol rating (0 to 10 scale: 10=dead); Rating of four leaf sheaths on a 0 to 4 scale (4=dead) and adding up ratings of the three outside leaves); and Mitter index, which was a crown rot index calculated by taking the (seedling height / disease lesion length) x the number of leaf sheaths with symptoms. The inoculation method that resulted in the most consistent difference between resistant (‘2-49’) and susceptible (‘Seri’) cultivars and least amount of variation were the millet and conidial slurry. Of these, the colonized millet seed method gave the most reliable phenotype ratings and was used to screen two mapping populations from Sunco/Macon and Sunco/Otis crosses. The Nicol rating of 0 to 10 yielded the most conclusive results with the least amount of variation and can be most easily applied to a high-throughput QTL mapping study. The millet seed inoculation utilizing the Nicol rating on a scale from 0 to 10 was used to screen mapping populations for identification of crown rot resistance QTL.

## **INTRODUCTION**

*Fusarium* crown rot (*F. pseudograminearum* (O’Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*) and *F. culmorum* (Wm. G. Sm.) Sacc.) is

one of the most detrimental soil-borne diseases of dry land wheat in the PNW (Cook, 1992; Smiley and Patterson, 1996; Smiley et al. 2005a). Cook (2001) estimated that soil-borne root diseases cost the U.S. about 10 million tons in wheat production annually. *F. pseudograminearum* and *F. culmorum* have been documented as the dominant species causing crown rot in wheat in the PNW (Cook, 1968; Smiley and Patterson, 1996; Smiley et al. 2005b; Strausbaugh et al. 2004).

In an economic analysis conducted in 1994 from 13 fields evaluated for the impact of crown rot, yield loss was estimated to represent 97 million kg (3.5 million bu.) in production at a value of \$13 million for the PNW region of the USA (Smiley et al. 2005). Yield from crown rot has been documented to range from 9% to 89%, with average of 9% in the PNW, and world-wide losses exceeding 30% (Cook, 1992; Kane, 1987; Burgess et al. 2001; Dodman and Wildermuth, 1987; Klein et al. 1991; Hekimhan et al., 2004). Smiley et al. (2005) documented yield loss in the PNW as high as 61% resulting following artificial inoculation with *F. pseudograminearum*. Yield loss estimates for bread wheat from surveys in Turkey have ranged from 24% to 42% from natural field infections (Hekimhan et al. 2004; Nicol et al. 2006).

Crown rot is typically associated with wheat production in semi-arid areas where wheat matures under hot-dry conditions (Burgess et al., 2001). Symptoms and adverse affects of crown rot are much more pronounced under drought conditions in dry-land wheat producing regions throughout Australia, Europe, North America, North and South Africa, and West Asia (Burgess et al. 2001; Smiley et al., 2005a; Nicol et al. 2006). The disease causes a rotting of the stems, crown, and root tissue and a chocolate brown to

reddish brown lesions on the crown roots and the basal portion of stems (Cook and Veseth, 1991; Paulitz et al. 2002).

Cook (2001) reported that host plant resistance was the most effective and affordable approach to combating soil-borne diseases such as crown rot. Genetic resistance to crown rot has been well documented (Purss, 1966; Wildermuth and McNamara, 1994; Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006; Nicol et al. 2006; Smiley and Yan, 2009). However, Smiley and Yan (2009) reported that while spring wheat phenotypic tolerance to *Fusarium* crown rot (caused by *F. pseudograminearum*) could be accurately described in inoculated field trials in the PNW, those for winter wheat could not easily be described due to year and location effects. Although winter wheat cultivars have been screened for tolerance to crown rot in naturally infested soils for four decades, due to a high degree of variation in the disease over years and test sites cultivars exhibiting consistent tolerance have not been identified (Smiley and Yan, 2009).

Wallwork et al. (2004) reported that screening of plants in field-sown trials often is complicated by uneven natural infection, contamination by other pathogens, or adverse environmental conditions. The authors developed an improved method for screening wheat plants grown in open ended tubes in an outdoor sand-based terrace allowing for the inoculation of plants in a semi-controlled environment. The outdoor terrace screening system was used to successfully detect adult wheat plant resistance to *F. pseudograminearum* and *F. culmorum* and a crown rot resistance QTL located on chromosome 4B.

From evaluations of cultivars for crown rot resistance over the years, a phenomenon of adult and seedling resistance has been documented (Wallwork et al. 2004; Mitter et al. 2006). Wallwork et al. (2004) postulated that the advantage of adult plant screening systems were that more intermediate levels of genetic resistance could be reliably detected, whereas seedling screens detected only high levels of resistance. Wildermuth and McNamara (1994) reported that field screening techniques were time consuming and laborious and that selections in controlled greenhouse environments were more efficient for screening large amounts of germplasm efficiently. Mitter et al. (2006) reported that outdoor screening systems developed by Wallwork et al. (2004) were difficult to reproduce.

Although adult plant screening systems are useful for detecting moderate levels of resistance, seedling screens are an attractive alternative because they are not limited by growing season time-constraints and can be evaluated in a relatively short time (22 days) (Wildermuth and McNamara, 1994). Evaluation of wheat seedlings in the greenhouse has been reported (Purss, 1966; Liddell et al. 1986; Wildermuth and McNamara, 1994; Smiley et al. 2005b; Mitter et al. 2006; Li et al. 2008). Because the main objective of resistance screening is to discover durable field resistance, there have been extensive efforts with this research to validate seedling assays in the greenhouse with adult plant assays in the field (Nicol et al. 2006).

Although Purss (1966) did not report significant confirmations of crown rot resistance between greenhouse and field trials, later reports indicated significant correlations (Wildermuth and McNamara, 1994; Mitter et al. 2006). Liddell et al. (1986) showed that with greenhouse screening systems, sufficient inoculum was needed to cause

uniform infection, but excessive amounts of inoculum often skewed results by causing pre-mature death of wheat plants. Wildermuth and McNamara (1994) showed significant correlations between relative susceptibility in the field (% diseased tillers of test cultivars / % diseased tillers of susceptible 'Puseas') with seedling disease ratings with an  $r=0.78$  ( $p<0.01$ ) when plants were inoculated with *F. pseudograminearum*. Others have continued seedling evaluations to improve greenhouse screening efficiency and validate results with field assays.

Mitter et al. (2006) developed a high throughput method for screening a greater number of seedlings for *F. pseudograminearum* resistance in the greenhouse. Single seedlings were grown in square seedling pots, inoculated 13 to 14 days after planting with *F. pseudograminearum*, and incubated in nearly saturated relative humidity conditions for 48 hours at 25 °C. Following the incubation period, the original growing conditions were restored. Crown rot severity was assessed 35 days after inoculation. Sixteen cultivars were compared using this technique in the greenhouse followed by in-field evaluations where the plants were rated at maturity. Field and greenhouse screenings were highly correlated according to Spearman's rank correlation coefficient ( $r_s = 0.987$ ), proving that the greenhouse screening technique is sufficient to appropriately screen cultivars for resistance to *F. pseudograminearum* (Mitter et al., 2006).

Li et al. (2008) conducted the most recent research evaluating a recent inoculation method to evaluate crown rot resistance. Experiments were conducted in the greenhouse to evaluate the soaking of seedlings (age 1 to 4 days old) in a conidial suspension varying in concentration ranging from  $5 \times 10^5$  to  $1 \times 10^7$  conidia per mL of solution (water + 0.1% tween (v/v)). In addition to these treatments, the duration of inoculation (1 to 10

minutes of soaking), incubation period (0, 24, and 48 hours at 100% relative humidity and darkness), the effect of water stress on crown rot symptom development, and comparisons to the Mitter et al. (2006) stem droplet method were conducted. Plants were rated on the basis of a 0 to 5 scale (0=no disease; 5=severe crown rot symptoms). Results showed no statistical differences in the duration of inoculation and incubation period but increasing disease development up to a conidial concentration of  $1 \times 10^6$  conidia per mL. Overall disease severity was significantly greater than plants inoculated with the stem droplet method described by Mitter et al. (2006). The authors concluded that the seedling dip inoculation was a simple reliable method for screening resistant wheat germplasm, but did note that there was a high mortality among the most susceptible cultivars evaluated.

Others have used other methods of inoculation utilizing liquid conidial suspensions with *Fusarium* spp. and *Pseudocercospora* sp. to inoculate wheat plants (Mitter et al. 2006; de la Peña and Murray, 1994). De la Peña and Murray (1994) developed an improved method for screening wheat plants for resistance to *P. herpitrichoides* by placing 250  $\mu$ L of a conidial agar slurry in 1.5-cm drinking straws (6-mm diam.) placed at the base of seedlings to improve the uniformity of contact with the stem base. Another potential inoculation method done by placing a relatively high volume (1 mL) of conidial suspension on the soil surface at the base of the seedling stem had not been previously reported. There remains the need for evaluating other methods of inoculating seedlings in the greenhouse to reduce variation in results and increase reproducibility for screening large numbers of genotypes efficiently.

Mitter et al. (2006) reported the need for a robust greenhouse screening system that had reasonable control over the environment and the use of well characterized pathogen isolates. The authors also stressed the need for standardized inoculation, incubation, and rating assessment systems to adequately quantify genetic resistance to crown rot and ability to screen a large amount of germplasm in the greenhouse. Furthermore they reported that the nature of crown rot resistance is partial and requires well refined screening systems with the ability to detect small differences in resistance.

The objectives of this series of experiments were to; 1. evaluate the relative virulence of five PNW isolates collected (Smiley and Patterson, 1996) and characterized by Smiley et al. (2005b) in the greenhouse on the Australian cultivars ‘2-49’ (partially resistant) and ‘Seri’ (susceptible) and PNW cultivars with unknown levels of resistance in the greenhouse in three replicated experiments; 2. compare the current method of inoculation using colonized millet outlined by Smiley et al. 2005b with the stem base droplet method outlined by Mitter et al. (2006), the application of the conidial agar slurry placed in a 1.5-cm drinking straw at the stem base outlined by de la Peña and Murray (2004) to *F. pseudograminearum* isolates, the seedling dip method outlined by Li et al. (2008) at different conidial concentrations, and the soil-based 1-mL inoculation method on the aforementioned cultivars using three different rating systems; the sum of three leaf sheaths rating (0 to 12) outlined by Wildermuth and McNamara (1994), the 0 to 10 rating system outlined by Nicol et al. (2001), and the crown rot severity index outlined by Mitter et al. (2006); 3. identify the most reliable *F. pseudograminearum* isolate, inoculation method, and rating system for identifying plant resistance QTL in two recombinant inbred line mapping populations (Sunco/Macon and Sunco/Otis).

## **MATERIALS AND METHODS**

A series of greenhouse-growth room assays were conducted to evaluate the resistance of several spring wheat cultivars with known *Fusarium* resistance or susceptibility to assess crown rot resistance in seedlings (Table 1). A total of seven growth room screening assays (labeled AE-1, AE-2, AE-3, AE-4, AE-5, AE-6, and AE-7 respectively) were conducted at the Plant Growth Facility in Pullman, WA during 2007, 2008, and 2009. The dimensions of the growthrooms and growth chambers for these experiments were 384 ft<sup>3</sup> and 69 ft<sup>3</sup>, respectively (Convicon, Winnipeg, Canada).

### **Seed preparation**

Seeds for AE-1 and AE-5 were not surface disinfested or pre-germinated. For AE-2, AE-3, AE-4, AE-6, and AE-7, seeds of each cultivar were surface disinfested in 95% EtOH (ethanol) for 6 min, rinsed in de-ionized distilled water for 1 min, then placed in 0.5% NaOCl for 10 min, followed by six rinses (1 min each) in de-ionized distilled water. Following surface disinfestation, seeds were placed on moist filter paper and placed in direct sunlight for germination for 3 days. Following germination seedlings were placed at 4 °C for 4 to 5 days to obtain consistent germination.

### **Planting and growth room conditions**

For all greenhouse assays seedlings were grown for 52 to 60 days in a growth chamber at 60/80 ( $\pm 5$ )% day/night RH, a 12- hour photoperiod, and temperatures of 25/15 °C day/night temperatures for the duration of the experiment according to Mitter et al. (2006). Seedlings were planted into a medium comprised of 50% sand and 50% peat (v/v) (Greensmix Sphagnum Peat Moss, Wuapaca Northwoods llc., Wuapaca, WI) in 3.8-cm diameter x 20.5-cm long “cone-tainers” (Stuewe and Sons Inc, Corvallis, OR)



arranged in plastic trays (7 rows x 14 columns), unless otherwise specified. To more appropriately mimic field conditions, inoculation methods experiments AE-4, AE-5, AE-6, and AE-7 utilized a 70:29:1 mix of Thatuna silt loam field soil:sand:peat (Peat source was Greensmix Sphagnum Peat Moss, Wuapaca Northwoods llc., Wuapaca, WI).

### Inoculum preparation

In all greenhouse experiments where millet was used as an inoculum source, with the exception of AE-5, inoculum preparation was done similar to Smiley et al. (2005b) using the following method. Inoculum of five respective PNW *F. pseudograminearum* isolates (082-03, 006-13, 044-09, 081-11, 032-06) collected and described by Smiley and Patterson (1996) was prepared on sterilized autoclaved white pearl millet seed (Table 1).

**Table 1.** Background information for five *Fusarium pseudograminearum* isolates used in greenhouse and terrace screening experiments.

Isolate <sup>1</sup>	Disease index in greenhouse: 0-7 scale <sup>2</sup>	Host, site and year of collection <sup>3</sup>				
		Host tissue	Nearest town	County	State	Year
1. 082-03	5.0	WW crown	Waitsburg	Walla Walla	WA	1993
2. 006-13	6.0+	WW crown	Dufur	Wasco	OR	1994
3. 044-09	5.3	WW crown	Moro	Sherman	OR	1994
4. 081-11	6.3	WW crown	Prescott	Walla Walla	WA	1994
5. 032-06	6.4	WW crown	Moro	Sherman	OR	1994

<sup>1</sup> Isolates were collected in a survey conducted by Smiley and Patterson, 1996 and reported to have significant virulence on wheat according to Smiley et al. 2005b.

<sup>2</sup> The disease rating scale was used in Smiley et al. 2005b in the greenhouse on the basis of 0 to 7; 0 = apparently healthy plant with no discoloration of any tissue; 1 = browning of scutellar node; 2 = browning of scutellar node and either the sub-crown internode or leaf sheaths; 4 = dying plant with browning of sub-crown internode, leaf sheaths, and lower culm; 5 = dying plant with rotted culm base; 6 = dead plant with rotted stem base (plants had matured to produce more than 3 leaves); and 7 = plant death by rotting of seed or by pre-emergence damping-off of seedling. Isolates were chosen due to a high degree and variation in virulence to function in inoculation mixtures for future field and greenhouse studies to quantify resistance.

<sup>3</sup> Explains that isolates were collected from winter wheat (WW) crown tissue and the respective locations, Counties, States, and years of collection.

To prepare inoculum, 200 grams of millet seed was placed into 0.5 or 1-L Mason jars and autoclaved twice over a 2-day interval at a temperature of 121 °C for 45 minutes (15 atm. psi.). The five isolates of *F. pseudograminearum* were grown in 20% potato dextrose agar (PDA) for 10 days under fluorescent lights set at a 12-hour photoperiod at 26 °C. Eight 1-cm squares were cut from each PDA plate for each PNW *F. pseudograminearum* isolate and added to the jars then incubated at 22 °C for 21 days prior to growth chamber inoculations. Jars containing inoculated millet grain were stored at 4 °C until use.

To prepare liquid macroconidia inoculum for AE- 4 and AE-5 PNW isolates were grown on Synthetischer Nährstoffärmer agar (SNA) plates for 10 days at 20-22 °C under a combination of cool white and black fluorescent lights with a 12-h photoperiod (Nirenberg, 1976). Macroconidia were harvested by flooding plates with distilled water, which were then filtered through several layers of Kimwipes<sup>®</sup> and the concentration adjusted to  $1 \times 10^6$  conidia per mL (Dodman and Wildermuth, 1987; Mitter et al. 2006). For AE-6 and AE-7 liquid conidial suspensions were made by placing inoculated millet grain in three layers of cheesecloth within a funnel. The millet was flushed with distilled water in 50 to 100 mL aliquots. The concentration of macroconidia in subsequent suspensions was confirmed with a haemocytometer. For AE-4 suspension fluids for the seedling dipping method and stem droplet method were deionized-distilled water and methylcellulose (1%), respectively. For AE-5, AE-6, and AE-7 deionized-distilled water served as the liquid suspension base.

For AE-4, AE-6, and AE-7 an agar conidial slurry was made by mixing an agar suspension with a distilled water-based conidial suspension. The agar slurry was made

by grinding water agar plates containing 1.5% water agar in a blender. The conidial slurry containing  $1 \times 10^6$  and  $1.6 \times 10^6$  conidia per mL was made in distilled water. The water based conidial solution was added to the heated ground slurry of water agar at a ratio of 50:50 to make a final concentration of  $0.5 \times 10^6$  or  $0.8 \times 10^6$  conidia per ml in the agar suspension (Table 3).

### **Rating systems**

Experiments (AE-1 through AE-7) were rated for crown rot severity at 35 days after inoculation according to three rating systems. The first rating system known as the 'leaf sheath sum' was outlined by McNamara and Wildermuth (1994) by taking the ratings of three leaf stems (LS1, LS2, and LS3) from 0 to 4 (4=dead leaf) and summing them to make a total leaf sheath summed rating of 0 to 12 (12=dead plant). The second system utilized a crown rot severity index calculated according to Mitter et al. (2006) in the following equation: Crown rot severity index = (length of stem discoloration/seedling height) x (number of leaf sheath layers with necrosis). The third rating system was devised by Nicol et al. (2001) based on an overall rating of the seedling base crown region from 0 to 10. The symptom rating system was based on the following scale; 0=no disease; 1 to 2 = minor symptoms on crown within the first internode region; 3 to 4 = obvious symptoms on crown within the first internode region; 5 to 6 = pronounced symptoms on crown with obvious darkened plant tissue due to infection; 8 to 9 = advanced darkened symptoms with severe stunting and near death due to disease infection; 10 = dead plant with severe disease symptoms) for crown rot severity.

### **Assay Experiment 1 (AE-1)**

The objective of AE-1 was to evaluate 11 cultivars with previously determined levels of resistance and susceptibility to crown rot under growth room conditions (Table 2). In AE-1 each cone-tainer was inoculated using one of two inoculation methods. The first method, denoted 'Early Inoculation Method', was inoculated at the time of planting by placing the seed into moist planting medium, 2.5-cm of soil over the top, then inoculum of each of the 5 respective PNW isolates covered by another 2.5-cm of soil. The second method, denoted by 'delayed inoculation method' adapted from Wallwork et al. (2004), was inoculated as outlined in the 'Early Inoculation Method', but delayed 10 days after seedling emergence. Plants from AE-1 were assessed according to the leaf sheath sum rating outlined by McNamara and Wildermuth (1994). Plants from the delayed inoculation method were allowed to mature in the growth chamber and overall stem ratings were assigned from 0 to 4 (4=dead plant). All methods of inoculation using the colonized method in all subsequent experiments (AE-2 through AE-7) were made using the delayed inoculation method adapted from Wallwork et al. (2004).

### **Assay Experiment 2 (AE-2)**

The objective of AE-2 was to evaluate the virulence of the five PNW *F. pseudograminearum* isolates on four different cultivars Sunco (PR), Macon (S), 2-49 (PR), and Seri (S) with a greater number of replications (n=17). Six inoculated millet seeds were placed according to the previously outlined delayed inoculation method adapted from Smiley et al. (2005b). Plants from AE-2 were rated according to Wildermuth and McNamara (1994) and Mitter et al. (2006).

**Table 2.** List of cultivars screened in AE-1 for Fusarium crown rot resistance with their respective market class and relative resistance / susceptibility.

<b>Cultivar<sup>1</sup></b>	<b>Market Class<sup>2</sup></b>	<b>Resistance/Susceptibility to Fusarium crown rot<sup>3</sup></b>
1. OR942504	Hard white winter	Unknown
2. KS93U161	Hard red winter	Unknown
3. Sunco	Hard white spring	Partially Resistant
4. Macon	Hard white spring	Susceptible
5. Pelsart	Hard white spring	Partially Resistant
6. Seri	Hard white spring	Susceptible
7. 2-49	Hard white spring	Partially Resistant
8. Gluyas Early	Hard white spring	Partially Resistant
9. Gala	Hard white spring	Partially Resistant
10. Baxter	Hard white spring	Partially Resistant
11. Chinese Spring	Hard red spring	Unknown

<sup>1</sup> Cultivars were chosen on the basis of prior greenhouse screens according to Wildermuth and McNamara (1994) and R.W. Smiley (personal comm.) for varying degrees of resistance to crown rot to quantify the ril mapping population parents ‘Sunco’ and ‘Macon’.

<sup>2</sup> The market class of each respective variety was hard white or hard red spring wheat.

<sup>3</sup> The degree of resistance was estimated based on previous published results and preliminary greenhouse experiments from R.W. Smiley and J. Nicol (personal comm.), Wildermuth and McNamara (1994), Wallwork et al. (2004), and Mitter et al. (2006).

### **Assay Experiment 3 (AE-3)**

AE-3 was designed to evaluate consistent performance of the most virulent isolates from previous AE-1 and AE-2 studies for PNW *F. pseudograminearum* Isolates 2 (006-13), 4 (081-11), and 5 (032-06). The second objective of AE-3 was to find the maximum threshold number of replications (n=20). Plants were rated according to Wildermuth and McNamara (1994) and Mitter et al. (2006).

### **Assay Experiment 4 (AE-4)**

The objective of AE-4 was to evaluate methods of inoculation with *F. pseudograminearum* on crown rot symptom development and performance of four cultivars (Seri (S), 2-49 (PR), Sunco (PR), and Macon (S)) (Table 3). The experiment was planted on January 8 and harvested March 4, 2008.

**Table 3.** Description of six inoculation methods for AE-4 to evaluate the effects of inoculation methods on the performance of four cultivars (Seri (S), 2-49 (PR), Sunco (PR), and Macon (S)) inoculated with two PNW *F. pseudograminearum* isolates 4 (081-11) and 5 (032-06) during 2008.

