Molecular and ecological studies of fungal biodiversity on durum wheat grown in rotation with pulses and canola

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ABSTRACT

Fungi contribute to key processes in the sustainable function of terrestrial ecosystems including nutrient cycling and transport of water to plants. However, some fungal species are of interest because their infection of a susceptible host crop results in diseases negatively affecting food supply and quality. These diseases are expected to be influenced by rotation crops which could impact the development of plant pathogens and their genetic biodiversity. The objectives of this study were to assess the biodiversity of fungal pathogens in durum wheat, to identify the naturally occurring fungi that could have biocontrol potential, and to define the impact of crop rotation with non-cereal crops on fungal populations in durum wheat. For this purpose, in 2004, 2005, and 2006, soil and durum wheat plant samples were collected after preceding crops of *Pisum sativum* L. (pea), *Lens culinaris* Medik (lentil), *Cicer arietinum* L. (chickpea), *Brassica napus* L. (canola) and *Triticum turgidum* L. (durum) in a long term experimental site in Swift Current, Sakatchewan. Samples were analyzed using a combination of traditional cultivation techniques and polymerase chain reaction (PCR), sequencing, and denaturing gradient gel electrophoresis (DGGE) techniques.

Fusarium species, known as the causal agent of *Fusarium* head blight (FHB) and Fusarium damaged kernels (FDK) were among the most ubiquitous and abundant in durum tissues. The most prevalent of all *Fusarium* at the study site were *F. avenaceum*, *F. reticulatum*, and *F. tricinctum*. Other recovered potential fungal pathogens belonged to the genera *Bipolaris*, *Phaeosphaeria*, *Pyrenophora*, *Cladosporium*, *Epicoccum*, *Alternaria*, *Cladosporium*, *Arthrinium*, *Nigrospora*, and

Microdochium. Principal component analysis revealed negative correlations between *Acremonium, Chaetomium*, *Penicillium*, and pathogenic *Fusarium*, *Bipolaris*, *Pyrenophora*, and *Alternaria*. These isolates could be antagonistic, and their potential as biocontrol agents against pathogens colonizing durum wheat in the semiarid Saskatchewan should be assessed.

Crop rotation had a limited impact on the abundance of fungal pathogens. *Fusarium torulosum* was less abundant in durum following canola while *Bipolaris sorokiniana* was less abundant in durum following pea. Even if no single crop rotation reduced significantly the prevalence of *F. avenaceum* in durum wheat, results suggest that a successful control of this important pathogen requires an integrated approach using diversified rotations.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Canada is a major producer of durum wheat (*Triticum turgidum* L. var. *durum*). Fluctuations in production due to weather or diseases in Canada have a significant impact on the world market of cereal products. Saskatchewan is the largest durum producing province in Canada where the annual average of durum export is worth $662 million (Anonymous, 2008). Durum wheat can be colonized by many fungal species. Some of these fungal inhabitants are severe pathogens causing diseases that reduce grain yield and quality.

Several surveys indicated that *Fusarium* species make up a large proportion of the fungal community in durum wheat in Saskatchewan (Fernandez and Jefferson, 2004; Clear at al., 2005). Fungi in the genus *Fusarium* have a cosmopolitan distribution and pose a threat to durum production which is multifaceted. Firstly, Fusarium head blight (FHB) reduces grain quality and causes difficulties when it comes to marketing, exporting and processing grain. The disease symptoms manifest initially through dark purple to black lesions on the external part of florets; later, the inflorescence turns chlorotic, and the grain presents atrophies. During prolonged wet periods pink spore masses of the fungus can also be seen on infected spikelets, glumes and kernels (Goswami and Kistler, 2004). In addition, *Fusarium* species cause crown and root-rot diseases threatening cereal production worldwide. *Fusarium* diseases cost the North America's cereal industry approximately US $5 billion annually as a result of reduced yields and price discounts (Bai and Shaner, 2004b). Secondly, cereal grain contamination with

mycotoxins and other biologically active metabolites is a serious threat to food and feed products (Desjardins and Proctor, 2007). In addition to reduced yields, the presence of Fusarium damaged kernels (FDK) results in reduced germination and poor quality of grains. Milling, baking, and pasta-making properties are seriously affected. Domestic and export markets can also suffer from lost sales due to low customer tolerances for FDK. Additionally, a few fragmentary reports on fungal communities from durum crops grown in Saskatchewan revealed the presence of other important pathogens such as: *Bipolaris sorokiniana*, *Microdochium bolleyi*, *Pyrenophora tritici-repentis*, *Septoria avenae*, *Septoria tritici*, and *Septoria nodorum* (Fernandez and Jefferson, 2004; Singh et al., 2007). Certain isolates of these fungi are responsible for some agriculturally important cereal diseases across the world (Duczek et al., 1991; Fernandez et al., 2000).

This study aimed at understanding the fungal diversity in durum. For this purpose, the distribution of fungal communities in soil and plant samples through durum growth stages, and in different plant organs was examined. Known fungal pathogens, and naturally occurring fungi that might have biocontrol activity against these pathogens were identified. Because *Fusarium* species are known to pose the greatest danger to durum wheat production, the biodiversity of *Fusarium* in durum

wheat was thoroughly analyzed by combining a culture-dependent and a culture-independent approach (Leslie and Summerell, 2006; Yergeau et al., 2005). In addition, the impact of a preceding crop of pea, chickpea, lentil, canola, and durum wheat on fungal populations in durum wheat was evaluated. Finally, the susceptibility of durum wheat to damping-off disease caused by pathogenic *Arthrinium sacchari* and *Nigrospora oryzae* fungal species was tested.

1.1 Study rationale

In Canada durum wheat is grown primarily in the province of Saskatchewan. The impact of a preceding pulse and oil seed crop on fungal pathogens prevalence in durum wheat crop grown in Saskatchewan is poorly understood. In order to test the efficacy of preceding crops in reducing pathogens' prevalence in durum wheat a correct identification and quantification of fungal durum inhabitants is required. The 3rd chapter describes fungal species associated with durum wheat throughout the entire growing season and in relation with five rotation systems. *Fusarium* spp. were expected to make up a large part of the fungal community in durum wheat. The high biodiversity and prevalence of *Fusarium* spp. in durum wheat was confirmed by both culture-dependent (chapter 3) and independent (chapter 4) approach. Chapter 5 examines how a culture independent approach can be applied to study other predominant fungal species than *Fusarium*. For this purpose, PCR amplified ITS regions of six *Pyrenophora* isolates were used to construct a genetic marker that can be useful when combined with the DGGE for the identification of *Pyrenophora* species directly from environmental wheat samples. Lastly, the 6th chapter characterizes the damping-off potential of two unexpectedly recovered from durum wheat fungal species: *Arthinium sacchari* and *Nigrospora oryzae*. Known as pathogens of several agricultural important crops these fungi were never mentioned before as pathogens of durum wheat.

2. LITERATURE REVIEW

2.1 The biodiversity of pathogenic fungi in wheat crops

Among 1.5 million estimated fungal species only 5% or 75 000 have been described (Havksworth, 2001). Their relationships with the plant hosts are not well understood since many of them can not be cultured by current standard laboratory techniques (Giller et al., 1997). Fungi live in soil, detritus, plants, animals or other fungi. Many of the fungi that colonize wheat crops are pathogenic and mycotoxigenic, causing diseases symptoms with serious economic consequences.

2.1.1 Fungi causing crown and root rot diseases

Crown and root diseases are characterized by brown spots appearing on roots and subcrown internodes (Alberta Agriculture and Food, 2007). Damping-off can be a consequence of the crown and root disease complex. Pre-emergence damping-off kills seeds and germinants before they emerge, while post-emergence damping-off affects young seedlings after emergence. Infected plants are more susceptible to dying when stressed, and tend to break over in strong winds or produce a stunted, unmarketable crop (Alberta Agriculture and Food, 2007). The causal agents of these diseases are *Cochliobolus*, *Fusarium,* and *Rhizoctonia* species.

Cochliobolus and its asexual forms *Bipolaris* and *Curvularia* comprise over 90 species (Sivanesan, 1987), but *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) is considered one of the most important root rot pathogen of common

and durum wheat crops worldwide (Berbee at al., 1999, Ledingham et al.,1973). Different mycotoxins levels produced by *C. sativus* were associated with alteration in colony morphology, and with the ability of the fungus to infect plant tissues *in vitro* (Marder et al., 2006).