Method of Inoculation <sup>1</sup>	Description
1. Seedling dip <sup>2</sup>	Pre-germinated 4-day-old seedlings were placed in a conidial suspension (concentration = $1 \times 10^6$ ) of distilled water for one minute then planted.
2. Seedling dip <sup>2</sup>	Pre-germinated 4-day-old seedlings were placed in a conidial suspension (concentration = $1 \times 10^6$ ) of 1% methyl cellulose for one minute then planted.
3. Stem base droplet <sup>3</sup>	A 10 $\mu$ L droplet of conidial suspension (concentration = $1 \times 10^6$ ) of distilled water was placed at the base of 10-day-old seedlings (14 days after emergence).
4. Stem base droplet <sup>3</sup>	A 10 $\mu$ L droplet of conidial suspension (concentration = $1 \times 10^6$ ) of 1% methyl cellulose was placed at the base of 10-day-old seedlings (14 days after emergence).
5. Colonized millet <sup>4</sup> grain	Grain millet (white pearl) was colonized with each respective isolate of <i>F. pseudograminearum</i> . Approximately 8 to 10 grains were placed at the base of each seedling 4 days after emergence covered with the potting mix and water applied.
6. Agar straw conidial suspension <sup>5</sup>	An agar conidial solution was made with 50% (1.5%) agar and 50% conidial suspension (concentration $1$ to $1.6 \times 10^6$ ). 250 $\mu$ L of solutions consisting of $5.0 \times 10^5$ and $8.0 \times 10^5$ conidial concentrated agar were placed in straws positioned at stem bases of 10-day-old seedlings.

<sup>1</sup>Control treatments complimented each inoculation method and consisted of sterile distilled water, 1 % sterile MC (methyl cellulose), or agar solution without any conidia for the seedling dip, stem base droplet, and agar straw treatments, respectively. For the colonized millet, control treatments consisted of millet not colonized with *F. pseudograminearum*.

<sup>2</sup> The seedling dip method was adapted from Li et al. (2008).

<sup>3</sup> The stem base droplet method was adapted from Mitter et al. (2006).

<sup>4</sup> The colonized grain millet method was adapted from Smiley et al. (2005b).

<sup>5</sup> The agar straw conidial suspension method was adapted from de la Peña and Murray (1994).

For the stem based droplet treatments, the plastic tube racks were placed on their side at 10 days following seedling emergence and placed in large storage tubs (76 cm x 91 cm) (Sterilite Co., Townsend, MA). A 10  $\mu$ L droplet of each respective water-based and methyl cellulose (MC) based treatment was placed at the base of each stem. Covers were immediately placed on the tubs and water added to the base of the tubs to ensure high relative humidity for 48 hours according to Mitter et al. (2006). For the agar straw inoculation method, plastic drinking straws were cut to a length of approximately 4 to 5 cm with a slit in the side to allow straw removal at seedling harvest. Straws were placed around germinating seedlings around 1 week after planting. Each method of inoculation treatment outlined in Table 3 was accompanied by a non-inoculated control treatment arranged as another isolate treatment. Control grain colonization treatments consisted of sterilized millet without conidia. Seedling dip and stem based droplet non-inoculated control treatments were soaked in de-ionized distilled water and sterile methyl cellulose (1%) without conidia. For the stem base droplet method, control treatments consisted of placing a droplet of sterile distilled water or sterile methyl cellulose (1%) at the base of the stem 1 cm from the soil line (Table 3).

#### **Assay Experiment 5 (AE-5)**

The objective of AE-5 was to evaluate the effect of inoculating non-germinated seed with a liquid conidial solution. Treatments consisted of soaking non-germinated seeds in a conidial suspension of PNW Isolate 4 (081-11) (concentration of  $1 \times 10^6$ ) for 1 minute, soaking seeds in sterile distilled water for 1 minute, and non-soaked seeds. AE-5 was rated according to Nicol et al. (2001).

### **Experimental Assay 6 (AE-6)**

AE-6 was planted May 5 and harvested June 30, 2009 to confirm the results of AE-4. Two additional treatments were added including inoculation of non-germinated grain (similar to that of AE-5) and the application of 1 mL of conidial suspension (conidial concentration =  $1 \times 10^6$ ) to the soil at the base of the seedling (Table 4). The isolate used for this experiment was PNW Isolate 2 (006-13) selected on the basis of AE-3 for screening the recombinant inbred line (RIL) mapping populations Sunco / Otis and Sunco / Macon. The experiment was rated on March 4, 2009 according to Wildermuth and McNamara (1994), Mitter et al. (2006), and Nicol et al. (2001).

### **Assay Experiment 7 (AE-7)**

AE-7 was planted July 1 and rated September 1, 2009 to investigate additional conidial concentrations ( $2.5 \times 10^5$  and  $5.0 \times 10^5$ ) published by Li et al. (2008) for the seedling dip method (Table 4). This experiment was held at 4 °C in the growth chamber for 10 days following inoculation to slow plant growth and lengthen the time for infection. AE-7 was rated at approximately 45 days after inoculation on September 1, 2009 according to the three rating systems previously described (Wildermuth and McNamara 1994; Mitter et al. 2006; Nicol et al. 2001).



**Table 4.** Treatment methods of inoculation used in Experiment AE-6 and AE-7 to evaluate six inoculation methods with *F. pseudograminearum* PNW Isolate 2 (006-13) on four spring wheat cultivars (Sunco (PR), Otis (S), 2-49 (PR), and Seri (S)).

Method of Inoculation	Description
1. Seedling dip <sup>1</sup>	Pre-germinated 4-day-old seedlings were placed in a conidial suspension (AE-6 concentration = $1 \times 10^6$ ) of distilled water for one minute. Low conidial concentrations were $2.5 \times 10^5$ and high conidial concentrations were $5 \times 10^5$ in AE-7.
2. Stem base droplet <sup>2</sup>	A 10 $\mu$ L droplet of conidial suspension (concentration = $1 \times 10^6$ ) of distilled water was placed at the base of 10 after emergence (~14-day-old seedlings).
3. Colonized millet <sup>3</sup> grain	Grain millet (white pearl) was colonized with PNW Isolate 2 (006-13) of <i>F. pseudograminearum</i> . Approximately 8 to 10 grains were placed at the base of each seedling 4 days after emergence covered with the potting mix and water applied.
4. Dry seedling dip <sup>4</sup> (AE-6 only)	Non-pre germinated seeds were soaked in a conidial suspension (concentration $1 \times 10^6$ ) for 1 minute, allowed to dry for 30 minutes, and planted.
5. Soil based inoculation	1 mL of conidial suspension (concentration $1 \times 10^6$ ) was applied to the soil near the stem base approximately 10 days after emergence (~14-day-old seedlings).
6. Agar straw conidial suspension <sup>5</sup>	An agar conidial solution was made with 50% (1.5%) agar and 50% water-based conidial suspension (concentration $2 \times 10^6$ ). 250 $\mu$ L of solutions consisting of $1.0 \times 10^6$ conidial concentrated agar were placed in straws positioned at stem bases of 10-day-old seedlings.

<sup>1</sup> The seedling dip method was adapted from Li et al. (2008). Conidial suspension concentrations for AE-6 were  $1 \times 10^6$  while low concentration ( $2.5 \times 10^5$ ) and high concentration ( $5 \times 10^5$ ) conidial suspensions were evaluated in AE-7.

<sup>2</sup> The stem base droplet method was adapted from Mitter et al. (2006).

<sup>3</sup> The colonized grain millet method was adapted from Smiley et al. 2005b.

<sup>4</sup> The dry seedling dip method was conducted only on AE-6 and was adapted from Nicol et al. (2004).

<sup>5</sup> The agar straw conidial suspension method was adapted from de la Peña and Murray (1994).

## **Data Analysis**

Experimental units for all assay experiments (AE-1 through AE-7) were the individual plants grown in cones arranged in a split-plot experimental design. Cultivars were considered fixed effects because they did not represent a group or continuum of genotypes as they were specifically selected for their resistance and susceptibility to crown rot from previous research. Isolates were considered to be fixed effects as they were selected by R.W. Smiley on the basis of their virulence response.

AE-1 was arranged with isolate as the main plot and cultivar as the sub-plot with 2 replications. AE-2 was arranged with cultivar as the main plot and the isolate as the sub-plot with 17 replications. AE-3 was arranged with the cultivar as the main plot and the isolate as the sub-plot, since the isolate effect of PNW Isolates 2 (006-13), 4 (081-11), and 5 (032-06) was of greatest interest in the assay. AE-3 had 20 replications. AE-4 was arranged with cultivar as the main plot and factorial subplots of 6 methods x 2 isolates x 2 controls for a total of 24 subplots with 10 replications. AE-5, AE-6, and AE-7 were arranged with method of inoculation as the main plot and cultivar as the subplot with four, ten, and seven replications, respectively. The data were analysed using PROC GLM SAS statistical software (Version 6.12; SAS Institute Inc., Cary NC). Least square means (lsmeans) were obtained using ANOVA and differences among means were determined using Fischer's least significant difference (LSD).

## RESULTS

### Assay Experiment 1

Significant differences were apparent in the level of crown rot severity between cultivars ( $p < 0.0001$ ) and PNW *F. pseudograminearum* isolates ( $p = 0.0001$ ) (Table 5). Much of the variation within the ANOVA was accounted for by the isolate main-plot effect. There was a significant isolate by cultivar interaction ( $p = 0.01$ ) (Table 6).

Within the partially resistant cultivars, Isolate 2 (006-13) had the greatest effect on Sunco, while Isolate 5 (032-06) had the greatest effect on 2-49 (Figure 1). Within the susceptible cultivars, Isolate 5 (032-06) had the greatest effect on Seri, while Isolate 1 (082-03) had the greatest effect on Macon (Figure 1). Isolates 1 (082-03) and 2 (006-13) were generally more virulent on Seri, Macon, Pelsart, KS93U161, and OR942504. Isolate 2 (006-13) resulted in the greatest disease severity on Gala, Sunco, and Chinese Spring. Isolate 4 (081-11) caused the most disease on Gluyas Early and the least on OR942504 and Chinese Spring. Isolate 5 (032-06) resulted in the most disease on Seri, 2-49, and Baxter. Isolate 2 (006-13) was rated as causing the most disease across cultivars (Table 5). The mean crown rot severity score for Sunco was significantly lower than Macon (Table 5).

The plants from the ‘late inoculation method’ were rated at maturity. Crown rot severity was significant for isolates ( $p < 0.0001$ ) but not for cultivars ( $p = 0.14$ ). The CV for crown rot severity was 49 for the late inoculation method. Overall crown rot severity was much lower for the ‘early inoculation method’ sub-experiment taken to maturity ( $\mu = 2.0$ ) than for the ‘delayed inoculation method’ ( $\mu = 4.8$ ), which was used as the standard for inoculating seedlings with colonized millet rated at the seedling stage.

**Table 5.** Crown rot severity of 11 wheat cultivars in screen AE-1 treated with five PNW *F. pseudograminearum* isolates with the ‘Early inoculation method’.

Cultivar Name <sup>1</sup>	Isolate 1 <sup>2</sup> (082-03)	Isolate 2 (006-13)	Isolate 3 (044-09)	Isolate 4 (081-11)	Isolate 5 (032-06)	Mean <sup>3</sup> LSD=1.9	Percent Infection of Seri <sup>4</sup>
1. Seri	8.5	9.5	2.0	9.5	11.0	8.1 a	100%
2. Macon	12.0	8.5	3.0	4.5	4.5	6.5 b	80%
3. Gala	4.0	10.0	1.5	7.5	7.5	6.1 bc	75%
4. Pelsart	7.5	6.0	2.5	5.5	6.0	5.5 bc	68%
5. Baxter	4.0	5.5	0.0	4.0	12.0	5.1 bcd	63%
6. KS93U161	8.5	5.5	2.0	4.5	1.5	4.4 cde	54%
7. Sunco	4.0	7.5	2.5	2.5	4.5	4.2 cde	52%
8. Gluyas Early	4.0	5.0	1.0	7.5	3.0	4.1 de	51%
9. OR942504	5.0	6.0	3.5	2.0	3.0	3.9 de	48%
10. Chinese Spring	2.5	7.5	1.5	1.0	3.0	3.1 de	38%
11. '2-49'	1.5	4.5	1.0	1.5	5.5	2.8 e	35%
<b>Mean</b>	5.59	6.86	1.86	4.55	5.59	4.89	60%

<sup>1</sup> Cultivars were selected from previous studies to evaluate for their degree of resistance or susceptibility to crown rot (Wildermuth and McNamara, 1994; Mitter et al. 2006).

Chinese spring was included as it is a common check in genetic studies.

<sup>2</sup> Isolates were collected during the survey conducted by Smiley and Patterson (1996) and rated for virulence in Smiley et al. (2005b) where they were assigned the respective isolate numbers (082-03, 006-13, 044-09, 081-11, and 032-06).

<sup>3</sup> Means and Fischers LSD values were calculated with SAS. Numbers followed by the same letter within a column are not significantly different at P=0.05.

<sup>4</sup> Proportions represent an infection percent of Seri, the susceptible check similar to that of Puseas in Wildermuth and McNamara (1994).

**Table 6.** The ANOVA results for AE-1, seedling screen of 11 cultivars with 5 PNW *F. pseudograminearum* isolates in a split plot experimental design.

Source	df <sup>1</sup>	Mean Square	F-Value	P-value <sup>2</sup>
Main Plot <sup>3</sup>				
Replicate block <sup>4</sup>	1	0.74	0.19	0.6662
Isolate	4	61.7	6.83	0.0001
Isolate*Replicate block	4	11.7	2.99	0.0358
Sub Plot <sup>3</sup>				
Cultivar	10	22.7	5.79	0.0001
Cultivar*Replicate block	10	10.1	2.57	0.0241
Cultivar*Isolate <sup>5</sup>	40	9.03	2.30	0.0118

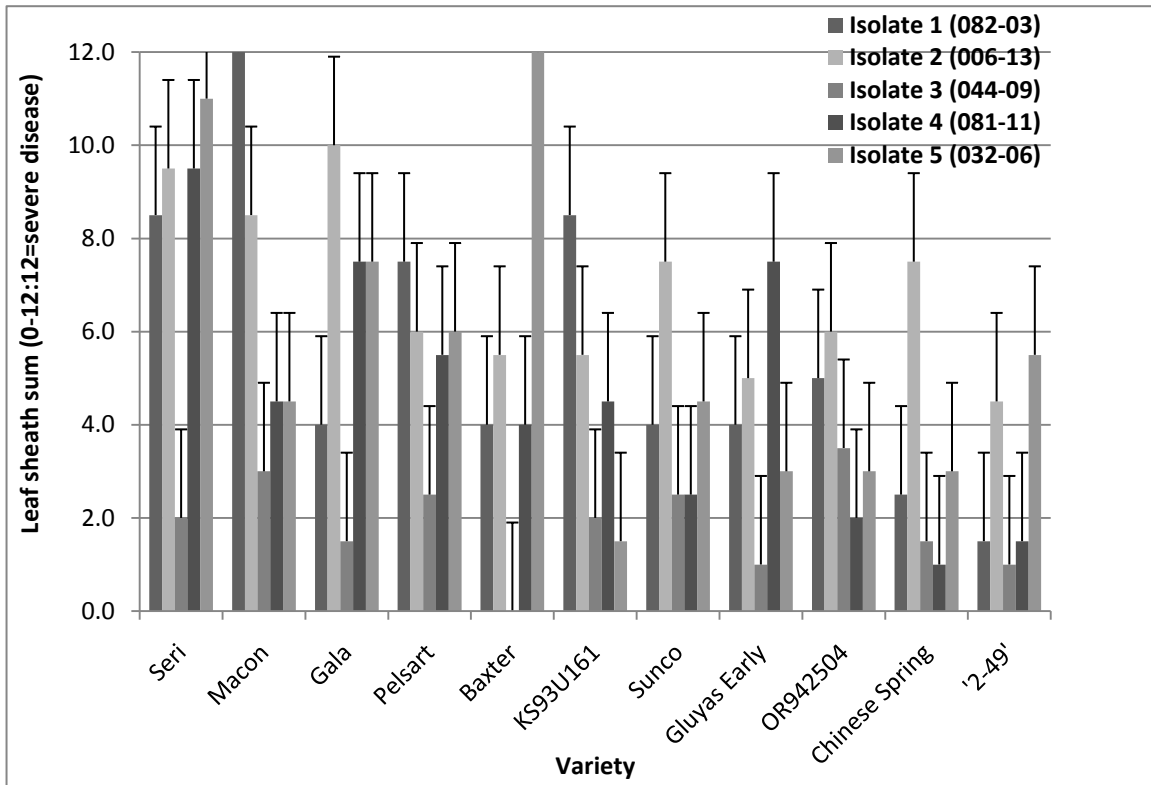
<sup>1</sup> df = degrees of freedom (number of samples (n) -1).

<sup>2</sup> P-value was considered significant at P=0.05.

<sup>3</sup> Experimental design was a split-plot with isolate as the main plot effect and cultivar as the subplot effect.

<sup>4</sup> Replicate block effect was the number of replications (n=2).

<sup>5</sup> Cultivar\*Isolate designates the cultivar by isolate interaction term (P=0.0118).

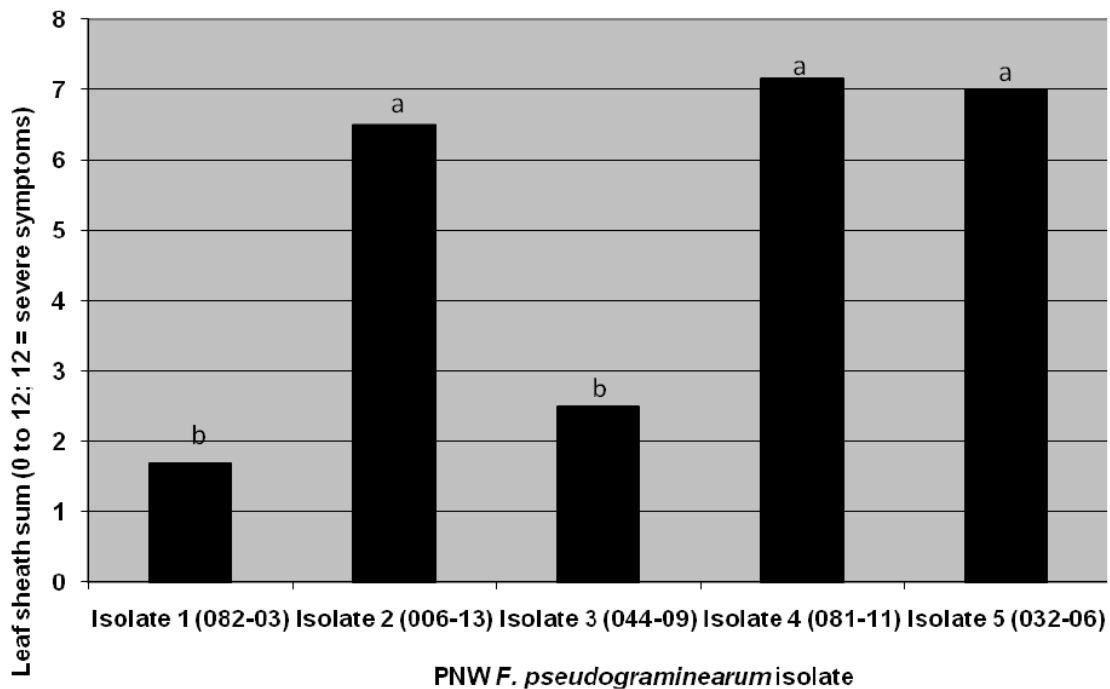


**Figure 1.** Crown rot severity of 11 wheat cultivars in screen AE-1 treated with five PNW *F. pseudograminearum* isolates rated according to the leaf sheath sum method. Bars represent Fischer's LSD value = 1.9 at P=0.05. PNW Isolate numbers are represented by the corresponding survey collection code outlined in Table 1 (Smiley and Patterson, 1996).

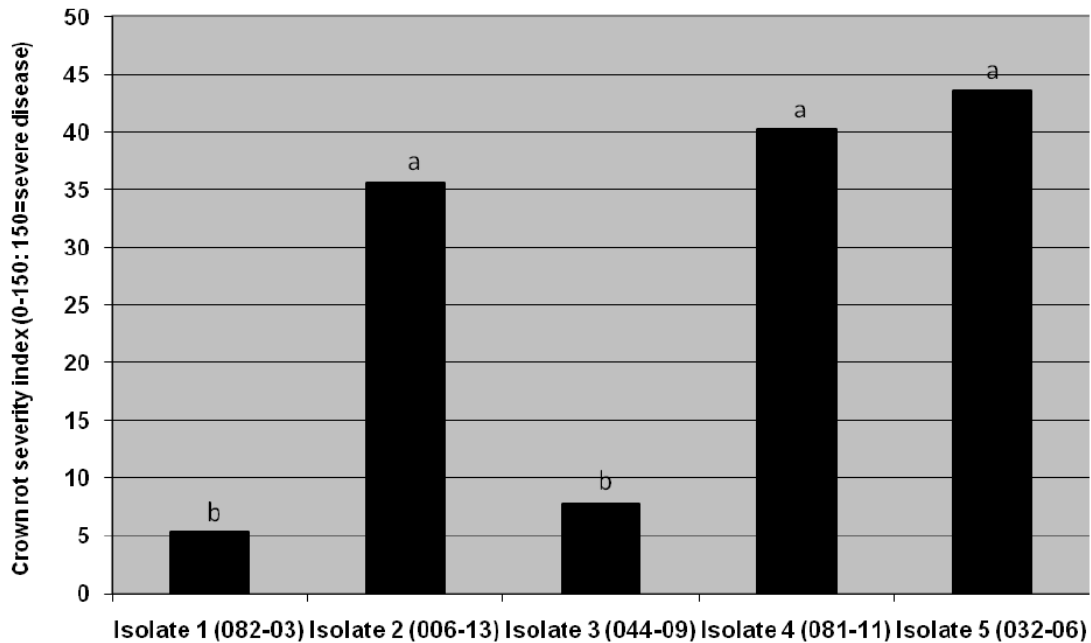
## Assay Experiment 2

Four cultivars were screened with the five PNW *F. pseudograminearum* isolates with 17 replications with significant differences between isolates ( $p < 0.0001$ ) but not cultivars ( $p = 0.73$ ) for both the leaf sheath sum rating (Figure 2) and crown rot severity index calculated according to Mitter et al. (2006) (Figure 3). All of the inoculation treatments had statistically more disease than the untreated checks. Over 95% of the overall variation was accounted for by the isolate random main effect. The CV was 59.4 for this experiment and within the acceptable range reported by Mitter et al. (2006). Isolates 2, 4, and 5 caused significantly more disease than isolates 1 and 3. Although

isolate 1 caused more disease in AE-1, it was not consistent in causing disease in AE-2 (Figure 2). There were consistencies in isolate performance and statistical differences between the Wildermuth and McNamara (1994) (Figure 2) rating method and that of the crown rot index calculated according to Mitter et al. (2006) (Figure 3). The CV for the Mitter crown rot severity index was substantially higher (CV = 153) than that of the leaf sheath sum calculated according to Wildermuth and McNamara (1994) (CV = 59). The reason for this was that the Mitter crown rot severity index is a much more specific and quantitative measure of disease levels than that of the Wildermuth and McNamara rating. Isolate 2 was consistent in virulence between experiments AE-1 and AE-2.