Fusarium spp. are also associated with root and crown rot. *Fusarium* spp. have specific geographic distribution, but species commonly recovered from durum wheat diseased roots and crowns are *F. graminearum*, *F. pseudograminearum*, *F. avenaceum*, *F. culmorum*, *F. crookwellense*, *F. acuminatum*, *F. equiseti*, and *F. oxysporum* (Fernandez et al., 2007; Fernandez and Jefferson, 2004; William et al., 2002). Rhizoctonia root and crown rot are mainly associated with *Rhizoctonia solani* and *Rhizoctonia oryzae*. These pathogens may cause distinctive damage, and their relative importance may vary with the developmental stage of the infected plant (Mazzola et al., 1996). Although durum wheat is known to be highly susceptible to *Rhizoctonia* infection (Smith et al., 2003), Rhizoctonia root rot, a serious problem in wheat growing regions of Australia, is less encountered in Canada (Thongbai et al., 1993).

2.1.2 Fungi causing leaf spotting diseases

There are several major leaf-spotting pathogens affecting durum wheat production worldwide. In the Canadian prairies *Pyrenophora* leaf diseases dominate although leaf diseases can also be caused by *Alternaria*, *Mycosphaerella*, *Phaeosphaeria*, and *Cochliobolus* (Fernandez et al., 1996; Fernandez et al. 2000). Pathogenic *Pyrenophora*, some of which have anamorphs in *Drechslera*, produce

leaf spots and leaf blights killing large areas of the leaf tissues. Infected areas, initially discrete, may expand as the infection progresses leading to reduction in the plant photosynthetic rate.

2.1.2.1 Biodiversity of *Pyrenophora* spp. on cereals

Several species of *Pyrenophora* were found in association with cereal crops worldwide. Pyrenophora leaf blotch, a disease that has been reported from most areas of the world where oats are grown is caused by the fungus *Pyrenophora chaetomioides*. This fungus was first named *P. avenae*, so today, both names are in use. In Brazil, *P. chaetomioides* is considered the main pathogen associated with oat crops (Blum, 1997); in India the fungus is widespread from the seedling stage to plant maturity (Harder and Haber, 1992).

Several species of *Pyrenophora* infect barley and cause disease, while only one species is known to infect oats. *P. graminea* causes a seed-borne disease of barley called leaf stripe. This fungus, which survives within wheat kernels as mycelium, enters the plant after seed germination, and leads to important yield reductions (Pecchioni et al., 1999). *P. teres* a species closely related to *P. graminea* causes net and spot blotch diseases of barley (Bates et al., 2001). Two forms of the pathogen exist, each producing characteristic disease symptoms. Smedegard-Petersen (1971) could not distinguish the two pathotypes of *P. teres* using morphological characteristics, and therefore proposed them as two forms, namely *P. teres* f. *teres* (net-blotch) and *P. teres* f. *maculata* (leaf-spot). Furthermore, he reported that net-blotch and leaf-spot isolates could mate in culture, thereby

confirming their being the same species. Although seeds can harbor this pathogen, the most important inoculum for initiating epidemics is the infected barley stubble from a previous crop (Brown et al., 1993). However, seed-borne inoculum is probably important for introduction of these diseases in non-contaminated fields. Net and spot blotch of barley are distributed worldwide, but it appears that the net form of the disease is most prevalent in cool and wet growing areas. In such environments yield losses of 40 percent have been reported (Shipton et al., 1973).

P. japonica and *P. hordei* are two other fungal species producing leaf spots on susceptible barley cultivars. The correct identification of *P. hordei*, a newly identified barley pathogen isolated firstly from Australia (Wallwork et al., 1992), is troublesome since colony morphology and shape of conidia are almost identical to those of *P. teres* (Stevens et al., 1997). Such a high level of morphological similarities indicates their close genetic relatedness. Another concern exists in the literature with regard to the morphological and molecular similarity that is found between *P. japonica* and *P. teres* f. sp. *maculata*. Crous et al. (1995), using restriction fragment banding patterns showed that these two fungal species were almost identical, further suggesting that *P. japonica* should be treated as a synonym of *P. teres*. Additionally, Campell et al. (1999) who worked with *Pyrenophora* spp. infecting barley in South Africa, supported the above statement and showed by using mating tests that isolates identified as *P. japonica* were in fact *P. teres* f. sp. *maculata*. According to Stevens et al. (1997), the phylogenetic analysis of ITS (internal transcribed spacer) regions of *Pyrenophora* spp. pathogenic on barley indicated low interspecific variation, showing a need for clarification of the

taxonomic status of this genus. Furthermore, the authors suggested that it may be appropriate to classify *P. japonica*, *P. teres* f. sp. *teres*, *P. teres* f. sp. *maculata*, and *P. hordei* as variants of the same species. However, a greater range of *Pyrenophora* isolates should be investigated using different cultural and molecular tools to help clarify taxonomical issues for this genus.