**Figure 2.** Crown rot severity rated according to the leaf sheath sum (0 to 12) according to Wildermuth and McNamara (1994) of five PNW *F. pseudograminearum* isolates for four spring wheat cultivars (Seri, 2-49, Sunco, and Macon) evaluated in AE-2 during September and October 2007 (LSD = 0.81; CV = 59). Bars with the same letter are not significantly different according to Fischers LSD at P=0.05.



**Figure 3.** Crown rot severity index calculated [(seedling height/disease lesion length) x total number of leaf sheaths with lesions] according to Mitter et al. (2006) of five PNW *F. pseudograminearum* isolates for four spring wheat cultivars (Seri, 2-49, Sunco, and Macon) evaluated in AE-2 (LSD = 13.3; CV = 153). Bars with the same letter are not significantly different according to Fischers LSD at P=0.05.

### Assay Experiment 3

A seedling screen was conducted to evaluate three PNW *F. pseudograminearum* isolates with 20 replications for four spring wheat cultivars (Sunco, Macon, 2-49, and Seri) with the leaf sheath sum rating documented by Wildermuth and McNamara (1994) and the crown rot index calculated according to Mitter et al. (2006) (Table 7). With the leaf sheath sum rating significant differences were realized only in isolates ( $p < 0.0001$ ). There were significant differences in the crown rot severity index among cultivars ( $p = 0.04$ ) and isolates ( $p < 0.0001$ ). Sunco and Macon had significantly higher crown rot severity indices than 2-49, while Seri was not significantly different from either grouping (data not shown).

**Table 7.** Mean crown rot severity of three PNW *F. pseudograminearum* isolates averaged over four wheat cultivars (Sunco, Macon, 2-49, and Seri) for two rating systems in screen AE-3.

Isolate treatment <sup>1</sup>	Leaf Sheath Sum (0 to 12) <sup>2</sup>	Crown rot severity index <sup>3</sup>
Isolate 2 (006-13)	5.5 a <sup>1</sup>	0.22 a
Isolate 4 (081-11)	5.1 a	0.24 a
Isolate 5 (032-06)	2.9 b	0.11 b
Untreated control	0.0 c	0.00 c
LSD <sup>4</sup>	1.05	0.06
CV	79	111

<sup>1</sup> Isolates were collected during the Smiley and Patterson (1996) field survey and collection data is outlined in Table 1.

<sup>2</sup> A total of 3 leaf sheaths was rated from 0 to 4 and summed for a total of 0 to 12 according to Wildermuth and McNamara (1994).

<sup>3</sup> A crown rot severity index was calculated by (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>4</sup> According to the least significant difference numbers within columns followed by the same letter are not significantly different at P=0.05.

PNW Isolates 2 and 4 were significantly more virulent than Isolate 5 for both rating systems. The disease level with inoculation was statistically greater than that of the untreated control. Isolate 2 consistently caused the most disease compared to other isolates screened across greenhouse assays AE-1, AE-2, and AE-3. Although there were not any statistical differences between cultivars with the leaf sheath sum rating across AE-2 and AE-3, Isolate 2 resulted in the most consistent and logical numeric mean separation between the parents of the RIL mapping population, Sunco and Macon. For this reason it was chosen to screen the Sunco / Macon ril mapping population.

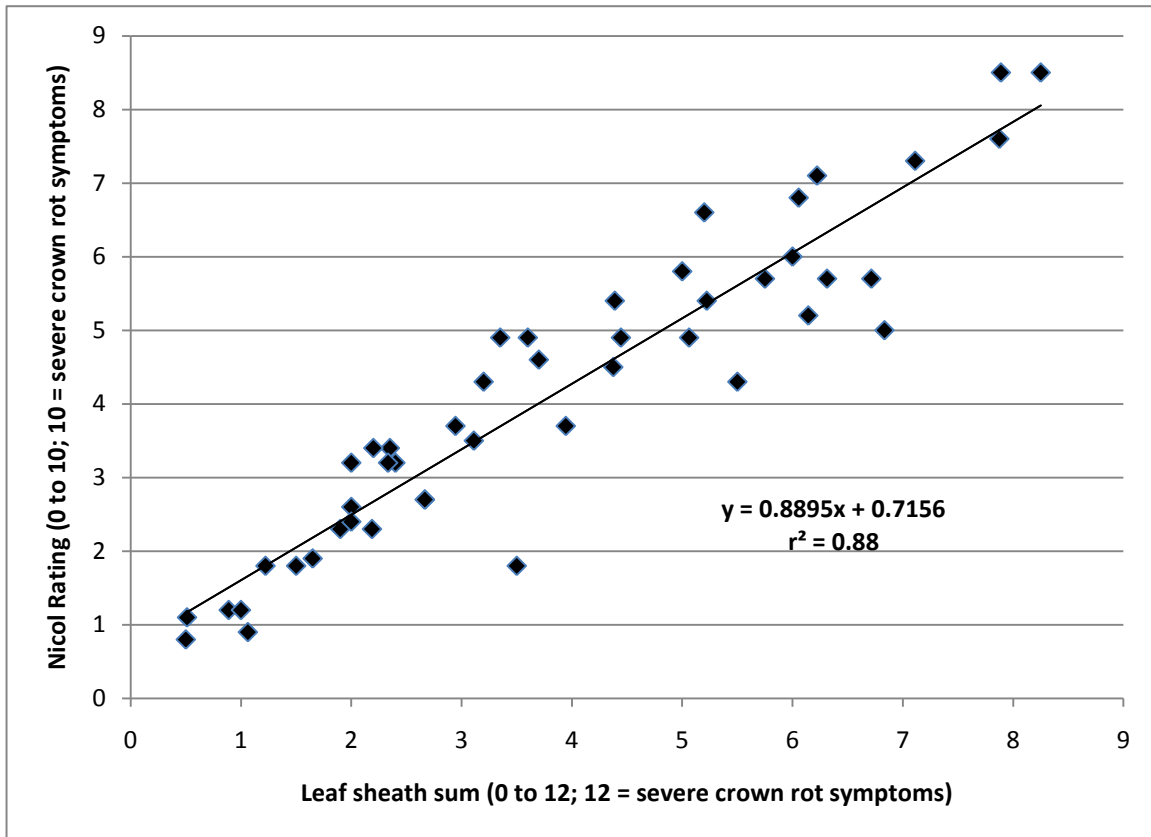


#### **Assay Experiment 4**

Significant differences were apparent between methods of inoculation between all three rating systems ( $p < 0.0001$ ) (Table 7). Several methods of inoculation for seedling assays were evaluated using two PNW isolates (4 and 5) on four spring wheat cultivars (Sunco, Macon, Seri, and 2-49) using three rating systems (Table 8). There was a significant cultivar by method interaction for the Nicol rating (0 to 10) ( $p = 0.04$ ) (Table 9; Figure 5).

The overall CV for the Nicol rating was 75, which was substantially lower than that of the leaf sheath sum (CV=95) and Mitter crown rot severity index (CV=161) rating systems (Table 8). The first leaf sheath rating (0 to 4) had the lowest CV=57 compared to the other rating systems. ANOVA indicated significant isolate effects for the leaf sheath sum ( $p = 0.01$ ) and Nicol rating systems ( $p = 0.007$ ), but not for the Mitter et al. (2006) rating system (Table 8). Differences between cultivars were apparent for the leaf sheath sum and the Nicol rating (0 to 10) systems, but not for the Mitter et al. (2006) crown rot severity index (Table 9).

There was a significant correlation between the independent variable leaf sheath sum and the dependent variable Nicol rating (0 to 10) ( $r^2 = 0.88$ ;  $p < 0.0001$ ) (Figure 4). Mean separations according to LSD tests were similar between leaf sheath sum and the Nicol (0 to 10) rating (data not shown). It appears that the rating of the first leaf sheath also gave acceptable mean separation comparable to that of the sum of three leaf sheaths (i.e. leaf sheath sum) (Table 8). The Mitter et al. (2006) crown rot index showed significant differences between methods of inoculation with an extremely high CV=161 (Table 8).



**Figure 4.** Linear regression of the leaf sheath sum (0 to 12) rating outlined by Wildermuth and McNamara (1994) and the Nicol rating (0 to 10) systems ( $r^2 = 0.88$ ;  $p < 0.0001$ ) averaged across four cultivars and six method of inoculation treatments in the AE-4 experiment.

**Table 8.** Summary of methods of inoculation for three rating systems with two PNW *F. pseudograminearum* isolates 4 (081-11) and 5 (032-06) evaluated in AE-4.

Method of Inoculation	(0 to 4)		(0 to 12)		Crown rot severity index <sup>3</sup>
	First leaf sheath <sup>1</sup>		Leaf sheath sum <sup>2</sup>		
	Is. 4	Is. 5	Is. 4	Is. 5	
1. Seedling dip <sup>5</sup> Water suspension	2.9 a <sup>4</sup>	2.8 a	5.3 ab	5.4 ab	0.94 a
2. Seedling dip <sup>5</sup> 1% MC suspension	2.6 ab	2.4 abc	6.0 ab	4.4 bc	0.87 a
3. Stem droplet <sup>6</sup> Water suspension	1.9 cd	0.9 e	3.6 cd	1.5 e	0.15 c
4. Stem droplet <sup>6</sup> 1% MC suspension	1.6 cd	0.9 e	2.5 de	1.5 e	0.05 c
5. Colonized Millet grain <sup>7</sup>	0.4 e	2.8 a	0.9 e	6.2 a	0.56 b
6. Agar straw 8.0 x 10 <sup>5</sup> conc. <sup>8</sup>	2.1 bcd	2.1 bcd	4.9 abc	4.7 abc	0.27 c
7. Agar straw 5.0 x 10 <sup>5</sup> conc. <sup>8</sup>	1.5 d	-----	3.3 cd	----	0.14 c
Mean	2.1	1.9	4.3	3.9	0.39
LSD <sup>8</sup> (P=0.05)	0.5	0.5	1.7	1.7	0.25
CV <sup>9</sup>	57		95		161

<sup>1</sup> First leaf sheath rating was taken as a subset of the Wildermuth and McNamara (1994) 0 to 4 rating of the first leaf sheath only.

<sup>2</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>3</sup> A crown rot severity index was calculated (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>4</sup> Numbers followed by the same letter within a column are not significantly different at P=0.05.

<sup>5</sup> The seedling dip method was adapted from Li et al. (2008) at a conidial concentration of 1 x 10<sup>6</sup> in water and 1% methyl cellulose suspensions, respectively.

<sup>6</sup> The stem droplet method was the placement of a 10 µL droplet at the stem base at 10 days after emergence according to Mitter et al. (2006).

<sup>7</sup> The colonized grain millet method was conducted according to Smiley et al. (2005b) in the greenhouse.

<sup>8</sup> The agar straw method was done by placing a cut plastic straw at the stem base and filling it with a 0.75 % agar conidial solution.

<sup>9</sup>CV = Coefficient of variation calculated by  $\sqrt{(\text{sample variance}/\text{mean})} \times 100$ .

**Table 9.** Mean crown rot severity for four cultivars evaluated in AE-4 over seven methods of inoculation using three rating systems and the first leaf sheath rating (0 to 4) of the leaf sheath sum.

Cultivar <sup>1</sup>	First leaf sheath <sup>2</sup>	Leaf sheath sum <sup>3</sup>	Crown rot severity index <sup>4</sup>
1. Seri (S)	2.2 a <sup>5</sup>	4.6 a	0.47 a
2. 2-49 (PR)	1.8 b	3.6 b	0.33 a
3. Sunco (PR)	1.7 b	3.3 b	0.34 a
4. Macon (S)	2.3 a	5.0 a	0.42 a
LSD (P=0.05)	0.29	0.82	0.24 (ns) <sup>6</sup>

<sup>1</sup> Two check varieties 2-49 (PR=partially resistant) and Seri (S=susceptible) and two ril mapping population parents Sunco (PR) and Macon (MS=moderately susceptible) were selected on the basis of their varying degrees of susceptibility and partial resistance.

<sup>2</sup> First leaf sheath rating was taken as a subset of the Wildermuth and McNamara (1994) 0 to 4 rating of the first leaf sheath only.

<sup>3</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>4</sup> A crown rot severity index was calculated by (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>5</sup> Numbers followed by the same letter within a column are not significantly different at P=0.05.

<sup>6</sup> (ns) = not statistically significant at P=0.05.

The seedling dip and colonized millet grain methods of inoculation resulted in the greatest disease severity for crown rot severity across the three rating systems, with the exception of the colonized millet grain treatment with Isolate 4 (Table 8). For the leaf sheath sum rating the seedling dip, colonized millet grain, and agar straw treatments were not significantly different from each other in crown rot severity. For the Mitter et al. (2006) crown rot severity index the seedling dip methods of inoculation had significantly greater severity than all other treatments, followed by the colonized grain treatment. For

this rating system the agar straw and stem drop methods of inoculation were not significantly different from each other in crown rot severity.

For all three rating systems the stem droplet method of Mitter et al. (2006) resulted in the least amount of disease severity, with the exception of the colonized grain treatment with Isolate 4 (Table 8). For the first leaf sheath rating the seedling dip and colonized grain method treatments resulted in the greatest disease severity and were not significantly different from each other for Isolate 5. For the first leaf sheath and the leaf sheath sum ratings the agar straw method ( $8 \times 10^5$  conidial concentration) resulted in significantly greater disease than that of the  $5 \times 10^5$  conidial concentration. Isolate 4 generally produced a greater amount of disease than Isolate 5, with the exception of the colonized grain inoculation method (Table 8).

There were statistical differences between cultivars for the leaf sheath sum and first leaf sheath rating systems, but not for the crown rot severity index (Table 9). There was a distinct mean separation between partially resistant and susceptible cultivars for the leaf sheath sum and first leaf sheath rating systems. For the leaf sheath sum rating, the susceptible check Seri and susceptible RIL mapping population parent Macon were not statistically different in disease severity. Likewise the partially resistant check 2-49 and parent Sunco were not statistically different from each other, but were significantly different from both susceptible checks (Table 9).

There was a significant cultivar by method of inoculation interaction ( $p=0.04$ ) for the Nicol rating (0 to 10) system (Figure 5; Table 10). The seedling dip and stem droplet methods of inoculation resulted in greater disease severity in Seri than 2-49, whereas that of the colonized millet grain and agar straw treatments produced numerically greater

disease severity in 2-49 over Seri for this rating system only (Figure 5). The colonized millet grain and agar straw treatments produced more disease with Macon compared to Sunco (Table 10). The CV was the lowest for the seedling dip with a water based conidial suspension (CV=51), colonized grain millet (CV=61), and agar straw methods ( $8 \times 10^5$  conidial concentration) of inoculation (CV=68). As disease severity decreased among the inoculation treatments, the CV seemed to increase, especially in the stem droplet treatments. Data from the colonized grain millet treatment was eliminated from further analyses for Isolate 4 due to a non-typical low level of disease severity.

Significant differences between cultivars within inoculation treatments were observed for the seedling dip with water-based conidial suspension (P=0.04) and marginally with the stem droplet in the 1% methyl cellulose conidial suspension (P=0.06), rated according to the Nicol rating system (Table 10). With the seedling dip water based conidial suspension treatment, the partially resistant check 2-49 had significantly less disease than the susceptible check Seri. However, for the seedling dip treatment, neither Sunco nor Macon were significantly different from the susceptible check Seri. There were significant numbers of dead or non-emerged plants for Sunco and Macon from the seedling dip treatments (data not shown). Although differences between Sunco and Macon were not statistically significant for the colonized grain millet treatment, numerical differences were pronounced with Macon showing substantially more crown rot severity than Sunco (Table 10).

**Table 10.** Summary statistics for crown rot severity of seven methods of inoculation evaluated with four cultivars rated according to the Nicol rating system (0 to 10) in AE-4.

Method of Inoculation	CV <sup>2</sup>	Variety <sup>1</sup>			
		Sunco (PR)	Macon (S)	2-49 (PR)	Seri (S)
1. Seedling dip Water suspension <sup>4</sup>	51	6.16 a <sup>3</sup>	6.34 a	3.66 b	8.27 a
2. Seedling dip 1% MC suspension <sup>4</sup>	76	4.08	3.73	4.44	7.06
3. Stem droplet Water suspension <sup>5</sup>	131	1.40	2.05	1.84	2.86
4. Stem droplet 1% MC suspension <sup>5</sup>	79	1.05 b	1.50 ab	1.38 ab	2.18 a
5. Colonized Millet grain <sup>6</sup>	61	4.44	7.89	6.00	5.75
6. Agar straw 8.0 x 10 <sup>5</sup> conc. <sup>7</sup>	68	4.02	4.45	4.16	3.66
7. Agar straw 5.0 x 10 <sup>5</sup> conc. <sup>7</sup>	98	2.00	3.35	2.66	2.40

<sup>1</sup> Two check varieties 2-49 (PR=partially resistant) and Seri (S=susceptible) and two rice mapping population parents Sunco (PR) and Macon (S) were selected on the basis of their varying degrees of susceptibility and partial resistance.

<sup>2</sup> CV = Coefficient of variation calculated by  $\sqrt{(\text{sample variance}/\text{mean})} \times 100$ .

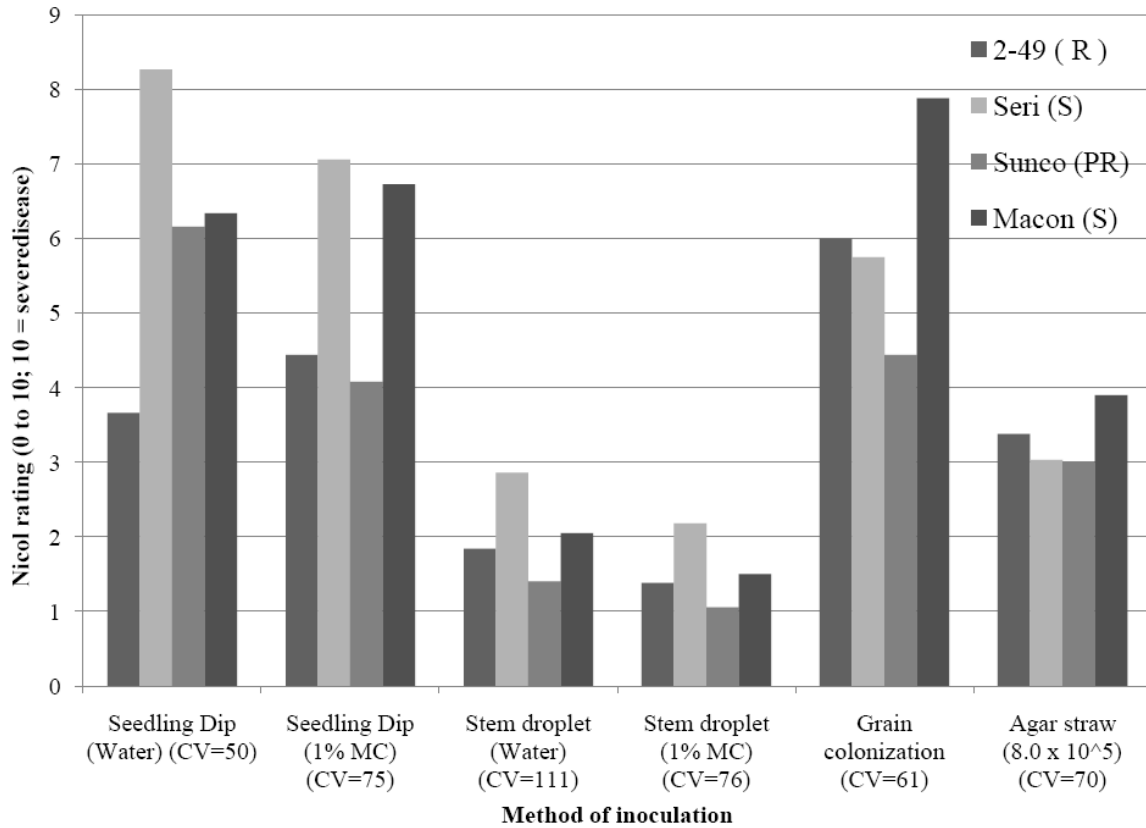
<sup>3</sup> Numbers followed by the same letter within a row are not significantly different at P=0.05.

<sup>4</sup> The seedling dip method was adapted from Li et al. (2008) at a conidial concentration of  $1 \times 10^6$  in water and 1% methyl cellulose suspensions, respectively and was significant for cultivars with the water based suspension (LSD = 2.44; P=0.04).

<sup>5</sup> The stem droplet method was the placement of a 10  $\mu$ L droplet at the stem base at 10 days after emergence according to Mitter et al. (2006). The stem droplet treatment with the 1% methyl cellulose suspension was significant for cultivars (LSD = 0.82; P=0.06).

<sup>6</sup> The colonized grain millet method was conducted according to Smiley et al. (2005b) in the greenhouse method.

<sup>7</sup> The agar straw method was done by placing a cut plastic straw at the stem base and filling it with a 0.75 % agar conidial solution.



**Figure 5.** Response of four spring wheat cultivars to six different methods of inoculation using the Nicol (0 to 10) rating system. The interaction between cultivars and methods of inoculation was significant ( $P=0.04$ ) according to ANOVA.



## Assay Experiment 5

ANOVA indicated significant differences between seedling soaking inoculation treatments ( $p < 0.0001$ ) but not for cultivars (Table 11). ANOVA showed that the inoculation effect accounted for 96% of the total variation. The seedling soaking method was significantly greater than those of the sterile control treatments as expected. Although there were not any statistical differences in crown rot severity between cultivars, Sunco had lower numerical values of crown rot severity than Macon. There were a high degree of missing non-emerged plants in the Seri and Macon treatments.

**Table 11.** Crown rot severity of four spring wheat cultivars evaluated with a non-germinated seedling dipping method in a conidial suspension of  $1.0 \times 10^6$  conidia per mL distilled water with *F. pseudograminearum* PNW Isolate 4 (081-11) in AE-5.

Cultivar <sup>4</sup>	Leaf Sheath Sum <sup>1</sup> (0 to 12) (ns) <sup>3</sup>	Nicol rating <sup>2</sup> (0 to 10) (ns) <sup>3</sup>
1. Sunco (PR)	7.0	4.8
2. Macon (S)	9.3	8.3
3. 2-49 (PR)	9.5	7.0
4. Seri (S)	12.0	10.0
CV <sup>5</sup>	30	38

<sup>1</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>2</sup> The Nicol rating represented a single objective rating of disease severity on the entire stem from 0 to 10 according to Nicol et al. (2001).

<sup>3</sup> (ns) = treatments were not significantly different at  $P=0.05$ .

<sup>4</sup> Two check varieties 2-49 (PR=partially resistant) and Seri (S=susceptible) and two ril mapping population parents Sunco (PR) and Macon (MS=moderately susceptible) were selected on the basis of their varying degrees of susceptibility and partial resistance.

<sup>5</sup> CV = Coefficient of variation calculated by  $\sqrt{(\text{sample variance}/\text{mean})} \times 100$ .

### **Assay Experiment 6**

Six methods of inoculation were evaluated on four spring wheat cultivars using 3 different rating systems and only the method of inoculation was significant for the first leaf sheath ( $p < 0.0001$ ), leaf sheath sum ( $p = 0.024$ ), and the Nicol rating systems ( $p = 0.020$ ) (Table 12). Mean disease levels were lower than in previous experiments. The seedling dip and agar straw methods resulted in significantly greater disease severity than the stem droplet method for the three significant rating systems.

### **Assay Experiment 7**

The methods of inoculation were significantly different for the first leaf sheath subset ( $p = 0.002$ ), leaf sheath sum ( $p = 0.002$ ), and Nicol rating ( $p = 0.003$ ) systems, but not the Mitter et al. (2006) crown rot severity rating (Table 13). There were also significant differences between cultivars for the first leaf sheath ( $p = 0.005$ ), leaf sheath sum ( $p = 0.0002$ ), Nicol rating ( $p < 0.0001$ ), and the Mitter crown rot severity ( $p = 0.0015$ ) (Table 13). Disease severity and CV values were within the acceptable range of past experiments. The first leaf sheath, leaf sheath sum, and Nicol rating systems had CV values within the acceptable range of 61%, 72%, and 75%, respectively.

Crown rot severity was significantly greater for the seedling dip method using the higher conidial concentration of  $5 \times 10^5$  than the lower concentration of  $2.5 \times 10^5$  or the colonized grain millet treatments for the first leaf sheath, leaf sheath sum, and Nicol rating systems. The colonized grain millet treatment and agar straw treatments had significantly greater virulence than the stem base droplet method for the Nicol rating and Mitter crown rot severity rating. The colonized grain millet and agar straw methods were

moderate in disease severity and not significantly different from each other for any of the rating methods (Table 13).

**Table 12.** Mean crown rot severity for six methods of inoculation used in AE-6 inoculated with *F. pseudograminearum* PNW Isolate 2 (006-13) on four spring wheat cultivars (Sunco (PR), Otis (S), 2-49 (PR), and Seri (S)).

Method of Inoculation <sup>1</sup>	(0 to 4) First Leaf Sheath <sup>2</sup>	(0 to 12) Leaf Sheath Sum <sup>3</sup>	(0 to 10) Nicol Rating <sup>4</sup>	Crown rot severity rating <sup>5</sup>
1. Seedling dip	1.1 a <sup>6</sup>	2.5 a	1.8 a	0.120 a
2. Stem base droplet	0.3 c	0.6 b	0.3 c	0.009 a
3. Colonized millet grain	0.4 bc	0.7 b	0.5 bc	0.014 a
4. Dry seedling dip	0.7 abc	1.5 ab	0.9 abc	0.035 a
5. Soil based inoculation	0.7 abc	1.6 ab	1.2 abc	0.100 a
6. Agar straw conidial Suspension	0.8 ab	2.2 a	1.4 ab	0.060 a
Mean	0.60	0.54	1.03	0.059
LSD <sup>7</sup>	0.47	1.28	0.96	0.76

<sup>1</sup> The seedling dip method was adapted from Li et al. (2008) at a conidial concentration of  $1 \times 10^6$  in distilled water. The stem base droplet method was the placement of a 10  $\mu$ L droplet at the stem base at 10 days after emergence according to Mitter et al. (2006). The colonized grain millet method was conducted according to Smiley et al. (2005b) in the greenhouse. The agar straw method was done by placing a cut plastic straw at the stem base and filling it with a 0.75 % agar conidial solution of  $1 \times 10^6$  conidia per mL.

<sup>2</sup> First leaf sheath rating was taken as a subset of the Wildermuth and McNamara (1994) 0 to 4 rating of the first leaf sheath only.

<sup>3</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>4</sup> The Nicol rating represented a single objective rating of disease severity on the entire stem from 0 to 10 according to Nicol et al. (2001).

<sup>5</sup> A crown rot severity index was calculated by taking (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>6</sup> Numbers followed by the same letter within a column are not significantly different at  $P=0.05$ .

<sup>7</sup> LSD = Fischers LSD calculated with SAS.

**Table 13.** Mean crown rot severity for six methods of inoculation used in AE-7 inoculated with *F. pseudograminearum* PNW Isolate 2 (006-13) on four spring wheat cultivars (Sunco (PR), Otis (S), 2-49 (PR), and Seri (S)).

Method of Inoculation <sup>1</sup>	(0 to 4) First Leaf Sheath <sup>2</sup>	(0 to 12) Leaf Sheath Sum <sup>3</sup>	(0 to 10) Nicol Rating <sup>4</sup>	Crown rot severity rating <sup>5</sup>
1. Seedling dip (Conidial conc. $5 \times 10^5$ )	2.4 a	5.9 a	4.6 a	0.07 b
2. Seedling dip (Conidial conc. $2.5 \times 10^5$ )	1.5 c <sup>6</sup>	3.4 bc	2.7 bcd	0.07 b
3. Stem base droplet	1.2 c	2.6 c	1.8 d	0.07 b
4. Colonized millet grain	1.8 bc	4.1 bc	3.2 bc	0.24 a
5. Soil based inoculation	1.5 c	2.9 c	2.3 cd	0.10 ab
6. Agar straw conidial Suspension	2.1 ab	5.0 ab	3.7 ab	0.19 ab
Mean	1.7	3.8	2.9	0.14
LSD <sup>7</sup>	0.6	1.6	1.3	0.16

<sup>1</sup> The seedling dip method was adapted from Li et al. (2008) at two conidial concentrations of  $5 \times 10^5$  (treatment 1) and  $2.5 \times 10^5$  (treatment 2) in distilled water. The stem base droplet method was the placement of a 10  $\mu$ L droplet at the stem base at 10 days after emergence according to Mitter et al. (2006). The colonized grain millet method was conducted according to Smiley et al. (2005b) in the greenhouse. The agar straw method was done by placing a cut plastic straw at the stem base and filling it with a 0.75 % agar conidial solution of  $1 \times 10^6$  conidia per mL adapted from de la Peña and Murray (1994).

<sup>2</sup> First leaf sheath rating was taken as a subset of the Wildermuth and McNamara (1994) 0 to 4 rating of the first leaf sheath only.

<sup>3</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>4</sup> The Nicol rating represented a single objective rating of disease severity on the entire stem from 0 to 10 according to Nicol et al. (2001).

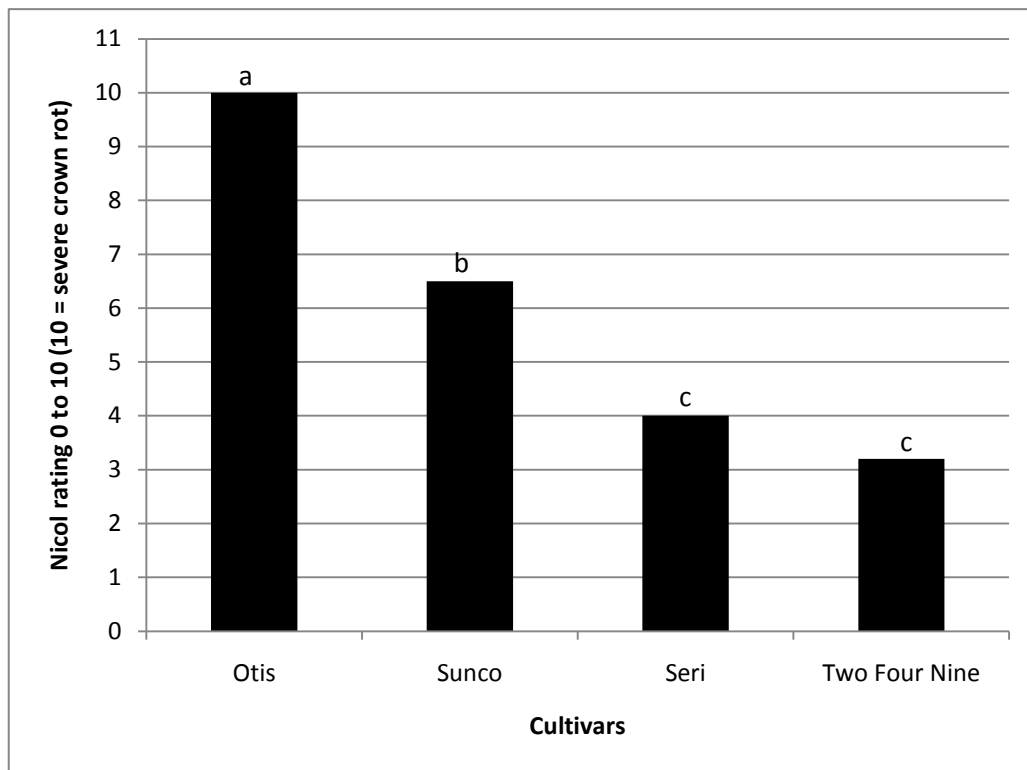
<sup>5</sup> A crown rot severity index was calculated by (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>6</sup> Numbers followed by the same letter within a column are not significantly different at  $P=0.05$ .

<sup>7</sup> LSD = Fischers LSD calculated with SAS.

The soil based inoculation method had significantly less disease than the agar straw method, but was not significantly different from the grain colonization method.

There were significant differences between cultivars for all of the rating systems. Sunco was not significantly different from Otis in disease severity for any of the rating systems. However, Seri had significantly more disease severity than all the other cultivars for all of the rating systems (Table 14). When cultivar means were evaluated with the high conidial concentration ( $5 \times 10^5$ ) seedling dip treatment, Sunco and Otis had significantly greater disease than the susceptible check Seri (Figure 6). There were many missing and non-emerged plants with both the Otis and Sunco cultivar treatments using the seedling dip method of inoculation.



**Figure 6.** Relative ranking of cultivars for the seedling dip method with a conidial concentration of  $5 \times 10^5$  according to the Nicol rating scale (0 to 10) in AE-7. Bars with the same letter are not significantly different according to Fischers LSD at  $P=0.05$ .

**Table 14.** Mean crown rot severity for four cultivars in AE-7 inoculated with *F. pseudograminearum* PNW Isolate 2 (006-13) averaged across six methods of inoculation treatments.

Cultivar <sup>1</sup>	(0 to 4) First Leaf Sheath <sup>2</sup>	(0 to 12) Leaf Sheath Sum <sup>3</sup>	(0 to 10) Nicol Rating <sup>4</sup>	Crown rot severity rating <sup>5</sup>
1. Sunco (PR)	1.5 b <sup>6</sup>	3.4 b	2.7 b	0.10 b
2. Otis (S)	1.4 b	3.2 b	2.4 b	0.07 b
3. 2-49 (PR)	1.4 b	2.9 b	2.1 b	0.07 b
4. Seri (S)	2.3 a	5.5 a	4.3 a	0.28 a
Mean	1.6	3.7	2.8	0.13
LSD	0.5	1.4	1.2	0.17

<sup>1</sup> Two check varieties 2-49 (PR=partially resistant) and Seri (S=susceptible) and two ril mapping population parents Sunco (PR) and Otis (S) were selected on the basis of their varying degrees of susceptibility and partial resistance.

<sup>2</sup> First leaf sheath rating was taken as a subset of the Wildermuth and McNamara (1994) 0 to 4 rating of the first leaf sheath only.

<sup>3</sup> Leaf sheath sum rating was conducted by rating the three oldest stems from 0 to 4 and adding each stem rating to total 0 to 12 according to Wildermuth and McNamara (1994).

<sup>4</sup> The Nicol rating represented a single objective rating of disease severity on the entire stem from 0 to 10 according to Nicol et al. (2001).

<sup>5</sup> A crown rot severity index was calculated by taking (length of stem discoloration/seedling height) x total number of leaf sheaths with lesions according to Mitter et al. (2006).

<sup>6</sup> Numbers followed by the same letter within a column are not significantly different at P=0.05 according to Fischers LSD.

## DISCUSSION

Smiley and Patterson (1996) reported that the dominant species isolated from PNW wheat fields in association with crown rot symptoms was *F. pseudograminearum*. Smiley et al. (2005b) evaluated the relative pathogenicity of *F. pseudograminearum* isolates collected in that survey in the greenhouse and field. The authors reported that *F. pseudograminearum* isolates were the most pathogenic species in greenhouse screens compared to the five major species involved in the crown rot infection complex. Smiley et al. (2005b) also found that there was a high level of variation in pathogenicity of these isolates and reported that recognition and management of this variation was important for evaluating resistance or tolerance in wheat.

Results from the current study agree with that of Smiley et al. (2005b). The degree of disease severity response of 11 cultivars with varying levels of partial resistance in AE-1, varied significantly. There was a significant variety x cultivar interaction ( $P=0.04$ ) indicating that the degree of disease severity caused by *F. pseudograminearum* isolates varied among cultivars. However, according to ANOVA the isolate effect accounted for the greatest amount of variation in AE-1, suggesting that isolates have a greater effect on variation than cultivars. Across all the cultivars evaluated in two other greenhouse screens (AE-2 and AE-3), the most pathogenic isolates were Isolate 2 (006-13), Isolate 4 (081-11), and Isolate 5 (032-06). These results agree with the degree of pathogenicity of these isolates performed in the greenhouse (Table 1; Smiley et al. 2005b). Mitter et al. (2006) also described significant isolate x cultivar interactions where similar cultivars (Sunco, Baxter, and Puseas) were evaluated in inoculated greenhouse seedling screens conducted in Australia.

The ultimate objective of this research was to quantify the current sources of resistance, and unknown PNW sources, against *Fusarium* spp. important to the PNW region in greenhouse screens for QTL identification. These results report the first relative response of Australian resistant sources (Sunco and 2-49) to *F. pseudograminearum* isolates collected in the PNW in greenhouse evaluations. The relative partial resistance of Sunco and partial susceptibility of two recombinant inbred line (ril) mapping population parents Macon and Otis, were quantified with various PNW *F. pseudograminearum* isolates. While the leaf sheath sum assessment and the crown rot severity index showed similar cultivar responses to isolates in mean separations (Fischer's LSD) in the first three greenhouse screens (AE-1, AE-2, and AE-3), the latter was a much more quantitative measure of disease severity. Isolate 2 (006-13) caused the most consistent crown rot severity in three greenhouse screens (AE-1, AE-2, and AE-3) utilizing a colonized millet grain method of inoculation method developed by Smiley et al. (2005b) and was used to screen two ril mapping populations to identify resistance QTL.

Reliable screening systems are required for accurate quantification of resistance and identification of resistance QTL (Wildermuth and McNamara, 1994; Wallwork et al. 2004; Mitter et al. 2006). Wildermuth and McNamara (1994) suggested that disease assessments must be accurate as not all rating systems and assessment methods permit detection of resistant lines/cultivars. Other methods of inoculation have been developed with different objectives and potential applications with the overall intent to screen a high number of individuals with the least degree of variation (Wallwork et al. 2004; Mitter et al. 2006; Li et al. 2008).



Three greenhouse experiments (AE-4, AE-6, and AE-7) were performed to evaluate the effect of five methods of inoculation including the seedling dip (at varying concentrations), stem droplet (10 µL droplet on the stem base), colonized millet grain, soil based inoculation, and agar conidial slurry placed in a straw at the stem base from previous reports (Li et al. 2008; Mitter et al. 2006; Smiley et al. 2005b; de la Peña and Murray, 1994). These experiments were assessed with three rating assessment systems that had been previously reported. Results showed that there can be significant variation introduced by different inoculation methods and rating assessments that can affect the measured cultivar response.

The cultivar response in crown rot severity in AE-4 was logical for the first leaf sheath and leaf sheath sum rating systems where the checks 2-49 and Seri were significantly different from each other. In addition Sunco was significantly different from Macon but not 2-49. In AE-7 the overall cultivar response was similar in that Seri had significantly greater disease than all other cultivars, but Sunco was not significantly different from Otis or 2-49 regardless of the rating system. There was a significant isolate effect in AE-4, mainly due to decreased pathogenicity of the colonized grain millet method with Isolate 4 (081-11). It is unknown why Isolate 4 failed to result in causing disease in AE-4, but could have had to do with inoculum age and lack of viable conidia.

The seedling dipping method of inoculation described by Li et al. (2008) resulted in the greatest crown rot severity on wheat seedlings in all three greenhouse screens. There was not an enhanced effect of adding 1% methyl cellulose to the conidial suspension for the seedling dip treatment in AE-4. When conidial concentrations were

reduced from  $1 \times 10^6$  conidia per ml (AE-4) to  $5 \times 10^5$  conidia per ml (AE-7) with the seedling dip method, the mean crown rot severity with the leaf sheath sum rating was 6.2 (AE-4 - Isolate 5) and 5.9 (AE-7 - Isolate 2), respectively. However, the cultivar response was compromised with the seedling dip method. While Seri had significantly greater crown rot severity than 2-49 at conidial concentrations of  $1 \times 10^6$  conidia per mL suspension in AE-4, Sunco and Macon were not significantly different in disease severity. In AE-7 a similar response resulted at the conidial concentration of  $5 \times 10^5$  where Sunco and Otis both had greater levels of disease severity than the susceptible check Seri. At lower conidial concentrations of  $2.5 \times 10^5$ , the cultivar response in disease severity was not significant ( $P=0.56$ , data not shown).

Li et al. (2008) reported a crown rot severity rating of between 2 and 3 with Sunco based on a 0 to 5 scale using the seedling dip method with a spore suspension concentration of  $5 \times 10^6$  conidia per ml. The authors compared the seedling dip method to the stem drop method described by Mitter et al. (2006) (concentration of  $1 \times 10^6$  conidia/ml) and found that the stem drop method resulted in much less disease severity where Sunco was rated  $<1$  and Ballaroi, a susceptible check, was rated  $<2$  on the same 0 to 5 scale.

The seedling dip method treatment in the present study consistently resulted in a disproportionate number of non-emerged plants in the Sunco treatment. The seedling dip treatment in all three greenhouse screens resulted in significantly greater disease than the stem drop method described by Mitter et al. (2006). Evaluation of inoculation methods showed that while the seedling dipping method of inoculation reported by Li et al. (2008) was rapid to use in screening greater volumes of plant material and resulted in logical

cultivar responses with stronger plant resistance (i.e. 2-49 and Seri), moderate levels of disease resistance could not be quantified. Wallwork et al. (2004) described Sunco as carrying a partial level of resistance, less than that of 2-49. Screens AE-1, AE-2 and AE-3 of the current study showed that Macon and Otis may carry a slight level of resistance as certain isolates did not cause significant crown rot severity.

A fourth experiment (AE-5) was adapted from the seedling dip method by dipping non-germinated seedlings in a conidial suspension (suspension concentration =  $1 \times 10^6$ ). This inoculation method has been successfully used by Nicol et al. (2007) for field evaluation of cultivars. The theory was dipping non-germinated seeds would be less damaging than dipping pre-germinated seedlings with exposed root radical tissue. Although there were pronounced numerical differences with this method, treatments were not statistically different due to a high degree of plant mortality in the Seri and 2-49 treatments.

The colonized millet grain method has been successfully utilized by Smiley et al. (2005b) in field and greenhouse studies to inoculate plants. In AE-4 the colonized grain millet method did not statistically differ from the seedling dip method for Isolate 5 (032-06). In AE-6 the colonized grain millet method did not result in significant disease severity. In AE-7 the colonized grain millet method resulted in significantly less crown rot severity than the seedling dip method (conidial concentration =  $5 \times 10^5$ ) for two of the three main rating systems.

The colonized grain millet method resulted in the most reasonable and logical cultivar response. In AE-7 Seri had significantly greater crown rot severity than Sunco, Otis, and 2-49, in that order of decreasing severity. Although there was not a statistical

difference in cultivar response to the colonized millet grain method in AE-4 with any of the rating systems, numerical values showed a prominent difference in the mean crown rot severity between Sunco (4.4) and Macon (7.8) with the Nicol 0 to 10 rating system. However, with this inoculation method, 2-49 (6.0) had slightly greater numerical crown rot severity than Seri (5.7), which resulted in the significant cultivar by method interaction with the Nicol 0 to 10 rating system. The response of the mapping population parent Otis in crown rot severity seemed to be less with these seedling greenhouse screens and inoculation methods. QTL mapping for the Sunco/Otis revealed that Otis exhibited two minor QTL for crown rot resistance in greenhouse, terrace and field screens (unpublished data).