Worldwide, several species of *Pyrenophora* have been isolated from wheat crops. *P. tritici-repentis* is an ascomycete responsible for the foliar disease tan spot (synonym yellow leaf spot) of wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*) (Morall and Howard, 1975).

In the United States, the disease was first identified in New York in 1940. The disease was found in Kansas in 1947. Since then, the disease has increased in incidence and severity to the present (Ciuffetti and Tuori, 1999). In Canada the disease was identified in 1939, but the first serious outbreak occurred in 1974 in Manitoba and Saskatchewan (Tekauz, 1976). Yield losses caused by tan spot in Saskatchewan can be as high as 30 - 40% but generally vary from 3 - 15% (Anonymous, 2003a). Two typical disease symptoms are necrosis and chlorosis of the leaf tissue. However, the work of Lamari and Bernier (1989) demonstrated that particular interactions between certain isolates of the fungus and wheat genotypes resulted in the differential development of tan necrosis and chlorosis symptoms. In order to differentiate host reactions to *P. tritici-repentis*, they proposed a rating system based on lesion type. Under this system, the researchers identified isolates causing chlorosis and necrosis, chlorosis only, necrosis only, and those avirulent

causing neither chlorosis nor necrosis. Durum wheat cultivars grown in western Canada are moderately to highly susceptible to leaf spots (Anonymous, 2001).

Tan spot, caused by *P. tritici-repentis*, is the major leaf spotting disease affecting durum wheat production (Fernandez et al., 1996; Fernandez et al., 2000). The fungus causes tan spot on leaves, but can also infect wheat kernels producing red or pink smudge. Light-dark regimes, temperature, and water availability are some of the parameters that can affect disease expression. High tan spot disease levels were shown to occur despite relatively dry conditions in southern Saskatchewan (Fernandez et al., 1996). In a recent study (Fernandez et al., 2000), a correlation between tan spot severity and plant height was found. In field conditions, the most susceptible durum genotypes were found among the short genotypes, and the least susceptible among the conventional height genotypes. According to this study, increased disease severity in short genotypes could be attributed to the denser canopy and close proximity of leaves providing a suitable environment for tan spot development in the stands. To date, three specific *P. tritici-repentis* toxins have been identified: Ptr ToxA causes leaf necrosis while Ptr ToxB and Ptr ToxC cause leaf chlorosis in susceptible *Triticum* species (Lamari et al., 2003).

Although there are no reports of economic losses associated with *P. semeniperda*, the pathogen produces highly toxic compounds that can affect pastures and cereal crops (Medd et al., 2003). There is no evidence that *P. semeniperda* occurs in Asia or Europe. According to Medd (1992), who provides a review of the geographical distribution of this species, *P. semeniperda* is mainly

found in grasslands and cereal growing regions of Argentina, Australia, Canada, Egypt, New Zealand, South Africa, and the United States. In contrast to the extensive information available on wheat infection by *P. tritici-repentis*, little is known about the infection process by *P. semeniperda*. Campbell and Medd (2003) demonstrated that *P. semeniperda* is a seed borne pathogen, and its ability to cause economic damage as a leaf pathogen is extremely low.

Pyrenophora phlei is another pathogen producing leaf spot on cereals and small grains. Very little information regarding this pathogen is available in the scientific literature. According to Sivanesan (1987), the geographic distribution of this species extends over Europe and North America, and its hosts are *Triticum*, *Avena*, *Agrostis*, *Festuca*, and *Phleum* spp. Furthermore and according to a recent report, *P. phlei* is abundant in timothy fields across western Canada (Anonymous, 2003b). Since *P. phlei* survives in crop residues, other hosts could be infected.

Other *Pyrenophora* spp. can appear, though infrequently, on wheat crops; *P. avenae*, *P. teres*, *P. japonica*, *P. graminea*, and *P. dictyoides* have occasionally been recovered from *Triticum* species (Sivanesan, 1987).

2.1.3 Fungi causing kernel diseases

Various fungal organisms can cause diseases that affect the quality of durum wheat kernels. Black point caused by *Cladosporium*, *Alternaria*, *Epicoccum*, and *Cochliobolus*, as well as red smudge caused by *Pyrenophora* species are of concern,

because high levels of disease adversely affect the appearance of the flour and its final products (Conner et al., 1992; Nirenberg et al., 1995).