In the current study the agar straw method adapted from de la Peña and Murray (1994) resulted in reliable and consistent results in adequate infection and cultivar response. In all experiments where it was evaluated (AE-4, AE-6, and AE-7) crown rot severity was not significantly different from that of the seedling dip, with the exception of the AE-4 Isolate 5 (032-06) treatment with the leaf sheath sum rating system. In AE-7 the agar straw method was not significantly different from the colonized millet grain method with any of the three rating systems. Although cultivars were not significantly different within the agar straw inoculation treatment, rankings were logical. The soil-based inoculation method resulted in crown rot severity ratings that were not significantly different from the colonized grain millet method in AE-7. The agar straw and soil-based methods have adequate application for *Fusarium* crown rot screening as they are reliable and relatively easy to apply and should be evaluated further as a comparable method to the colonized millet grain for identifying intermediate or moderate sources of resistance.

Three rating assessment systems were reported for use with seedling screens in the greenhouse in this research. Several methods of assessment exist and have been used by others including crown rot severity indices (Mitter et al. 2006), discoloration of tiller bases, numbers of whiteheads (Nicol et al. 2007), yield (Smiley et al. 2005a), number of healthy seedlings, percent diseased tissue (Wildermuth and McNamara, 1994). For the purposes of this research the 0 to 10 assessment adapted from Nicol et al. (2001) was the most efficient rating assessment method in terms of time, ease of use, and repeatability. The 0 to 10 rating system was highly correlated to the Wildermuth and McNamara (1994) leaf sheath sum ( $r^2=0.88$ ;  $P<0.0001$ ) and was able to accurately detect the significant cultivar by method interaction in AE-4.

The assessment methods of leaf sheath sum described and crown rot index are very accurate and repeatable, but time consuming and tedious compared to the 0 to 10 rating. The leaf sheath sum rating requires that 3 leaf sheaths be peeled back and assessed, while the crown rot index requires that plant height, lesion length, and the number of leaves with lesions be recorded. The 0 to 10 assessment is a simple visual rating that takes seconds to visualize and assess infected plants. Time and accuracy is important in genetic studies that require the screening of thousands of lines.

For the purposes of screening moderate to partial resistance in a greenhouse environment the colonized grain millet and agar straw methods are the most reliable and preferred. Future research should focus on the refinement of the agar straw conidial slurry inoculation method on a wider range of cultivars. Future research also needs to be conducted in the areas of reducing conidial concentrations for non-pre-germinated seed inoculation to modify AE-5 and the application of the soil-based inoculation method.

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## **CHAPTER 5**

**CHAPTER 5**  
**IDENTIFYING QUANTITATIVE TRAIT LOCI FOR FUSARIUM CROWN ROT**  
**RESISTANCE (*F. PSEUDOGRAMINEARUM*) IN THE SPRING WHEAT**  
**CULTIVAR SUNCO**

Grant Poole<sup>1</sup>, Richard W. Smiley<sup>2</sup>, Timothy D. Murray<sup>1</sup>, Julie M. Nicol<sup>3</sup>, Timothy C. Paulitz<sup>4</sup>, and Kimberly Garland-Campbell<sup>4</sup>. <sup>1</sup>*Washington State University, Dept. of Plant Pathology, Pullman, WA, 99164-6430; E-mail: [gpoole@wsu.edu](mailto:gpoole@wsu.edu); FAX: 509-335-9581;* <sup>2</sup>*USDA-Agricultural Research Service, Pendleton, OR.;* <sup>3</sup>*CIMMYT (International Maize and Wheat Improvement Centre) P.O. Box. 39 Emek 06511 Ankara, Turkey.* <sup>4</sup>*USDA-Agricultural Research Service, Pullman, WA.*

**ABSTRACT**

Fusarium crown rot (FCR), caused by a complex of *Fusarium* species, of which *F. pseudograminearum* and *F. culmorum* are the most important, reduces wheat yields in the Pacific Northwest (PNW) of the U.S. by an average of 9%. Currently there is not any consistent durable resistant in PNW wheat cultivars and plant host resistance is the most reliable and efficient means of controlling root diseases on a wide geographic scale. The advent of DNA-based markers has facilitated the application of marker assisted selection. Several significant QTL for crown rot resistance have been documented on chromosomes 1A, 1D, 2B, 3B, and 4B from resistant cultivars in Australia. Our objective was to identify major quantitative trait loci for Fusarium crown rot resistance in the Australian

spring wheat cultivar ‘Sunco’. Two mapping populations consisting of 151 F<sub>5</sub>:F<sub>6</sub> and 219 F<sub>6</sub>:F<sub>7</sub> recombinant inbred lines (RIL), were derived from crosses between Sunco (partially resistant) by Otis (susceptible) and Sunco by Macon (susceptible), respectively. A single PNW *F. pseudograminearum* isolate (006-13) was used to inoculate trials conducted in growth chamber, outdoor terrace and field assays during 2008 and 2009. Stem base crown tissues of seedlings (for the growth chamber assay) or adult plants (the terrace bed and field assays) were rated for disease severity on a numeric scale from 0-10. Genotypes of the parents and Sunco/Otis RIL population were evaluated with 354 DArT (Triticarte<sup>®</sup>) and 30 microsatellite SSR polymorphic markers. The parents and Sunco/Macon RIL population were evaluated with 328 DArT and 18 microsatellite SSR polymorphic markers. A total of five significant QTL were identified on chromosomes 3B, 4B, 4A, and 7A with LOD scores ranging from 3.0 to 14.3. The most significant QTL was inherited from Sunco and identified on chromosome 3BL across all three seedling growth chamber assays for the Sunco/ Macon population and one of the two growth chamber assays for the Sunco/Otis mapping population with maximum LOD scores of 14.3 and 10.0 explaining 28% and 23% of the variation, respectively for each population. This QTL covered a 3.8 cM region and was verified in the same chromosomal location of 3BL with field data for the Sunco/Macon population. SSR microsatellite markers will be evaluated in this QTL region for potential use in marker assisted selection for improving Fusarium crown rot resistance in PNW germplasm.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) production was approximately 659.3 million tons in 2009, which was second to maize (796.3 tons) and higher than rice (433.5) according to Foreign Agricultural Service production statistics (USDA-FAS, 2009). Due to its diversity of uses, protein content, and storage qualities, it is the most common staple food crop for more than one-third of the world's population (Sleper and Poehlman, 2006). Cook (2001) estimated that soil-borne root diseases cost the U.S. about 10 million tones in wheat production annually. Crown rot is one of the most detrimental soil-borne diseases of dryland wheat throughout the world, and in the Pacific Northwest (PNW) of the United States (Cook, 1992; Burgess et al., 2001; Backhouse et al. 2004; Nicol et al., 2007). The disease is caused by a complex of species of which *F. pseudograminearum* (O'Donnell & Aoki) (= *F. graminearum* group I, = *Gibberella coronicola*) and *F. culmorum* (Wm. G. Sm.) Sacc.) are the most significant world wide (Burgess et al. 2001; Smiley et al. 2005a; Nicol et al. 2007). Yield loss from crown rot have been documented to be from 9% to 89%, with an accepted average of 9% in the PNW, with world wide losses exceeding 30% (Cook, 1992; Kane, 1987; Burgess et al. 2001; Dodman and Wildermuth, 1987; Klein et al. 1991; Hekimham et al., 2004)

Symptoms of crown rot are described by the dark brown discoloration (with an occasional pink tinge) of the crown region and lower one, two, or sometimes three internodes (Cook, 1968; Cook and Veseth, 1991). Crown rot is typically associated with wheat production in semi-arid areas where wheat matures under hot-dry conditions because the fungus colonizes stressed plants during the grain filling period (Burgess et al., 2001). Fusarium crown rot pathogens survive in the soil as chlamydospores and thick

walled conidial cells in dead organic matter and plant debris infecting plants in the fall or spring (Cook and Veseth, 1991; Paulitz et al. 2002). Control of the disease has relied on cultural practices including late planting and moderate nitrogen fertilization to discourage large vegetative plants that tend to experience more stress during grain filling (Cook and Veseth, 1991).

Host plant resistance has been documented as being the most efficient and reliable approach to reducing wheat yield loss due to crown rot (Cook, 2001). Evaluation of resistance to *Fusarium* crown rot dates back to the 1960's in Australia (McKnight and Hart, 1966; Purss, 1966). Evaluations of PNW germplasm by Smiley and Yan (2009) showed there is a high degree of variation in response to *Fusarium* crown rot over years and across sites. As a result no cultivars in the PNW have been identified as being consistently resistant to the disease. Thus there is a need to introgress resistance into cultivars adapted to the PNW.

Resistance to crown rot has been described as 'polygenic', or from several potential resistance genes (Purss, 1970; Burgess et al. 2001; Collard et al. 2005; Bovill et al. 2006; Ma et al. 2009). Adult and seedling resistance have each been separately documented (Wallwork et al. 2004; Mitter et al. 2006). It appears that the most resistant genotypes can be detected in seedling bioassays, but genotypes with adult-plant resistance (such as that of the Australian cultivar 'Sunco') cannot be detected in seedling assays. Adult plant screening systems were reported to be necessary to identify resistant germplasm and quantitative trait loci (QTL) useful for breeding and to reduce the potential for significant crown rot resistance to remain undetected in seedling screening programs (Wallwork et al. 2004). Seedling resistance can be evaluated during the first 45

days of the seedling phase of growth. Adult plant resistance is documented by evaluating mature plants for crown rot symptoms and damage. Wallwork et al. (2004) described an outdoor terrace system and supplemental irrigation to facilitate the inoculation of plants with *Fusarium* spp. and subsequent plant growth and disease development. This system can be utilized in a breeding program to effectively screen for adult plant resistance.

Because crown rot is difficult to evaluate and subject to environmental variability, the use of genetic markers as indirect selection tools will facilitate breeding efforts.

Genetic markers flanking QTL that are associated with resistance have been identified in four genetic studies (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006; Ma et al. 2009). Wallwork et al. (2004) used the outdoor terrace screening system and reported the first QTL for crown rot resistance in wheat on chromosome 4B near the dwarfing gene *Rht-B1* (within 20 cM) in a population generated from a cross between ‘Kukri’ and ‘Janz’. Kukri was the resistant parent. Collard et al. (2005) confirmed this QTL on 4B, 19.8 cM from the *Rht-B1* gene using a seedling assay similar to that developed by Wildermuth and McNamara (1994).

Collard et al. (2005) evaluated a doubled haploid population derived from a cross between ‘2-49’ (partially resistant) and ‘Janz’ (susceptible) using a growth chamber assay in which seedlings were rated (Wildermuth and McNamara, 1994). The largest QTL were located on chromosomes 1D and 1A, with LOD scores of 7.5 and 3.2 explaining 21% and 9% of the variation, respectively. These resistance alleles were inherited from 2-49, but additional alleles were discovered on chromosome 2B that were inherited from the susceptible parent ‘Janz’. Bovill et al. (2006) also utilized a seedling assay similar to that of Collard et al. (2005) to evaluate a doubled haploid population derived from a cross

between parental lines ‘W21MMT70’ (partially resistant) x ‘Mendos’ (moderately susceptible). Three putative QTL were identified and located on chromosomes 2B, 2D, and 5D with LOD scores of 5.6, 2.2, and 9.4, respectively, but were not significant for all three seedling screening assays. The authors postulated that the resistance locus on 2BS was possibly from an introgression from *T. timopheevii*. This introgression has been documented in ‘Sunco’ (Kammholz et al. 2001).

Ma et al. (2009) reported QTL for resistance to Fusarium crown rot using a seedling dip inoculation method followed by a seedling assay for both *F. pseudograminearum* and *F. graminearum* resistance as outlined in Li et al. (2008). Two significant QTL were identified in a RIL population derived from CSCR6 (resistant)/Lang (susceptible), on chromosomes 3BL and 4B.. Both resistant alleles were derived from CSCR6. The QTL on 3BL had a combined LOD score of 11.1, explaining 41.6% of the variation in the data, and was named ‘*Qcrs.cpi-3B*’. The flanking DArT markers for the major QTL on chromosome 3BL were wPt10505 and wPt2277. The authors postulated that crown rot resistance was not species-specific as both *Fusarium graminearum* and *F. pseudograminearum* isolates returned similar QTL with the same chromosomal location (3BL).

Sunco is an additional source of partial resistance that also possesses useful properties to introgress into PNW breeding programs. Currently there is not any information on the genetic location of the resistance in the partially resistant cultivar Sunco. Identification of QTL would allow for the potential use of marker assisted selection (MAS) for applications of gene pyramiding and rapid backcrossing of crown rot resistance genes into locally adapted PNW wheat cultivars. The objective of this research



were to identify QTL in the cultivar Sunco using two recombinant inbred line populations derived from Sunco by PNW cultivars.

## **MATERIALS AND METHODS**

### **Plant materials**

Two recombinant inbred line (RIL) mapping populations were developed between 2006 and 2008 in greenhouses at the Plant Growth Facility at Washington State University, Pullman, WA. Two hundred and nineteen F<sub>6</sub>:F<sub>7</sub> recombinant inbred lines were generated from the Sunco/Macon cross. One hundred and fifty-one F<sub>5</sub>:F<sub>6</sub> recombinant inbred lines were generated from a cross between the Sunco/Otis cross. Sunco is a hard white spring bread wheat (*Triticum aestivum* L.) released from Australia in 1986 with partial resistance to stem and leaf rust and carries partial resistance to Fusarium crown rot (Woolston, 2000; Wheat pedigree and identified alleles of genes on-line, 2006, <http://genbank.vurv.cz/wheat/pedigree/krizeni2.asp?id=48411>). Otis is a Pacific Northwest hard white spring bread wheat cultivar released in 2005 and known for its adaptation to arid and intermediate rainfall zones, high yield potential, and high temperature adult plant resistance to local races of stripe rust, partial resistance to Hessian fly, and dual purpose end use quality for noodle and bread making (Kidwell et al. 2006). Macon is a Pacific Northwest hard white spring wheat released in 2002 and adapted to intermediate to high rainfall non-irrigated growing conditions known for its Hessian fly resistance and bread and noodle making qualities (Kidwell et al. 2003).

### **Phenotypic screening**

A single *Fusarium pseudograminearum* isolate (006-13) collected near Duffur, Wasco County, Oregon by Smiley and Patterson (1996) and documented in field

screening assays according to Smiley and Yan (2009) was used for screening both RIL mapping populations in this study. This single isolate was used to screen both the Sunco/Macon RIL population and the Sunco/Otis RIL population in seedling growth chamber assays (similar to Wildermuth and McNamara, 1994 and Mitter et al. 2006), an outdoor terrace screening method similar to that of Wallwork et al. (2004), and inoculated field trials in North Central Washington.

The procedure for inoculum preparation for the seedling evaluation conducted in the growth chamber and adult plant evaluation using outdoor terrace screening trials was similar to that used by Smiley et al. (2005a) and Smiley and Yan (2009). To prepare inoculum, 100 grams of white pearl millet seed were placed into 0.5-Liter Mason jars and autoclaved twice over a two-day interval at a temperature of 121 °C at 15 atm. psi for 45 minutes. PNW isolate 006-13 of *F. pseudograminearum* was grown in 20% potato dextrose agar (PDA) for 10 days under fluorescent lights set at a 12-hour photoperiod at 26 °C. Four 1 cm squares of the PNW *F. pseudograminearum* isolate (006-13) was added to the jars and incubated at 22 °C for 21 days prior to growth chamber and terrace inoculations.

Field trials were inoculated with a liquid conidial suspension of the same single *F. pseudograminearum* isolate (006-13) (Nicol et al. 2007). To prepare liquid inoculum, the respective isolate was grown on colonized millet as described previously. Approximately 100 g of colonized millet was placed in cheesecloth and flushed with de-ionized distilled water to make approximately 125 mL of conidial solution. Conidial concentrations were checked with a haemocytometer and adjusted to 250,000 conidia per mL of solution (Mitter et al. 2006; Nicol et al. 2004). Seeds of each line were placed in plastic weigh

boats and soaked in 1 mL of the conidial suspension (500,000 conidia per mL) for 1 to 3 minutes and dried approximately two days prior to planting.

### **Growth chamber seedling screening**

Three runs of the growth chamber assay were conducted for the Sunco/Macon RILs; harvested and rated on July 7 (2008), Sept. 20<sup>th</sup> (2008), and Sept. 15<sup>th</sup> (2009). Two growth chamber assays were conducted for the Sunco/Otis RILs; harvested and rated on July 15<sup>th</sup> and Sept. 17<sup>th</sup>, 2009. For the growth chamber assays, seeds of each cultivar were surface disinfested in 95% EtOH (ethanol) for 6 minutes, rinsed in de-ionized distilled water for 1 minute, then placed in 0.5% NaOCl for 10 minutes, followed by six rinses (1 minute each) in de-ionized distilled water. Following surface disinfestation, seeds were placed on moist filter paper and incubated for 3 days. Seedlings were placed in a 4 C for 4 to 5 days to obtain consistent germination.

Seedlings were planted into a medium comprised of 50% sand and 50% peat (Greensmix Sphagnum Peat Moss, Wuapaca Northwoods LLC., Wuapaca, WI) (v/v) in 4 cm diameter x 20.5 cm long cone-tainers (Stuewe and Sons, Corvallis, OR) arranged in plastic trays and arranged in a randomized complete block design with 10 replications. The experiment was conducted in three 964 ft<sup>3</sup> (Convicon, Winnipeg, CA.) growthrooms located at the WSU Plant Growth Facility in Pullman WA. Each growth chamber could only contain ~1,200 plants. Therefore, the Sunco/Macon RILs were split into two groups for testing with common check and parental genotypes included in each growth chamber. Genotypes within growth room sets were arranged in a randomized complete block design, with 10 replications.

Seedlings were grown at 60/80 ( $\pm 5$ )% day/night RH, a 12 hour photoperiod, and 25/15 °C day/night temperatures for the duration of the experiment, 35 days after inoculation. Plants were allowed to dry and symptoms were rated on dried tissue. Crown rot disease symptoms were rated using a 0 to 10 scale (0=no disease observed, 10 = stunted and dead) according to Nicol et al. (2004). The 0 to 10 symptom rating system was based on the following scale; 0=no disease; 1 to 2 = minor symptoms on crown within the first internode region; 3 to 4 = obvious symptoms on crown within the first internode region; 5 to 6 = pronounced symptoms on crown with obvious darkened plant tissue due to infection; 8 to 9 = advanced darkened symptoms with severe stunting and near death due to disease infection; 10 = dead plant with severe disease symptoms) for crown rot severity. Seedling height from the base of the soil to the tip of the longest leaf was also recorded.

### **Outdoor terrace adult plant screening**

Two runs of the outdoor terrace screening assay were conducted for the Sunco/Macon RILs and one was conducted for the Sunco/Otis RILs from mid-May through late August during the 2008 and 2009 growing seasons. A single seed was planted into a medium comprised of 50% sand and 50% peat (Greensmix Sphagnum Peat Moss, Wuapaca Northwoods llc., Wuapaca, WI) (v/v) in 6 cm diameter x 24 cm long cone-tainers (Ray Leach Container, Canby, OR), similar to that of growth chamber screens. Genotypes were divided into four sets to control for variability in the sand bed. Each set of genotypes included parents and check genotypes. Genotypes within sets were arranged in a randomized complete block design, with 10 replications. Fertilizer

(Osmocote Classic grade 14-14-14 (%N-%P-%K)) was added to each pot at a rate of ~1.0 gram per pot.

Following seedling emergence, millet colonized with *F. pseudograminearum* isolate 006-13 was placed approximately 2 cm above the seed and covered with potting mix. Water was immediately applied to the pots to activate the inoculum and begin infection. Plots were irrigated on a daily schedule of 1 hour per day during emergence, then irrigations were scheduled over longer intervals of two to four hours and were infrequent to encourage root growth. The irrigation was discontinued in late July and disease symptoms were rated in late August 2008 and 2009 on dried plant tissue. The 0 to 10 scale was used for the outdoor terrace bed screening, and the number of nodes showing symptoms was also recorded.

### **Field screening**

A single field trial located near Mansfield WA was planted May 13<sup>th</sup> and 20<sup>th</sup> 2009 for the Sunco/Otis RILs and Sunco /Macon RILs, respectively. Following seed inoculation as described above, approximately 30 seeds were planted in a 1.5 m linear row per to a depth of 5 cm with a deep furrow hand planting apparatus. Plots were spaced 25 cm apart. The experiments were arranged as randomized complete blocks with three replications for Sunco/Otis and two replications for Sunco/Macon. The location was typical of dryland wheat growing conditions in North Central Washington with a sandy loam soil (type = Timentwa-Siweeka complex with bedrock: source <http://websoilsurvey.nrcs.usda.gov>) receiving approximately 25 cm of rainfall in a season. After the growing season, when the wheat had senesced, approximately 10 plants were harvested per plot from which 5 stems were randomly selected and rated for crown

rot severity on a scale from 0 to 10, similar to that of the growth chamber and outdoor terrace evaluations.

### **Statistical analysis for crown rot disease screening**

A single rating scale was used (0 to 10; 10 = dead plant) for the growth chamber, outdoor terrace and field screens. Experimental units were individual plant stems that were scored for all growth room, terrace, and field data sets. The mean growth room, terrace, and field ratings from each respective screen and year based on the 0 to 10 scale were used for QTL mapping.

#### **Growth room crown rot screening**

Growth room screens were blocked with all treatments and replications for individual lines within one room (i.e. all ten replications for lines 1 through 120 for Sunco/Macon were in one room). For each Sunco/Macon screen three growth rooms were used, whereas two were used for each Sunco/Otis growth room screen. Ten replications were used for the first two growth room screens for Sunco/Macon and first screen for Sunco/Otis. In the remaining screens seven replications were used for each respective population. The ANOVA model consisted of the independent variables genotypes (random effect), growth rooms (fixed effect), and replications within growthrooms. The dependent variable was the crown rot severity rating (1 to 10 scale). Least squares means were calculated for genotypes (growth room) to account for variation between growthrooms and used in QTL analysis. Proc General Linear Models analysis was conducted with SAS v. 6.12 (SAS Institute Inc., Cary, NC).

### Terrace crown rot screening

Experimental units for the outdoor terrace screening system were individual plants planted in cone-tainers. Ten replications were used for all data sets and all years involved in the analysis. All replications and genotypes were blocked within four locations located throughout the terrace screening area (i.e. block 1 included all replications for genotypes 1 through 80). The ANOVA model consisted of the independent variables genotypes, blocks, and replications within blocks. The dependent variable was the crown rot severity rating (1 to 10 scale). Least squares means were calculated for genotypes(blocks) to account for variation between blocks within the terrace plot with SAS.

### Field screening

Experimental units for the field screen were each of the five individual stems randomly subsampled from within each replicated plot and scored on a 0 to 10 scale for crown rot severity. Means were calculated for five stems within each plot. Data was analyzed across three replications from the Sunco/Otis field plot and two replications from the Sunco/Macon plots both arranged in a randomized complete block design. The independent effects genotypes (random) and replication were analyzed with Proc GLM ANOVA and least squares means (lsmeans) with SAS.

Pearson correlations were calculated on the basis of means for each parameter data set (i.e. growth room, terrace, and field) using Microsoft Office Excel<sup>®</sup> 2007 Ed. Regression analysis was calculated on significant correlations where terrace and field served as the independent Y variable and growth room served as the dependent X variable using Microsoft Office Excel<sup>®</sup> 2007 Ed. Broad sense heritability ( $H^2$ ) was

calculated for all locations using the formula:  $H^2 = \text{Var}(G)/\text{Var}(P)$  (where  $\text{Var}(G)$  is the variance of the genotypic effect and  $\text{Var}(P)$  is the variance of the phenotypic effect) using the SAS code provided by Holland et al. (2003).

### **Molecular marker analysis**

Fresh leaf tissue of each RIL from the F<sub>6</sub>:F<sub>7</sub> Sunco/Macon and F<sub>5</sub>:F<sub>6</sub> Sunco/Otis RIL populations, plus parents, were collected at the 5 leaf stage and stored at -80 °C until processing. Samples were ground to a fine powder in liquid nitrogen with mortar and pestle and DNA was extracted using the following protocol. DNA extraction buffer consisted of 625 mL ddH<sub>2</sub>O, 100 mL of 0.5-M EDTA (pH=8.0), 100 mL Tris (pH=8.0), 100 mL 5-M NaCl, 62 mL 20% SDS (sodium dodecyl sulphate), and 3.80 g sodium bisulfate. The pH of the buffer solution was adjusted to 8.0 with 4 M NaOH. Approximately 300 to 400 mg of plant tissue was placed in a 50 mL tube. DNA extraction buffer was added to a total volume of 25 mL in the tube. Samples were placed in a water bath set at 65 °C for 45 minutes and agitated every 10 minutes. After incubation in the water bath, approximately 25 mL of chloroform was added to make a total volume of 45 mL and mixed thoroughly by twisting. Tubes were centrifuged for 15 minutes at 10,000 g. Supernatant was transferred to a clean tube; 45 mL of chilled 95% EtOH was added to elute DNA and mixed gently. Tubes were incubated for 20 minutes at 4 °C, then extracted plant DNA was transferred to another clean tube and re-suspended in 70% EtOH then placed at -20 °C overnight. Precipitated DNA was collected and dried for 3 to 5 hours, then re-suspended in 500 mL pure water (water volume was adjusted according to DNA content) and placed at 4 °C until use.



Two marker systems, DArT and SSR, were utilized to generate a linkage map for each population. DArT genotyping was carried out on both mapping populations by Triticarte Pty. Ltd. (Yarralumla, ACT., Australia; <http://www.triticarte.com.au>) using the wheat DArT arrays of 1,500 and 1,200 random markers for Sunco/Otis and Sunco/Macon, respectively. Procedures for hybridization of genomic DNA to the DArT array were conducted according to the description in Akbari et al. (2006). In addition to DArT markers, 70 polymorphic SSR markers were screened across the parents and both RIL mapping populations.

SSR analysis was conducted according to PCR conditions modified by Röder et al. (1998) with the exception that primers carried the addition of M13 tails for amplification on an ABI 3130xl (Oetting et al. 1995). One  $\mu\text{L}$  of template sample DNA (100 ng/  $\mu\text{L}$ ) was added to a 10  $\mu\text{L}$  PCR master mix solution for the PCR reaction. The master mix solution consisted of 7.6  $\mu\text{L}$  of nanopure ddH<sub>2</sub>O, 1.2  $\mu\text{L}$  of loading buffer, 0.48  $\mu\text{L}$  of 25 mM MgCl<sub>2</sub>, 0.96  $\mu\text{L}$  of oligonucleotides (2.5 mM), 0.06  $\mu\text{L}$  of forward primer (M13 tail), 0.3  $\mu\text{L}$  reverse primer, 0.24  $\mu\text{L}$  of 10 mM of appropriate fluorophores for ABI (Applied Biosystems, Foster City, CA), 0.12  $\mu\text{L}$  (5 units per  $\mu\text{L}$ ) of Taq DNA polymerase. Samples were amplified with an initial denaturation at 94 °C for 5 minutes, followed by 43 cycles of 30 s denaturation at 94°C, 45 s annealing at 50 to 65 °C (depending on primers), and a 1 minute extension at 72 °C. Amplified samples were analyzed on an ABI 3130xl.

### **Linkage map construction and QTL analysis**

Segregation ratios of markers were tested using a Chi-square goodness-of-fit test to a 1:1 ratio at a significance level of  $P=0.05$ . Linkage analysis was conducted using

MapMaker v3.0 (Lander et al. 1987). For smaller linkage groups with markers of 15 or less, three point linkage analysis was performed using the “compare” command to determine the most likely order of markers. The “try” command was used to add markers to a framework map set of markers which was verified with the “ripple” command. For larger linkage groups, of 15 markers or more, three point linkage analysis was conducted on the entire group with the “three point” command and then ordered with the “order” command. Once a linkage group was established, it was verified with the “ripple” command. The Kosambi mapping function was used to calculate genetic distances in centimorgans (cM) between ordered markers (Kosambi, 1944). Each individual ordered linkage group was then assigned to a chromosome based collinearity with the map of Somers et al. (2004) and other previously published DArT maps made available by Triticarte Pty. Ltd. (available online at [http://www.triticarte.com.au/content/further\\_development.html](http://www.triticarte.com.au/content/further_development.html)).

QTL analysis was carried out utilizing WinQTL Cartographer v2.5 (NC State Univ., Statistical Genetics, Raleigh, NC) (Basten et al. 1997). For the analysis, trait values were expressed as means over the 10 replications within each screening system and run, adjusted for the effects of growth chamber or set (for the growth chamber and terrace bed assays). Single-marker analysis using one-way ANOVA with a comparison-wise probability level of  $p < 0.01$  was used to identify single markers linked to crown rot resistance. QTL locations were also determined using composite interval mapping (CIM). In order to detect QTL a LOD threshold of 3.0 was used.

## RESULTS

### Crown rot evaluation for Sunco/Macon

A total of three growth chamber, two terrace, and one field screening runs were carried out during 2008 and 2009 for the Sunco/Macon RIL population (Table 1). Significant genotype effects were evident across all screens ( $p < 0.0001$ ). There were also significant differences between growth chamber screening runs and between years for the terrace screens. Sunco/Macon growth chamber screen 3 had significantly less crown rot severity than screen 1 ( $p = 0.006$ ), however screens 1 and 2 were not significantly different (Table 1). For the terrace screens, year 1 had significantly less crown rot severity than year 2 ( $p < 0.0001$ ) (Table 1).

There were significant differences between growth chambers within each run for the three seedling screens as well as differences between four blocked locations within the terrace experiment (range in values from  $p < 0.0001$  to  $p < 0.0018$ ) which were partially accounted for by analyzing the results for genotypes within sets.

Mean crown rot severity was normally distributed across the three growth chamber screens around a mean of 2.8 and standard deviation of 0.9 (Figure 1). Transgressive segregation toward susceptibility was observed in the population (Table 1; Figure 1). Sunco had slightly less crown rot severity compared to Macon, with the exception of screen 1 where Sunco was not statistically different from Macon. The mean crown rot severity across the three growth chamber screens was less for Sunco than Macon (Table 1). The mean crown rot severity and range of ratings was the lowest for the field screen compared to that of the growth chamber and terrace systems. The coefficient of variation (36%) was the lowest for the field screening method compared to

those of the growth chamber and terrace screening methods. Overall heritabilities ( $H^2$ ) were very low due to the extreme amount of variation in disease expression characteristic of crown rot (Table 1).

In the 2008 terrace experiment crown rot severity was lower for Sunco compared to Macon, but significantly greater for Sunco in the 2009 terrace experiment. The mean crown rot severity was significantly greater (4.0) in 2009 for the terrace experiment compared to 2008 (2.9) due to uneven irrigation distribution within the terrace plots. The terrace 2009 data were not strongly correlated to that of 2008 (R-value = 0.10) (Table 2).

The growth chamber screening method was slightly correlated to that of the field ( $r=0.14$ ,  $p=0.03$ ) (Table 2; Figure 2). The mean terrace crown rot severity was moderately correlated to that of the growth chamber ( $r=0.33$ ,  $p<0.0001$ ), but not correlated to that of the field ( $r=0.08$ ,  $p<0.28$ ). There was a slight negative correlation between plant height and crown rot severity ( $r=-0.22$ ;  $p=0.0008$ ).

### **Crown rot evaluation for Sunco/Otis**

A total of two growth chamber, one terrace, and one field screen were carried out during 2008 and 2009 for the Sunco / Otis RIL population (Table 4). Significant genotype effects were evident across all screens ( $p<0.0001$ ). There were also significant differences between the two growth chamber screening runs ( $p<0.0001$ ). Sunco / Otis growth chamber screen 2 had significantly less crown rot severity than screen 1 ( $p<0.0001$ ). The mean crown rot severity was numerically similar between growth chamber screen 1 ( $\mu=3.9$ ) and the terrace screen in 2009 ( $\mu=4.0$ ) (Table 4).

Mean crown rot severity was normally distributed across the two growth chamber screens around a mean of 3.5 and standard deviation of 0.9 (Figure 3). Transgressive segregation was observed in the Sunco / Otis population (Table 4; Figure 3) toward susceptibility. Sunco had slightly less crown rot severity compared to Otis for growth chamber screen 1. The crown rot severity was slightly higher for Sunco than Otis for the mean of the two growth chamber screens, yet the two were not statistically different. Crown rot severity was numerically less for Sunco ( $\mu=1.8$ ) compared to Otis ( $\mu=2.5$ ) for the 2009 field screen.

The mean crown rot severity and range of ratings was the lowest for the field screen compared to that of the growth chamber and terrace systems, as was the case for Sunco / Macon. Mean crown rot severity ratings between the growth chamber and terrace screening systems were within similar ranges. Similarly to Sunco / Macon, the heritabilities were very low due to the extreme amount of variation in disease expression, which is characteristic of crown rot (Table 4). There were no significant correlations of the genotype means of the screening methods for the Sunco / Otis population.

### **Linkage map construction**

Over 1,225 and 1,544 DArT markers were polymorphic between the parents Sunco and Macon and Sunco and Otis, respectively. In addition 70 SSR markers polymorphic between the parents were analyzed as known chromosomal ‘anchors’, or markers from previously published regions at the distal ends of chromosomes and roughly every 50 cM of the 21 haploid wheat chromosomes (Somers et al. 2004). Over 530 and 832 DArT markers were eliminated from the mapping analysis for Sunco/Macon and Sunco/Otis, respectively, due to a failure of a Chi-square test fit to a 1:1 ratio. A

total of 695 DArT and 18 SSR markers were analyzed in the development of the Sunco/Macon linkage map. Of these a total of 328 DArT and 18 SSR markers (346 total) were included in the genetic linkage map construction to form 39 linkage groups at LOD = 5. Of these linkage groups 27 were assigned to all 21 haploid chromosomes with a total genetic distance of 1,778 cM for Sunco/Macon. For Sunco/Macon, three linkage groups were assigned to chromosome 1A. Chromosomes 3A, 3B, 7A, and 7B each were comprised of two linkage groups. The only under represented linkage group for the Sunco/Macon population contained only 4 markers was assigned to chromosome 4D.

A total of 712 DArT and 30 SSR markers were analyzed in the development of the Sunco/Otis linkage map. Of these a total of 354 DArT and 30 SSR markers were included in the genetic linkage map construction to form 39 linkage groups at LOD = 5, of which 26 were assigned to all 21 haploid chromosomes with a total genetic distance of 2,451 cM for Sunco/Otis. The average distance between markers was 5.1 and 6.3 for Sunco/Macon and Sunco/Otis, respectively. A total of 40 and 24 markers were mapped to the major linkage group on chromosome 3B for Sunco/Macon and Sunco/Otis, respectively. Chromosomes 2B, 3B, 4A, 6A, and 7D each were comprised of two linkage groups. In the Sunco/Otis population there were not any unrepresented chromosomes as all 26 linkage groups were assigned to the 21 chromosomes. However, chromosomes 3D, 4D, 5D, and 6D had only 5, 4, 4, and 5 markers within those respective linkage groups assigned to those chromosomes. These chromosomes could be considered to be underrepresented with so few markers.

## Crown rot resistance QTL identification

Sunco/Macon

Single marker analysis revealed significant QTL ( $P=0.05$ ) on chromosomes 1A, 1B, 1D, 2B, 3B, 4B, 4D, and 7A in growth chamber, terrace, and field screens (Table 5). Utilizing CIM, one major QTL, that was inherited from Sunco, was identified on chromosome 3B. The 3B QTL was detected in all 3 growth chamber screens and in the field screen. Across the three growth chamber screens LOD values for the 3B QTL ranged from 2.5 to 14.3 and explained from 5% to 28% of the variation in the growth chamber phenotypic data. This QTL was also identified in the field screen with a LOD = 2.2, explaining 4% of the variation in the phenotypic data (Figure 4; Table 5). Following convention, this QTL was named *Qcrs.wsu-3BL*, where ‘crs’ stands for “crown rot severity” and ‘wsu’ stands for “Washington State University”. To date we have not found any SSR markers linked to the crown rot resistance QTL *Qcrs.sm-3BL* but this work is in progress. Other less significant QTL were identified on chromosomes 1D, 2B, and 4B. These were only detected in a single assay but have been reported by others on those chromosomes (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006). Heritability ranged from 0.04 to 0.17 for the growth chamber screens.

There was a significant plant height QTL in the Sunco /Macon population on chromosome 4B (LOD = 8.8 explaining 31% of the variation for plant height). Wallwork et al. (2004) reported that the *Rht-B1* locus mapped to chromosome 4B in a Kukri/Janz background. Kukri and Sunco share similar parentage (cv. Cook) and could both carry the *Rht-B1* locus. The *Rht-B1* locus was originally located on chromosome 4B (McVittie et al. 1978; Somers et al. 2004).

## Sunco/Otis

Single marker analysis revealed significant QTL ( $P=0.05$ ) on chromosomes 1A, 2A, 2B, 3A, 3B, 4A, 4B, 5B, and 7A. CIM analysis identified one major QTL on chromosome 3B across both growth chamber data sets with LOD scores of 10 and 1.5 explaining 23% and 4% of the variation for growth chamber screen 1 and 2, respectively (Figure 5; Table 6). The mean growth chamber rating had a LOD = 8.8 explaining 20% of the variation (Table 6). Another QTL located 15 cM in proximal to the major QTL on 3BL was identified in the field screen. This second 3B QTL was inherited from Otis and had a LOD = 2.6 explaining 8% of the variation in the phenotypic data. The major seedling QTL identified in growth room assays inherited from Sunco was named by convention “*Qcrs.wsu-3BL*”, where ‘crs’ stands for crown rot severity and ‘wsu’ stands for “Washington State University” as it may be the same QTL as that identified in the Sunco/Macon population. However, this conclusion needs further research and validation with more growth room screens for the Sunco/Otis population. The SSR marker ‘*gwm247*’ is linked to this resistance QTL and appears to be a possible candidate for validation in wheat backgrounds for potential use in marker assisted selection.

Another major QTL on chromosome 7A was inherited from Otis and identified in one terrace screen from 2009 with a LOD = 5.1 and explained 20% of the variation and was located 31 cM from the SSR marker *wmc646*. Other significant QTL were inherited from Otis and identified on chromosome 4B from the terrace screen and growth chamber screen 2 with LOD scores ranging from 2.7 to 3.2 and both explained 7% of the variation in the data. The growth chamber data showed a slightly significant QTL on chromosome 4A with a LOD = 3.0 and 2.2 explaining 6% and 8% of the variation in the data for



growth chamber screens 1 and 2, respectively. The least significant QTL was inherited from Otis and identified in growth chamber screen 2 on chromosome 1A with a LOD = 2.9 and explained 7% of the variation. CIM showed other less significant QTL on chromosomes 2A, 2B, 3A, and 5B for the Sunco/Otis population (Table 6).

The markers in common on chromosome 3B between Sunco/Macon and Sunco/Otis were *wmc777*, wPt-3342, wPt-6945, and wPt-6239. These markers were in similar locations on each genetic linkage map. Markers were within approximately 1 to 12 cM from each other. The only DArT marker in common within the 3BL QTL region between the Sunco/Macon and Sunco/Otis populations was ‘wPt-3342’.

## DISCUSSION

The most recent and significant QTL identified for crown rot resistance was located at the distal end of chromosome 3BL and designated *Qcrs.cpi-3B* with an average LOD = 10.2 explaining 44% of the variation over two seedling growth chamber screens with an *F. pseudograminearum* isolate (Ma et al. 2009). The resistant source of this QTL was a cultivar known as ‘CSCR6’ and was of the species *T. spelta*. The authors reported that their intent was to discover novel sources of resistance by mapping wheat from a species other than *T. aestivum* which had been used to identify all of the resistance QTL to date (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006).

To date there has not been a report of significant crown rot resistance QTL on chromosome 3BL in the cultivated *T. aestivum* cultivar Sunco. This research reports a novel major crown rot resistance QTL with an average growth chamber mean (three screens) LOD score = 14.3 and 10.3 in Sunco/Macon and Sunco/Otis, respectively, explaining 28% of the variation in the phenotypic data on chromosome 3BL inherited from the *T. aestivum* source ‘Sunco’ in two mapping populations.

The potential does exist that the 3BL QTL inherited from ‘CSCR6’ published by Ma et al. (2009) could be in a similar location to that of the Sunco/Otis population in the current study as both QTL regions contain DArT markers ‘wPt-8206’ and ‘wPt-0324’ of each respective population. Neither of these markers were polymorphic in the 3BL QTL region of the Sunco/Macon population. In fact there were not any DArT or SSR markers that were polymorphic in both populations within the QTL region of chromosome 3BL between the Sunco/Macon and the CSCR6/Lang population published by Ma et al. (2009). DArT marker ‘wPt-3342’ was the only polymorphic marker in common between

the 3BL QTL region of Sunco/Macon and Sunco/Otis reported here. There were only four polymorphic markers in common between the entire chromosome 3B maps of Sunco/Otis and Sunco/Macon. Additional markers are being tested for that region and may reveal additional collinearity. In the meantime, crosses between resistant RILs possessing the 3BL QTL from both populations may reveal whether QTL are shared or different.

Collard et al. (2005) reported a major QTL located on chromosome 1DL (LOD = 8.5 explaining 21% of the variation) from a single growth chamber assay that was tightly linked (<1 cM) to the SSR marker *wmc149*. Results from the current study show a minor adult plant QTL from the Sunco/Macon terrace screen mean (LOD = 2.5 explaining 6% of the variation) on chromosome 1DL within 6 cM of the SSR marker *wmc149*.

The adult plant QTL reported by Wallwork et al. (2004) located on chromosome 4B (LOD = 54 explaining 48% of the variation) was 7.7 cM from SSR marker *gwm149*. Wallwork et al. (2004) reported a major QTL on chromosome 4B inherited from Kukri. The authors indicated that the nature of the partial resistance in Sunco was similar to that of Kukri as they both inherited their partial resistance from the cultivar Cook. However, the significant QTL reported here in the Sunco/Otis population was inherited from Otis. The minor QTL identified on chromosome 4B in this study were located approximately 49 cM and 41 cM from the SSR marker *gwm149* in the Sunco/Macon and Sunco/Otis populations, respectively. Therefore, these minor QTL appear to be in different locations than that reported by Wallwork et al. (2004).

Although Wallwork et al. (2004) reported a negative correlation of  $r = -0.59$  between crown rot severity and plant height, that is taller plants were more resistant and

lacked the *Rht-B1* allele for reduced plant height, only a slight negative correlation and significant regression ( $p=0.0008$ ) between plant height and crown rot severity was found in the Sunco/Macon population ( $r= -0.22$ ). Plant height was measured in the growth chamber and field and a significant QTL for those data sets were found on chromosome 4B (LOD = 8.8 explaining 31% of the variation for the growth chamber plant height) in the Sunco/Macon population. This correlation and QTL on 4B may further validate the accuracy of the Sunco/Macon genetic linkage map in this study (Wallwork et al. 2004).

Significant QTL found in similar locations to other minor QTL reported in previous studies on chromosome 2B cannot be easily assessed as they do not share similar markers of interest (Bovill et al. 2006). To date there have not been any reports of a significant QTL on chromosome 7A. This study reported a significant QTL on chromosome 7A (LOD = 5.1 explaining 20% of the variation) in the Sunco/Otis population from a single terrace data set (inherited from Otis). Further screening is needed to further validate this QTL.

Previous researchers utilized either adult plant terrace screening systems or growth chamber screening assays to identify QTL for crown rot resistance (Wallwork et al. 2004; Collard et al. 2005; Bovill et al. 2006; Ma et al. 2009). This study is the first to confirm the identification of the major 3BL seedling QTL from growth chamber screens with field screening in the Sunco/Macon population. Although these results may suggest the possibility that seedling and adult plant resistance could potentially coincide in some instances, the significant QTL identified with seedling growth chamber screens did not appear in either of the terrace screens for either Sunco/Macon or Sunco/Otis.

The concept of adult plant and seedling resistance requires further study with different sources of resistance. Although Wallwork et al. (2004) reported that the response of resistant cultivars was similar between *F. pseudograminearum* and *F. culmorum* in Australia, this needs further evaluation with PNW isolates of each respective species. The findings reported here and by others have confirmed the concept that resistance to crown rot is polygenic and subject to a great deal of environmental variation (Collard et al. 2005; Bovill et al. 2006). However, these current findings and those of Ma et al. (2009) (3BL QTL explaining 44% of the variation) and Wallwork et al. (2004) (4B QTL explaining 48% of the variation) do also suggest that strong single gene sources of host plant resistance may exist for crown rot.