Several *Fusarium* pathogens can cause kernel diseases of major economic importance. Fusarium head blight (FHB) is a fungal disease that stops kernel development that can result in the formation of Fusarium damaged kernels (FDK). FDK are light weight, shriveled, and pink in color. FHB is not a new disease. It was discovered in England in 1884 and it was first called wheat scab, and later, tombstone disease, because of the lifeless appearance of the infected kernels (Anonymous, 2007). Since then, FHB has become an important disease all over the world.

2.1.3.1 Biodiversity of *Fusarium* spp. on cereals

Studies of the geographic distribution of members of the *Fusarium* spp. present on cereals have been undertaken on isolates gathered from around the world. *Fusarium* spp. such as *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. equiseti*, *F. poae*, and *F. tricinctum* are common fungi which contaminate cereals worldwide (Kosiak et al., 2003; Nirenberg et al., 1994; Bentley et al., 2006). Reports from all continents demonstrate the wide distribution of these devastating fungi.

Surveys performed in Scandinavia, France, and other mid-European countries identified *F. graminearum*, *F. avenaceum* and *F. poae* as the most common species in wheat, durum-wheat and barley fields (Lukanovski et al., 2002,

Ioss et al., 2004). Some examples of *Fusarium* diversity and distribution over Europe are given below.

Over the period of 1994-1996, a post-harvest survey was conducted in 695 grain samples (wheat, barley and oats) to assess the occurrence and geographic distribution of *Fusarium* species in Norwegian cereals (Kosiak et al., 2003). The most prevalent *Fusarium* species recovered in this study were *F. avenaceum*, *F. poae*, *F. culmorum*, and *F. tricinctum*. Less prevalent species were *F. graminearum*, *F. equiseti*, and *F. torulosum*, while sporadically occurring species were *F. arthrosporioides*, *F. semitecum*, *F. proliferatum*, *F. sambucinum*, *F. sporotrichioides*, and *F. verticillioides*. According to this study *F. avenaceum* was the most dominant and ubiquitous species in all analyzed regions - Southeast, Southwest, Upper East and Mid-Norway. Additionally, two of the *Fusarium* species isolated occasionally - *F. semitectum* and *F. sambucinum* - were recovered from oats and barley. The Systematic Botany and Mycology Laboratory Fungus - Host Distribution Databases (Farr et al., 2008) refers to *F. semitectum* and *F. sambucinum* in association with 128 and 58 plant hosts respectively. *Triticum* species were never host of *F. sambucinum.* Moreover, temperature is considered as an important factor influencing species dominance in certain geographic areas. *F. avenaceum* and *F. culmorum* are seemingly better suited to cooler conditions (Anonymous, 2007), and therefore, their presence in the Norwegian provinces could be expected. In addition, studies completed in UK identified *F. culmorum*, and *F. avenaceum* as the most important cause of head blight on *Triticum* species (Parry et al., 1995) while in Germany, *F. graminearum* was identified as the most prevalent

of all *Fusarium* spp. (Obst et al., 2002). In many of these studies, *Fusarium* species were taxonomically identified based on micro- and macro-morphological characteristics, such as cultural morphologies including colony characteristics on specific culture media, and the size, shape and development of sexual and asexual spores.

Outbreaks of FHB have occurred sporadically in Australia. These have been associated with *F. pseudograminearum* and *F. graminearum* (Mitter et al., 2004). During the 1999 FHB epidemics, a total of 700 *Fusarium* isolates were recovered from infected wheat crops and 20 *Fusarium* spp. were identified by morphological and molecular methods. This study revealed that the two most dominant species in the Australian wheat crops were *F. pseudograminearum* (59% of all isolates) and *F. graminearum* (22% of all isolates). Other species such as *F. culmorum*, *F. avenaceum*, and *F. crookwellenses* have been reported to associate with wheat, barley and durum crops in eastern Australia (Backhouse et al., 2004).

Studies on the occurrence of FHB in Asian countries indicate that *F. graminearum* is abundant. Available information on severe epidemics in China describes *F. graminearum* as the fungal agent causing hundreds of cases of animal and human intoxications (Li et al., 2002). Studies on the biodiversity of fungi associated with common and durum wheat in Iran, Japan, and India have identified *F. graminearum* as the main contaminant *Fusarium* species in wheat (Heidarian and Ershad, 2001; Suzuki et al., 1980; Saharan et al., 2004).