Future work needs to be conducted to identify SSR markers in the regions of the major 3BL QTL identified in this study. Once markers are identified, there is a need to validate polymorphisms in the background of PNW germplasm for their potential use in marker assisted selection. Secondly, Sunco needs to be evaluated against a subset of *F. pseudograminearum* and *F. culmorum* isolates collected from a recent Fusarium crown rot survey of the PNW to measure the variation in resistance response at both the seedling and adult plant stage (Poole, PhD Doctoral Dissertation, 2010). The subject matter of the nature of adult plant and seedling resistance needs to be further evaluated with sources of resistance that exhibit each of these responses individually by evaluating them over several years in growth chamber , terrace, and field trials.

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**Table 1.** Crown rot severity summary statistics including minimum and maximum ranges, standard deviations, coefficient of variation (CV) and broad sense heritability ( $H^2$ ) for the Sunco/Macon RIL (219 lines) mapping population.

Trial	Parent <sup>1</sup>		Population <sup>2</sup>						
	Sunco	Macon	Min	Max	Mean	SD	CV	( $H^2$ ) <sup>3</sup>	SE <sup>3</sup>
Growth chamber screen July 2008 <sup>4</sup>	2.3	1.8	0.5	7.1	3.0	1.3	86	0.63	0.03
Growth chamber screen Sept. 2008 <sup>4</sup>	2.1	3.2	0.4	6.2	2.9	1.2	114	0.59	0.04
Growth chamber screen July 2009 <sup>4</sup>	1.4	2.0	0.7	6.0	2.7	1.2	71	0.07	0.10
Growth chamber mean <sup>5</sup>	1.9	2.3	0.9	5.8	2.8	0.9	90	0.60	0.04
Terrace screen 2008 <sup>4</sup>	2.8	3.3	1.1	4.9	2.9	0.7	66	0.22	0.07
Terrace screen 2009 <sup>4</sup>	5.1	3.3	1.4	9.7	4.0	1.2	53	0.61	0.04
Terrace mean <sup>5</sup>	4.0	3.3	1.7	5.0	3.4	0.7	59	-	-
Field screen 2009 <sup>6</sup>	2.5	2.3	0.2	4.2	1.9	0.5	36	0.17	0.11

<sup>1</sup> Parents of the ril mapping population (Sunco and Macon).

<sup>2</sup> Descriptive statistics of the Sunco/Macon recombinant inbred line mapping population consisting of 219 individuals.

<sup>3</sup> Broad sense heritability ( $H^2$ ) of each respective growth chamber, terrace, and field screen calculated by  $H^2 = \text{Var}(G)/\text{Var}(P)$  (where  $\text{Var}(G)$  is the variance of the genotypic effect and  $\text{Var}(P)$  is the variance of the phenotypic effect) using the SAS code provided by Holland et al. (2003). Each respective standard error (SE) of variance was calculated for heritability estimates.

<sup>4</sup> Growth room and Terrace screens were carried out in 2008 and 2009. Ten replications were included in the 2008 screens and 2009 terrace screen, while 7 replications were used in the 2009 growth room screen. Plants were rated from 0 to 10 according to Nicol et al. 2001; 0=no disease; 1 to 2 = minor symptoms on crown within the first internode region; 3 to 4 = obvious symptoms on crown within the first internode region; 5 to 6 = pronounced symptoms on crown with obvious darkened plant tissue due to infection; 8 to 9 = advanced darkened symptoms with severe stunting and near death due to disease infection; 10 = dead plant with severe disease symptoms) for crown rot severity. For all screens lines were blocked to include all replications within 3 growth rooms and 4 blocks in the terrace. Least square means (Lsmeans) were analysed using SAS. When data was analyzed including sets accounting for variation between four block sets within the terrace screening system, heritabilities were lower for 2008 and 2009 at 0.03 (SE=0.01) and 0.14 (SE=0.02) for each year respectively.

<sup>5</sup> Means were calculated for each set of respective growth room and terrace screens.

<sup>6</sup> A field screen was conducted in Douglas Co. WA with 5 sub-samples rated from 0 to 10 according to Nicol et al. (2001) from 2 replicate blocks as described for growth room and terrace screens.

**Table 2.** Correlation coefficients for the Sunco/Macon population assessed for crown rot severity among mean growth chamber screens, mean terrace screens, and one field screen.

	Growth chamber <sup>1</sup>	Terrace	Field
Growth chamber <sup>1</sup>	1.00		
Terrace <sup>2</sup>	0.33 (p<0.0001) <sup>4</sup>	1.00	
Field <sup>3</sup>	0.14 (p=0.03)	0.08	1.00

<sup>1</sup> Growth chamber value is based on a mean of 3 growth room screens carried out during 2008 and 2009. Plants were rated on a 0 to 10 rating scale developed by Nicol et al. (2001).

<sup>2</sup> Terrace value is based on a mean of 2 terrace screens carried out in 2008 and 2009. Plants were rated on a 0 to 10 rating scale developed by Nicol et al. (2001).

<sup>3</sup> Field value is based on one year of data. Means from 5 sub-sampled stems from 2 replicated plots were rated according to Nicol et al. (2001) on a 0 to 10 scale.

<sup>4</sup> P-values were reported only for significant correlations calculated according to regression analysis.

**Table 3.** Correlation coefficients for the Sunco/Macon population assessed for crown rot severity among three growth chamber screens, two terrace screens, and one field screen.

	Growth chamber 1	Growth chamber 2	Growth chamber 3	Terrace '08	Terrace '09	Field
Growth chamber 1	1.00					
Growth chamber 2	0.50 (p<0.0001) <sup>1</sup>	1.00				
Growth chamber 3	0.13 (p=0.07)	0.23 (p<0.0001)	1.00			
Terrace '08	0.08	0.10	0.39 (p<0.0001)	1.00		
Terrace '09	0.05	0.18 (p=0.005)	0.33 (p<0.0001)	0.10	1.00	
Field	0.07	0.09	0.14 (p=0.02)	0.06	0.06	1.00

<sup>1</sup>P-values were reported only for significant correlations calculated according to regression analysis.

**Table 4.** Crown rot severity summary statistics including minimum and maximum ranges, standard deviations, coefficient of variation (CV) and broad sense heritability ( $H^2$ ) for the Sunco/Otis RIL mapping population.

Trial	Parent <sup>1</sup>		Population <sup>2</sup>						
	Sunco	Otis	Min	Max	Mean	SD	CV	( $H^2$ ) <sup>3</sup>	SE <sup>3</sup>
Growth chamber screen July 2009 <sup>4</sup>	2.6	2.7	1.7	10.0	3.9	1.1	74	0.48	0.06
Growth chamber screen Sept. 2009 <sup>4</sup>	3.7	3.0	1.6	5.5	3.1	0.8	59	0.39	0.06
Growth chamber Mean <sup>5</sup>	3.1	2.8	2.0	5.2	3.5	0.7	67	0.60	0.07
Terrace screen 2009 <sup>4</sup>	6.4	5.8	1.5	7.0	4.0	1.1	51	0.28	0.09
Field screen 2009 <sup>6</sup>	1.8	2.5	1.3	3.5	2.3	0.5	30	0.29	0.10

<sup>1</sup> Parents of the ril mapping population were Sunco and Otis.

<sup>2</sup> Descriptive statistics of the Sunco/Otis recombinant inbred line mapping population consisting of 151 individuals.

<sup>3</sup> Broad sense heritability ( $H^2$ ) of each respective growth chamber, terrace, and field screen calculated by  $H^2 = \text{Var}(G)/\text{Var}(P)$  (where  $\text{Var}(G)$  is the variance of the genotypic effect and  $\text{Var}(P)$  is the variance of the phenotypic effect) using the SAS code provided by Holland et al. (2003). Each respective standard error (SE) of variance was calculated for heritability estimates.

<sup>4</sup> Growth room and Terrace screens were carried out in 2008 and 2009. Ten replications were included in the 2008 screens and 2009 terrace screen, while 7 replications were used in the 2009 growth room screen. Plants were rated from 0 to 10 according to Nicol et al. 2001; 0=no disease; 1 to 2 = minor symptoms on crown within the first internode region; 3 to 4 = obvious symptoms on crown within the first internode region; 5 to 6 = pronounced symptoms on crown with obvious darkened plant tissue due to infection; 8 to 9 = advanced darkened symptoms with severe stunting and near death due to disease infection; 10 = dead plant with severe disease symptoms) for crown rot severity. For all screens lines were blocked to include all replications within 3 growth rooms and 4 blocks in the terrace. Least square means (Lsmeans) were analysed using SAS.

<sup>5</sup> Means were calculated by averaging each respective growth room screen.

<sup>6</sup> A field screen was conducted in Douglas Co. WA with 5 sub-samples rated from 0 to 10 according to Nicol et al. (2001) from 2 replicate blocks as described for growth room and terrace screens.



**Table 5.** A summary of QTL for the Sunco/Macon population for crown rot resistance using composite interval mapping (CIM) from screens conducted during 2008 and 2009. Population parameters including the QTL inheritance origin, chromosomal location, likelihood of odds (LOD) score, percent phenotypic variation ( $R^2$ ), and the flanking markers of each QTL region is summarized.

Trial Screen	Inherited Origin <sup>2</sup>	Chromosome <sup>3</sup>	LOD <sup>4</sup>	% Variation ( $R^2$ ) <sup>5</sup>	Flanking Markers <sup>6</sup>
Terrace 2009	Macon	1A (Group 2)	1.5	3.0	wPt-2527 and wPt-666424
Terrace 2008	Macon	1A (Group 3)	2.2	6.0	wPt-731445 and wPt-731282
Terrace 2008	Sunco	1B	1.5	3.0	wPt-9809 and wPt-1403
Field 2009	Sunco	1B	1.7	4.0	wPt-9809 and wPt-7160
Terrace mean <sup>1</sup>	Sunco	1D	2.5	6.0	wmc429 and wPt-665814
Growth chamber mean <sup>1</sup>	Macon	2B	1.5	5.0	gwm429 and wPt-3949
Growth chamber mean	Sunco	3B	14.3	28.0	wPt-0668 and wPt-10537
Growth chamber Screen 1	Sunco	3B	14.1	28.0	wPt-0668 and wPt-10537
Growth chamber Screen 2	Sunco	3B	10.7	18.0	wPt-0668 and wPt-10537
Growth chamber Screen 3	Sunco	3B	2.5	5.0	wPt-6239 and wPt-10991
Field 2009	Sunco	3B	2.2	4.0	wPt-0668 and wPt 10537
Terrace 2008	Sunco	3B	0.8	1.0	wPt-0668 and wPt-10537
Terrace 2009	Sunco	3B	1.6	4.0	wPt-6239 and wPt-10991
Growth chamber mean	Sunco	4B	2.0	4.0	gwm251 and wPt-733363
Field 2009	Macon	4B	1.1	10.0	gwm251 and wPt-733363
Growth chamber Screen 1	Macon	4D	2.9	9.0	cfd084 and wPt-1940
Terrace 2009	Sunco	4D	2.7	8.0	wmc285 and wPt-8836
Terrace 2008	Macon	7A	1.3	3.0	wPt-8375 and wPt-8670
Terrace 2009	Macon	7A	1.1	4.0	wPt-6013 and wPt-5558

<sup>1</sup> Mean parameters for growth chamber and terrace screens represent averages across years 2008 and 2009.

<sup>2</sup> The inherited origin is the parent the QTL was inherited from based on CIM analysis.

<sup>3</sup> Chromosomal location of the QTL denoted by the chromosome number followed by the respective hexaploid bread wheat genome.

<sup>4</sup> LOD = Likelihood of odds score, a statistic that represents the likelihood that a particular QTL is associated with a map location.

<sup>5</sup>  $R^2$  value denotes the percentage of overall phenotypic variation described by the QTL of interest.

<sup>6</sup> Flanking markers are those markers (DArT or SSR) that are most closely linked to the QTL of interest based on CIM.

**Table 6.** A summary of QTL for the Sunco/Otis population for crown rot resistance using composite interval mapping (CIM) from screens conducted during 2008 and 2009. Population parameters including the QTL inheritance origin, chromosomal location, likelihood of odds (LOD) score, percent phenotypic variation ( $R^2$ ), and the flanking markers of each QTL region is summarized.

Trial Screen	Inherited Origin <sup>2</sup>	Chromosome <sup>3</sup>	LOD <sup>4</sup>	% Variation ( $R^2$ ) <sup>5</sup>	Flanking Markers <sup>6</sup>
Growth chamber Screen 2	Otis	1A	2.9	7.0	wPt-731445 and wPt-665379
Growth chamber mean <sup>1</sup>	Otis	1A	1.8	3.0	wPt-731445 and wPt-665379
Growth chamber Screen 2	Sunco	2A	2.7	9.0	wPt-9320 and wPt-6711
Growth chamber Screen 1	Sunco	2B	2.0	4.0	wPt-733641 and wPt-8916
Growth chamber mean <sup>1</sup>	Otis	3A	2.1	6.0	wPt-9422
Terrace 2009	Otis	3A	1.6	15.0	wPt-734079 and wPt-4859
Growth chamber Screen 1	Sunco	3B	10.0	23.0	gwm247 and wPt-0324
Growth chamber Screen 2	Sunco	3B	1.5	4.0	gwm247 and wPt-0324
Growth chamber mean <sup>1</sup>	Sunco	3B	8.8	20.0	gwm247 and wPt-0324
Field 2009	Otis	3B	2.6	8.0	wPt-7301 and wPt-672088
Growth chamber Screen 1	Otis	4A	3.0	6.0	wPt-0162 and wPt-800509
Growth chamber Screen 2	Otis	4A	2.2	8.0	wPt-3810 and wPt-671707
Growth chamber mean <sup>1</sup>	Otis	4A	1.4	3.0	wPt-0162 and wPt-800509
Terrace 2009	Otis	4B	3.2	7.0	wPt-1046 and wPt-732448
Growth chamber Screen 2	Otis	4B	2.7	7.0	wPt-1046 and wPt-732448
Terrace 2009	Otis	5B	2.3	6.0	wPt-0819 and wPt-5246
Terrace 2009	Otis	7A	5.1	20.0	wPt-3393 and wPt-3702

<sup>1</sup> Mean parameters for growth chamber screens represent averages across years 2008 and 2009.

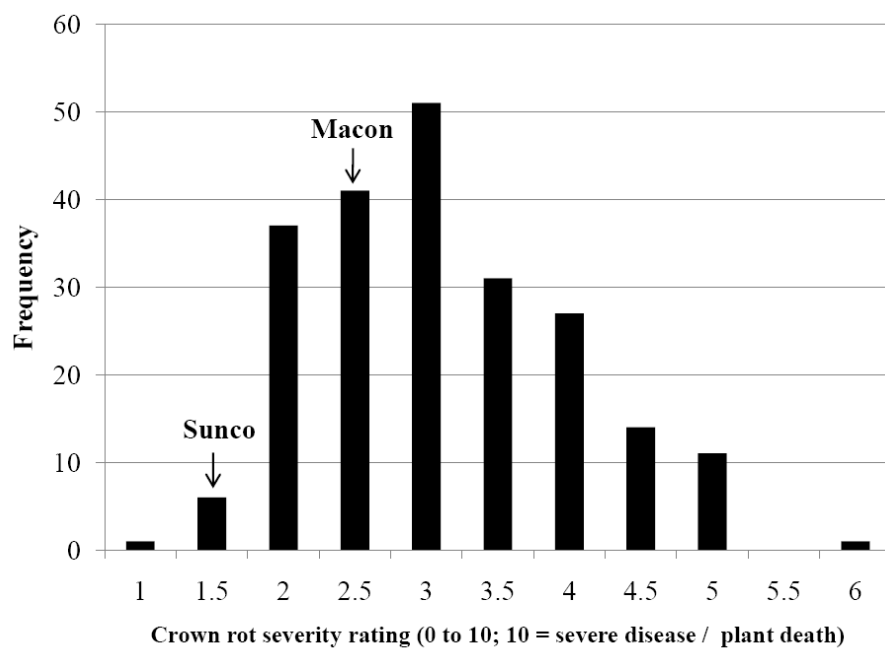
<sup>2</sup> The inherited origin is the parent the QTL was inherited from based on CIM analysis.

<sup>3</sup> Chromosomal location of the QTL denoted by the chromosome number followed by the respective hexaploid bread wheat genome.

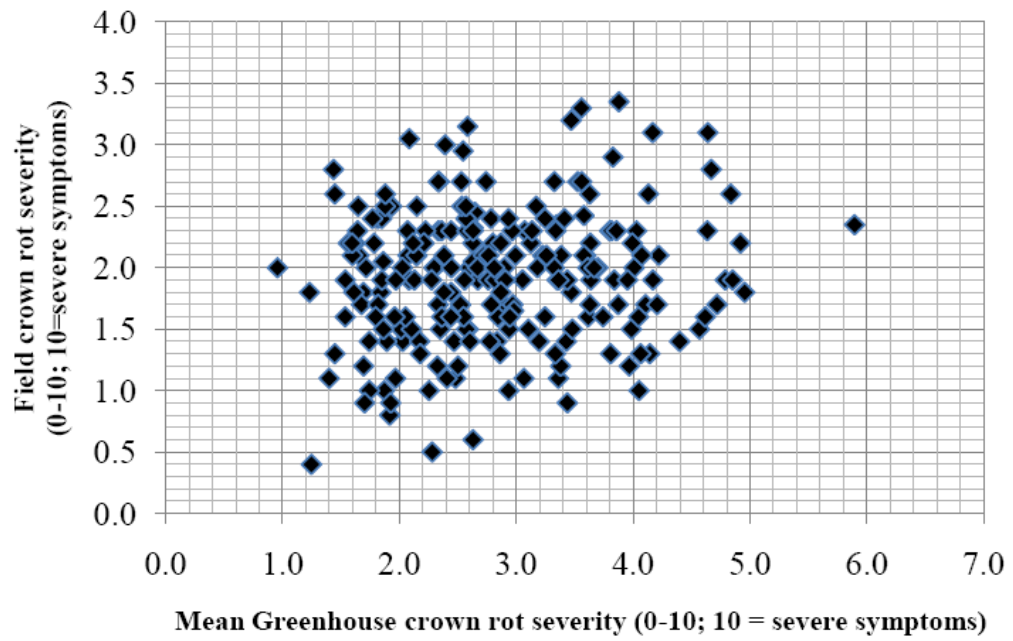
<sup>4</sup> LOD = Likelihood of odds score, a statistic that represents the likelihood that a particular QTL is associated with a map location.

<sup>5</sup>  $R^2$  value denotes the percentage of overall phenotypic variation described by the QTL of interest.

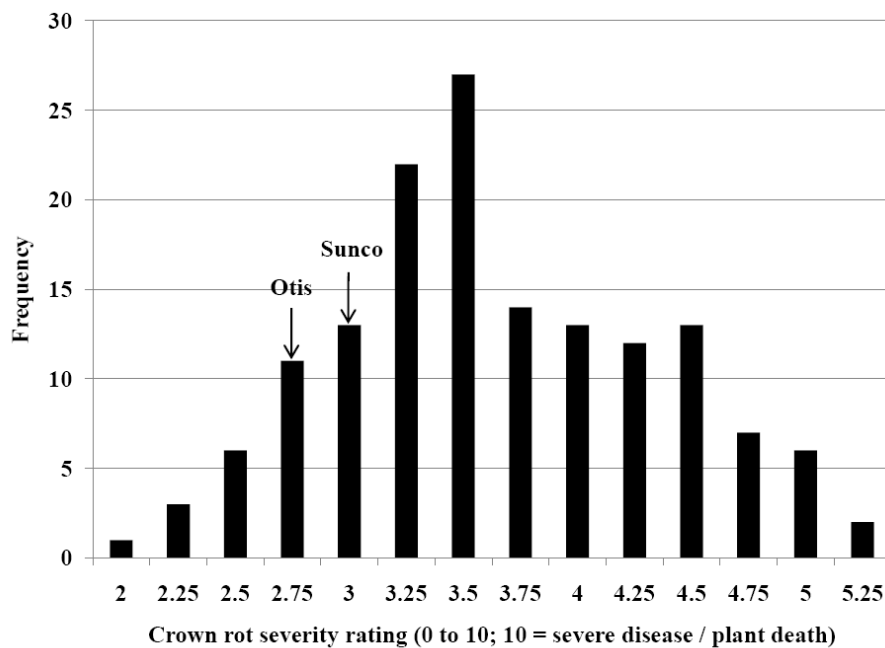
<sup>6</sup> Flanking markers are those markers (DArT or SSR) that are most closely linked to the QTL of interest based on CIM.



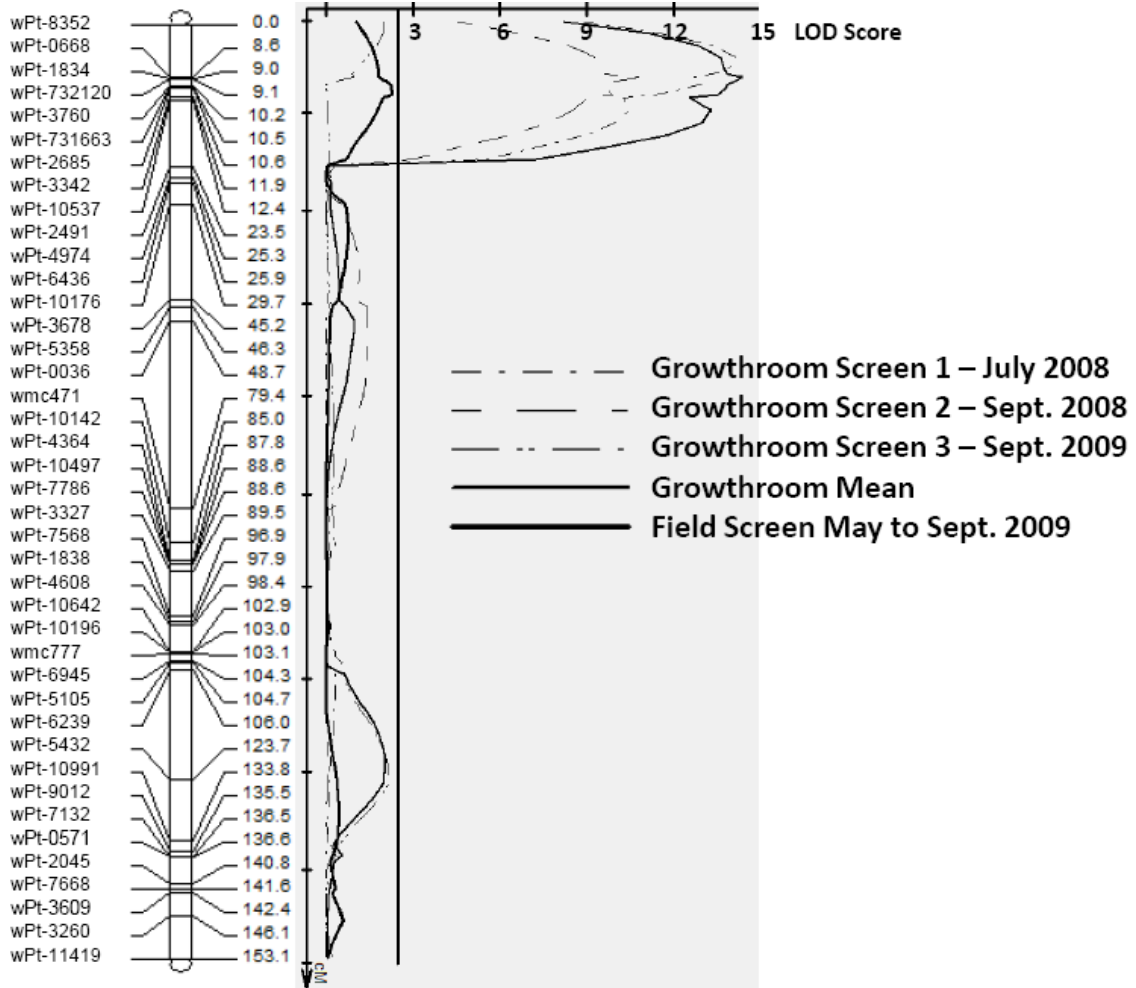
**Figure 1.** Frequency distribution of crown rot severity ratings (0 to 10; 10 = severe disease) based on a mean of the three growth chamber screens for the Sunco/Macon population.