There was less work done in Africa on the biodiversity of *Fusarium* species on wheat, but limited numbers of available studies tend to support the general

conclusion that *F. graminearum* is the most prevalent *Fusarium*. A recent survey of the fungal isolates in South African wheat and wheat-based products (Mashinini and Dutton, 2006) shows that the major fungal contaminants were *Fusarium* species and of these, *F. graminearum* was the most commonly isolated. In this study, two additional species -*F. oxysporum*, and *F. subglutinans*- were highly prevalent, while *F. nygami* and *F. verticillioides* were sporadically isolated. *F. verticillioides* is usually associated with maize (Marasas et al., 1979); however, the study of Mashinini and Dutton (2006) demonstrates that this fungus can also be an opportunist colonizing wheat, and its mycotoxins can affect human and animal health. In a different study (Boshof et al., 1999) from the south of Africa, *F. acuminatum*, *F. chlamydosporum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. moniliforme*, *F. oxysporum*, *F. solani*, and *F. subglutinans* were found on wheat in addition to *F. graminearum*. This study also shows that species which are usually not found in association with wheat - such as *F. solani* - could be occasionally recovered from wheat plants.

The fact that *F. graminearum* is the predominant pathogenic species in *Triticum* crops worldwide is also confirmed by studies conducted in the United States where large amounts of wheat are grown and FHB is a major problem. The United States Department of Agriculture (USDA) ranks FHB as the worst plant disease to hit the nation since 1950s (Windels, 2001). FHB has caused devastating losses to wheat, durum, and barley growers and industry in the USA, particularly in the 1990s (McMullen et al., 1997). The disease affects both common wheat and durum wheat and has caused serious losses in grain yield and quality (Bai and

Shaner, 1994a). Several *Fusarium* species were found in a survey conducted over a 3-year period (1999-2001) in multiple wheat growing regions of North America, (Markell and Francl, 2003). Of the seven *Fusarium* species recovered in 1999, *F. graminearum* abundance was high in early summer and declined later in the year as *F. sporotrichioides* abundance increased. *F. equiseti* and *F. sporotrichioides,* like *F. graminearum* were observed throughout 1999, while *F. moniliforme, F. semitectum, F. culmorum*, and *F. sambucinum* were found sporadically and in low numbers. *F. graminearum* and *F. moniliforme* were the most common species recovered in 2000, while in 2001, *F. graminearum* was dominant.

Several studies have reported the presence of *Fusarium* spp. and FHB outbreaks in Canada. According to the Canadian Grain Commission, FHB first appeared in Manitoba in 1923. The causal agent was identified as *F. graminearum*. Since then, *F. graminearum* has gradually moved north and west, and other smaller outbreaks have been identified in the province of Saskatchewan and Alberta (Anonymous, 2007). The presence of pathogenic *Fusarium* on *Triticum* species across Saskatchewan was reported in several studies. Gordon (1952), studying *Fusarium* spp. associated with roots rots of the *Gramineae* in the northern Great Plains discovered that the most common species were *F. avenaceum*, *F. equiseti*, *F. oxysporum* and *F. culmorum*. A more recent study (Fernandez et al., 2001) investigated the biodiversity of *Fusarium* spp. on durum wheat in 1998 and 1999. In both years, about 20% of the fungal isolates recovered were *Fusarium* spp., indicating that this genus accounts for a large proportion of the fungal community in wheat plants growing in Saskatchewan. Among these, *F. equiseti* was the species

most commonly isolated, followed by *F. culmorum*, *F. acuminatum*, *F. avenaceum*, and *F. oxysporum*. Less common species were *F. graminearum*, *F. pseudograminearum* and *F. poae*, while occasional species were *F. crookwellense*, *F. moniliforme*, and *F. subglutinans*.

The compilation of data sets from independent studies shows that *F. graminearum* and *F. avenaceum*, the most pathogenic *Fusarium*, are widely distributed on cereal crops throughout the world. *F. proliferatum*, *F. acuminatum*, *F. subglutinans*, *F. flocciferum*, *F. moniliforme* and a few others have a much more restricted geographic distribution, but still, can be part of FHB complex and thus do pose a real danger to cereal production.

2.2 Methods to control pathogenic fungi in wheat crops

Disease management approaches are mostly based on chemical pesticides, and therefore, are neither generically efficient nor ecologically safe (Bailey et al., 2002). Another approach is the development of varieties with disease resistance. The life of wheat resistant varieties released from breeding programs is relatively short due to the fact that they are susceptible to new races of pathogens (Leung et al., 2003). Coevolution of pathogens and their hosts is an ongoing process that results in new pathogen phatotypes that can overcome resistance.

On the other hand, genetically engineered resistant genotypes have offered hope, but it may take time for this approach to become socially and ecologically acceptable. Related issues involve the safety of food, horizontal gene transfer and public perceptions of genetically modified crops. Difficulties encountered with the

control of pathogenic fungi have led to the development of environment friendly biological control agents that may substitute for chemical fungicides and to the development of agronomic practices that result in decreased disease incidence (Krupinsky et al, 2002; Sturtz and Bernier, 1989; Sivasithamparam, 2002).

2.2.1 Crop rotation to reduce pathogens' inocula

Cultural practices may affect the relationship between plant pathogens and their hosts. Monocultures increase the severity and incidence of many diseases, whereas breaks in the cropping sequence with nonhost crops often decrease disease incidence (Ledingham, 1961; Sturtz and Bernier, 1989). Some nonhost crops are more effective in reducing the incidence of disease than other crops (Chinn, 1976). Crop rotations with non-cereal species are believed to be the most efficient way of decreasing the inoculum levels of wheat pathogens that persist in crop residues or in the soil from one year to another. However, fungal growth on or in residues of alternative host crops planted in rotation with cereals may also allow these wheat pathogens to be carried from one season to the next. The benefits of pulse and oil seed crops in a rotation system are not completely understood. The effect of rotation system on several fungal pathogens is discussed below.

The effects of crop rotation on *Fusarium* populations vary. Crop rotation with nonsusceptiple species may decrease *Fusarium* infection according to some reports (Sturz & Bernier, 1987; Wilson and Hamblin, 1990), while other studies found no effect of rotation on *Fusarium* inoculum density (Conner et al., 1996; Bailey et al. 2000).

Latta et al. (1891) were the first to report high *Fusarium* inoculum level in plots where wheat had been grown continuously or in rotation with maize crops. The importance of rotating wheat with noncereal species was later reported by Bolley (1913) and since that time, numerous experiments were performed in order to clarify the role of rotation system in decreasing *Fusarium* inoculum in wheat crops or crop debris. During 1985-1986, an experiment was conducted to assess the impact of cropping sequence on the survival and development of cereal diseases in Manitoba. This research (Sturz and Bernier, 1987) reports on the survival and abundance of fungal crown and root-rot pathogens in the stubble and soil-fractions of a variety of cereal and noncereal crops grown in rotation with winter wheat. A year of winter wheat was followed by a second of either barley, winter wheat, oat, flax, or canola. Seven fungal species pathogenic to winter wheat seedlings were recovered. Three of them were *F. culmorum*, *F. equiseti*, and *F. avenaceum*. The *Fusarium* spp. were isolated at a higher frequency from barley, winter wheat and oat, than flax and canola. Based on these findings, the researchers concluded that a 1-year rotation with canola or flax can reduce the levels of *Fusarium* pathogens in winter wheat crops. On the other hand, Conner et al. (1996) showed that flax had to be grown for at least two years to reduce common root rot in subsequent wheat crops.

In another experiment (Wilson and Hamblin, 1990), the severity of root rot caused by *Fusarium* spp. in spring wheat was greater following another wheat crop than after pea or lupin. In contrast, Bailey et al. (2000) found that rotation with noncereals crops such as canola and pea did not affect root rot severity or *Fusarium*

abundance in spring wheat, suggesting that the environment is the only factor influencing disease severity and the prevalence of virulent species in the population structure of pathogenic *Fusarium*.

The role of crop diversification in reducing FHB severity was also assessed in a study by Fernandez (2004) in which canola, flax, lentil and pea crops were grown in rotation with wheat and barley in south-east and east-central Saskatchewan. *Fusarium* was the most common genus isolated from the roots of the non-cereal crops tested. The most abundant *Fusarium* species isolated from the roots of pulse crops was *F. avenaceum*, which is one of the most common FHB and root rot pathogens of cereal crops in Saskatchewan. *F. equiseti* was the second most abundant *Fusarium* species, while other important cereal pathogens, such as *F. culmorum* and *F. graminearum*, were isolated only sporadically. The results of this study suggested that these non-cereal crops would help to maintain or increase *Fusarium* inoculum in crop residues, and they might contribute to root rot and FHB in subsequent cereal crops.