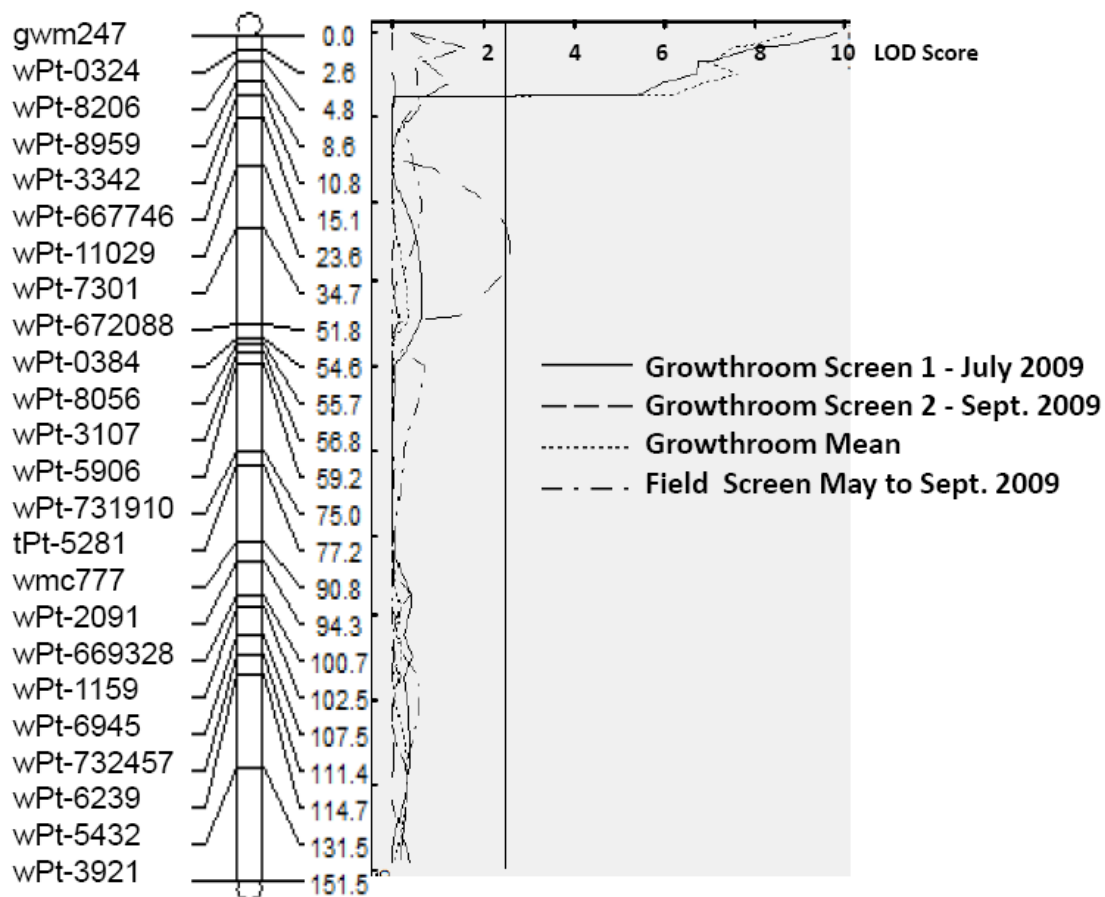
**Figure 2.** Regression scatter plot of crown rot severity for the field versus the independent variable growth chamber for the Sunco/Macon population ( $R=0.14$ ;  $p=0.03$ ).



**Figure 3.** Frequency distribution of crown rot severity ratings (0 to 10; 10 = severe disease) based on a mean of the three growth chamber screens for the Sunco/Otis population.



**Figure 4.** Fusarium crown rot resistance quantitative trait locus on chromosome 3B identified in the Sunco / Macon population across 3 growth chamber and one field screen utilizing composite interval mapping.



**Figure 5.** Two crown rot resistance quantitative trait loci on chromosome 3B identified in the Sunco/Otis population across two growth chamber (inherited from Sunco) and one field screen (inherited from Otis) utilizing composite interval mapping.

## **CHAPTER 6**



## CHAPTER 6

### SUMMARY OF FINDINGS AND CONCLUSIONS

Crown rot is a disease of world wide distribution and importance. Prior to this research crown rot had been identified and surveyed throughout the PNW region with an average yield loss to wheat of 9% reported (Cook 1968; Smiley et al. 2005). The causal fungi (*Fusarium* spp.) are so widespread because they are able to overwinter in dryland wheat residue as fungal hyphae and thick walled chlamydospores in wheat stubble residue throughout the dryland fallow period of production common to the wheat producing areas of the PNW region. Although growers are aware of the disease, especially in drought areas where the disease persists (i.e. white heads during grain ripening), previous control measures have focused on adjusting cultural practices including planting date and controlling nitrogen fertility to maintain plant vigor and prevent moisture stress during grain filling. Plant host resistance seems to be the most reliable and effective control measure for a disease such as crown rot with such a wide-spread distribution and occurrence.

Smiley and Patterson (1996) conducted field surveys (288 fields from 19 counties) during 1993 and 1994 and began to document the wide-spread distribution of crown rot in the PNW region. They found that *F. pseudograminearum* and *F. culmorum* were the most dominant pathogens associated with crown rot symptoms. Following these results there was a need to survey an expanded region to encompass additional climatic areas to better compare and characterize the geographic distribution of crown rot in the region.

Intensive surveys were conducted during 2008 and 2009 covering 210 dryland and irrigated spring and winter wheat fields in 13 Washington and 5 northern Oregon counties. *Fusarium* spp. were isolated from 99% and 97% of the fields surveyed in 2008 and 2009, respectively. Crown rot symptoms were reported in every field in both years with a crown rot severity rating (0 to 10 scale) ranging from 0.2 to 5.1 in 2008 and 0.5 to 5.4 in 2009. *F. culmorum* and *F. pseudograminearum* were the two fungal species isolated most frequently from symptomatic stem tissue at proportions of 31% and 30%, respectively, averaged over both survey years. Other minor species isolated from infected stems, included *F. crookwellense*, *F. acuminatum*, *F. equisiti*, and *Bipolaris sorokiniana* at 13%, 1%, 1%, and 2%, respectively.

These results agree with that of Smiley and Patterson (1996) and other surveys in the PNW and the world showing the wide-spread distribution of crown rot symptoms and the association of the species *F. pseudograminearum* and *F. culmorum* with disease occurrence. In addition to these two main species causing crown rot, *F. crookwellense* was isolated from 13% of the fields surveyed over both years according to conidia morphology outlined by Lesley et al. (2006). Pending confirmation of these morphological identifications with conventional PCR primers, as this finding would be the first report of *F. crookwellense* in the PNW.

Burgess et al. (2001) reported that *F. crookwellense* is often isolated regularly in association with crown rot in Australia. Others have reported isolating *F. crookwellense* from crown rot surveys in Turkey and Australia (Tunali et al. 2006; Bentley et al. 2006). In a genetic characterization of *F. pseudograminearum* isolates using AFLPs and sequence data for *F. crookwellense* isolates from Australia, Bentley et al. (2006) reported

that several *F. crookwellense* isolates were misidentified as *F. pseudograminearum* based on conidia morphologically. In comparative studies in Australia on durum wheat, *F. crookwellense* was reported to have caused more severe symptoms than *F. pseudograminearum*. Lesley et al. (2006) reported that conidia morphology of *F. crookwellense* also resembles that of *F. culmorum*. Although there is a potential that this species could exist in the PNW region as it was identified in equal proportions across both survey years (12% and 13% in 2008 and 2009, respectively) and play a minor role in the crown rot complex, the conidia morphological identifications in this study need to be confirmed with conventional PCR primers (CRO-C) designed to amplify *F. crookwellense*.

The occurrence of crown rot and the two main causal *Fusarium* spp. was significantly different between agronomic zones of the PNW (Douglas et al. 1990). Regions 1 and 4, characterized by cool winters with sufficient winter moisture and relatively dry summers had significantly less crown rot severity on the stems and a greater frequency of *F. culmorum* isolations. Region 5 characterized as predominantly summer-fallow transition zones, had significantly greater levels of crown rot severity and a greater frequency of *F. pseudograminearum* isolations according to t-tests in the current study. Regions 2 and 6, characterized by high moisture and irrigation, respectively, had greater proportions of both species isolated within a field. Backhouse et al. (2004) reported the association between the occurrence of *F. culmorum* with cooler areas receiving more precipitation (>500 mm). Sitton and Cook (1981) reported that *F. pseudograminearum* occurred in drier and warmer counties (temperatures at 31 °C) in Washington state. Smiley and Patterson (1996) suggested similar correlations. Findings

of the current study suggest that *F. culmorum* may persist in cooler wetter regions whereas *F. pseudograminearum* may persist in hotter and drier regions. However, further study is needed with the appropriate experimental design to further characterize the occurrence of causal *Fusarium* spp. of crown rot within certain climatic zones in the PNW.

Another association of interest for future research would be the occurrence and location of crown rot symptoms. Cook (1968), Strausbaugh et al. (2004), and others suggest that *F. culmorum* causes symptoms and is associated with colonization on the roots whereas *F. pseudograminearum* causes symptoms and is more associated with the crown and subsequent internodes. The average number of symptomatic nodes in this study was 1.3, suggesting that crown rot symptoms persisted within the first internode. *F. culmorum* and *F. pseudograminearum* were equally isolated from a 2 cm section of the first internode region in nearly equal proportions over the course of the two year survey. In analyzing the climatic region results it appeared that where *F. culmorum* isolations were most frequent, crown rot severity symptoms on the stems were less. On the contrary, where *F. pseudograminearum* isolations were most frequent, crown rot severity symptoms on the stems were greater. The infection area, symptom development, and activity of these two fungal species in the crown rot complex need further research. Cook (1968) reported that *F. culmorum* “enters crowns of cereal plants via infected crown root and wounds made by crown root emergence 4-6 weeks after planting”. Q-PCR techniques could be utilized to further localize infection points for these two fungi to confirm root infections with *F. culmorum* versus stem infections with *F. pseudograminearum*.

The advent of Q-PCR technology and PCR-based primers that are species-specific has allowed for quick and efficient identification and quantification of the fungi which cause crown rot. Morphological identifications are tedious and require mycological expertise to isolate pathogenic fungi into culture and to identify conidial morphology. Prior to this study there was a single report of the use of Q-PCR technology with Taq-Man based primers to quantify and identify *Fusarium* spp. causing crown rot. The method utilized a DNA extraction kit (FastDNA) where the extraction protocol was modified. In the current study we used this method to attempt to extract and amplify target pathogenic *Fusarium* spp. utilizing species-specific primers. The final objective of this research was to successfully quantify plant DNA extract from infected wheat stems from the 2008 Fusarium survey and evaluate the correlation of DNA concentrations to visual crown rot severity ratings. Preliminary attempts to use the FastDNA kit (designed for extracting plant DNA) to extract and amplify pathogen DNA from wheat stems infected with crown rot from the 2008 Fusarium survey were not successful. Later, it was discovered that several inhibitors (phenolic compounds) exist in wheat stems that limit Q-PCR amplification which are not removed by the FastDNA extraction kit.

Hogg et al. (2007) modified the extraction protocol with dilutions and an extra step utilizing a SpeedVac to vacuum dry samples at the binding matrix stage to remove residual ethanol that could inhibit Q-PCR amplification. These modifications were attempted in extracting DNA from survey wheat stems infected with crown rot and were not successful. The Ultra Clean Soil DNA kit manufactured by Mo-Bio produced the most consistent DNA extractions and subsequent Q-PCR amplifications in eight DNA extraction optimization experiments.

To correlate DNA concentrations from infected stems to crown rot severity scores an experiment was conducted with 24 stems from 8 rating categories and 3 replicate samples within each rating category. DNA extractions were performed on 3 cm sections of single stems. Results showed 7 positive amplifications with the FPG primer set that were positively correlated to crown rot severity ratings of 0 to 10 ( $R^2=0.61$ ). Another experiment was conducted to further examine this correlation by pooling infected stems from 3 randomly selected samples within 48 fields, for a total of 144 DNA extractions with the Mo-Bio kit. Results showed 49% amplification with the FPG primer set and 21% amplification with the OPT primer set. Although there were 16 amplifications with both primer sets from the same extracted sample, the amplifications were weakly (negatively) associated ( $r=-0.14$ ) suggesting that one species dominated over another within a stem. DNA concentrations amplified with either primer set (FPG or OPT) were not correlated to crown rot severity ratings in this study for. Results showed substantial variation in species occurrence within a field and sample. These studies did not show a strong association between morphological identification and Q-PCR species amplification from separate stems within a field sample.

Although Hogg et al. (2007) was the first to report strong correlations between DNA concentration from Q-PCR and visual crown rot disease severity rating scores, their experiments were conducted under inoculated conditions in the field. Smiley et al. (2005) reported yield loss as high as 61% under inoculated field conditions. Strausbaugh et al. (2005) reported an association between disease severity root ratings and DNA concentrations under inoculated greenhouse conditions but also reported the difficulty in finding significant correlations between visual crown rot severity ratings and DNA

concentrations from naturally occurring inoculum in the field. It is important to note that the current study isolated DNA from non-inoculated naturally infected wheat stems from a survey of random fields. Hogg et al. (2010) reported that DNA concentrations in naturally occurring field soils were 0.4% that of inoculated field soil.

Currently, the use conventional PCR techniques to diagnose and identify field samples to the species level would suffice for genetic identification of samples. The ability to quantify and correlated DNA concentrations with visual ratings with Q-PCR techniques would be beneficial in studies depending on accurate disease quantification, such as those required for QTL analysis. Future research on the use of Q-PCR to quantify disease expression should focus on the isolation and extraction of pathogenic fungal DNA from crown rot infected wheat stems from inoculated and non-inoculated naturally occurring field and greenhouse trials for the advancement of the use of Q-PCR as a diagnostic tool. Further research could also include a comparison between fungal isolation and subsequent morphological identification with Q-PCR fungal DNA amplification.

Additional research could be done to develop *Fusarium* spp. specific primers sequenced from local PNW isolates to ensure more accurate Q-PCR amplification of local isolates as was conducted by Schroeder et al. (2006) for *Pythium* spp. Regardless of the potential limitations, results from the current studies with Q-PCR optimized the methods of extracting pathogen DNA from crown rot infected wheat stems for diagnosing *Fusarium* crown rot in the field. Two other objectives of this research focused on quantifying host plant resistance QTL to aid in marker assisted selection for *Fusarium* crown rot.

Cook (2001) stated that the most effective means for control of many wide-spread soil-borne pathogens is host plant resistance. Research began in Australia in the 1960's to find plant host resistance in wheat. Eventually resistant cultivars were released in the 1980's, including Sunco the resistant used in the current study. The cultivars currently grown on a wide-spread scale in the PNW region do not show significant levels of resistance to the disease. Recently efforts have been made in the local PNW region to begin introgressing crown rot resistance into PNW cultivars. Recent advances in molecular biology have allowed for the use of microsatellite SSR markers to function as a tool to aid in selection for traits such as Fusarium crown rot resistance. The first step in identifying molecular markers linked to disease resistance is to identify QTL, or the genomic DNA region where the trait of interest (i.e. disease resistance is to lie).

In this study two RIL mapping populations (Sunco/Macon and Sunco/Otis) were developed at Washington State University in cooperation with Oregon State University. Prior to screening the mapping populations for resistance to Fusarium crown rot to map subsequent QTL there needed to be effective and efficient screening systems developed in the greenhouse and outdoor terrace growing systems to quantify seedling and adult plant resistance. The seedling screening system is done in a relatively short period of time and requiring efficient inoculation and disease infection to adequately quantify disease severity for QTL mapping. Several greenhouse inoculation trials were conducted during 2007, 2008, and 2009 to evaluate the virulence of local PNW isolates on the parents (Sunco, Macon, and Otis) and partially resistant (2-49) and susceptible checks (Seri) as well as different inoculation methods. PNW Isolate 2 (006-13) collected near Duffur, OR. was selected as the most consistently virulent isolate across optimization



experiments to screen the RIL mapping populations. The parents were significantly different from each other in crown rot susceptibility, where Macon was more susceptible than Sunco in initial screens.

Several methods of inoculation for use in the greenhouse were evaluated including dipping seedlings in a conidial suspension (seedling dip method), placing a droplet of conidial suspension on the plants (stem droplet method), placing a conidial agar based solution in a plastic straw around the base of the seedling (agar straw method), and placing colonized millet under the soil 4 days after emergence (colonized grain millet method). The most consistent and efficient method of inoculation that logically separated the partially resistant and moderately susceptible parents with the most reasonable CV was the colonized grain millet inoculation method.

The colonized grain millet method was used to screen both RIL mapping populations in the greenhouse and terrace during 2008 and 2009. During 2009 an additional field trial location was placed in Douglas County that utilized the seed dip method of inoculation in a conidial suspension of  $2.5 \times 10^5$  conidia per mL. There were significant differences between genotypes within each mapping population for crown rot severity with PNW Isolate 2 (006-13). Differences were more pronounced in crown rot severity between the parents Sunco and Macon in seedling screens compared to Sunco and Otis.

Plant genomic DNA was extracted from the parents and both RIL mapping populations. A genetic linkage map was developed for the Sunco / Otis RIL population using 354 DArT and 30 microsatellite SSR polymorphic markers while the genetic linkage map for Sunco / Macon RIL population was developed with 328 DArT and 18

microsatellite SSR polymorphic markers. A total of five significant QTL were identified on chromosomes 3B, 4B, 4A, and 7A with LOD scores ranging from 3.0 to 14.3. The most significant QTL was inherited from Sunco and identified on chromosome 3BL across all three seedling greenhouse assays for the Sunco / Macon population and one of the two greenhouse assays for the Sunco / Otis mapping population with maximum LOD scores of 14.3 and 10.0 explaining 28% and 23% of the variation, respectively for each population. This QTL covered a 3.8 cM region and was verified in the same chromosomal location of 3BL with field data for the Sunco / Macon population. SSR microsatellite markers will be evaluated in this QTL region for potential use in marker assisted selection for improving Fusarium crown rot resistance in PNW germplasm.

The most significant QTL identified in this study was the one on 3BL inherited from the parent Sunco in both RIL populations that described 28% and 23% of the variation respectively. Future research needs to be conducted to verify whether or not these are the same QTL or different, although they appear to be in similar chromosomal locations on 3B. There was a high degree of heterogeneity between markers that mapped to chromosome 3B between the Sunco/Otis and Sunco/Macon populations. Other major crown rot resistance QTL have been identified in a similar location on chromosome 3BL in other genomic backgrounds with an average LOD=10.2 explaining 44% of the variation in greenhouse seedling data (Ma et al. 2009). The potential does exist that the 3BL QTL inherited from 'CSCR6' published by Ma et al. (2009) could be in a similar location to that of the Sunco/Otis population in the current study as both QTL regions contain DArT markers 'wPt-8206' and 'wPt-0324' of each respective population.

Future research in the area of plant host resistance as it pertains to Fusarium crown rot in the PNW region should focus on; 1.) finding SSR markers in the 3BL QTL region of Sunco/Macon and Sunco/Otis to be used as potential markers in MAS; 2.) screening recent Fusarium survey isolates from 2008 and 2009 for virulence on current mapping population parents and resistant checks. With the confirmation of the 3BL QTL in the field data from Douglas County, field screening at various locations should be explored as an option for screening mapping populations in addition to growth chamber and the outdoor terrace systems.

From the results of this study and others it appears that seedling greenhouse screens still serve as the quickest and most reliable screening method for quantifying crown rot resistance. However, in my opinion this would not negate the need for mature plant field screening systems to verify seedling greenhouse evaluations. The outdoor terrace data returned few significant QTL. This could have been due to a high degree of variation within the terrace plots. The terrace system needs to be optimized to reduce variation by utilizing sub-irrigation in the bed earlier in the season. Also different levels of millet grain inoculum could be explored to find an optimal level of inoculation in the outdoor terrace system.

It appears that the colonized millet grain inoculation method returns the best results for the parents Sunco, Macon, and Otis. However, if future parents carrying crown rot resistance are considered for crossing they should be evaluated with the inoculation methods outlined herein this research as cultivars can respond differentially to different inoculation methods, especially if they are going to be used for QTL mapping. From this research the seedling dip method was the quickest most efficient

method of inoculation for cultivars carrying a higher degree of resistance, such as 2-49, but caused too much disease to differentiate reactions on the moderately resistant cultivar Sunco. Those are the considerations for future crown rot resistance screening systems.

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