Whether or not rotation with nonsusceptible crops can reduce inoculum potential of *Fusarium* pathogens below critical levels is not well understood. Levels of *Fusarium* inoculum could depend on other factors such as the sporulation potential of these fungi, the potential for survival in the soil or plant residues, and environmental conditions.

The effect of crop rotation on the severity of diseases caused by *Pyrenophora* spp. on wheat in the mixed grass prairie ecozone of the semiarid Canadian prairies has been the field of study of many researchers for the last 25

years. In general results suggest that rotations with non-cereal crops can reduce tan spot severity in cereals.

Whether rotation diversity reduces tan spot disease was tested by Sutton and Vyn (1990). Disease severity in continuous wheat, soybeans-wheat-wheat, corn-barley-wheat, corn-soybean-wheat and alfalfa-alfalfa-wheat was compared over a three-year period (1984 to 1987). In the second stage of rotations, more of tan spot was observed in wheat grown after wheat; in the third stage, tan spot was severe only in continuous wheat, and in wheat after soybeans or wheat. Density of pseudothecia of *Pyrenophora tritici-repentis* increased with density of wheat residues and tan spot was moderate or severe only when the crop management practices resulted in accumulation of wheat residues on the soil surface.

Dynamics of foliar diseases were also studied in durum wheat to determine the effect of summerfallow and continous wheat on tan spot severity at Indian Head, Saskatchewan, from 1987 to 1990 (Bailey et al.,1992). Disease ratings (0-9) of leaf spots were greater on winter wheat following a cereal than following pea or summer fallow. Hot and dry weather did not enhance tan spot development, and the effect of tillage on leaf disease ratings was inconsistent. Other studies have demonstrated that including pulses in wheat-based rotations can reduce tan spot severity. For example, tan spot infection was reduced when spring wheat followed lentil rather than wheat in a field experiment conducted in Swift Current, Saskatchewan (Zentner et al., 2001).

The effect of crop rotation systems on the incidence of leaf spot diseases in spring wheat was also assessed over a three-year period (1993 to 1996) in

southwestern Saskatchewan (Fernandez et al., 1998). Ten crop rotations were established with various sequences of spring wheat, fall rye, flax, lentil, and summer fallow. The most prevalent fungus isolated from lesioned leaf tissue was *P. tritici-repentis*, and its occurrence in the fallow-wheat rotation was not significantly different than in continuous wheat. Crop rotations including flax or lentil reduced the occurrence of *P. tritici repentis*. The severity of leaf spots in wheat after fallow was greater than in continuous wheat. Percent area with leaf spots in wheat grown after wheat was higher than in wheat grown after flax or lentil in years with high disease level (1995-1996), but not in 1993 or 1994 when overall disease levels were low.

Some researchers suggest that crop rotation has no effect on the intensity of tan spot of wheat, and give results completely different from those of the studies described previously. In Manitoba, from 1993 to 1999, leaf-spot diseases were related to crop sequences (Gilbert and Woods, 2001). The pathogens most commonly isolated were *P. tritici-repentis*, *Phaeosphaeria nodurum*, *Cochlibolus sativus*, and *Mycosphaerella graminicola*. No significant effects of rotation with other noncereal crops were observed on *P. tritici-repentis* abundance. Bailey et al. (2001) analyzed the impact of tillage and crop rotation on diseases in wheat over a period of four years in Saskatchewan. The rotation systems under study were: summerfallow - spring wheat - spring wheat - winter wheat, spring wheat - spring wheat – flax - winter wheat, and pea -spring wheat - flax - winter wheat. Although the inoculum levels of some pathogens such as *Stagnospora nodorum* and *Bipolaris sorokiniana* were lower in the rotation with greatest crop diversity, no significant

impact was registered for the *P. tritici-repentis* inoculum. Crop rotations, in this study, had a limited impact on fungal pathogens. Once more, environmental conditions may have modified the effect of rotations.

While a great deal of literature describes the influence of diversified rotations on tan spot incidence and severity in common wheat fields, little is known about how noncereal crops influence *P. tritici-repentis* prevalence in durum wheat. More research is needed to better understand the effect of rotations with noncereals on the prevalence and aggressiveness of this species in a subsequent durum crop.

2.2.2 Beneficial fungi

In the past few years numerous studies have demonstrated the ability of certain microorganisms to antagonize pathogenic fungi. Mechanisms by which the antagonists suppress diseases include hyperparasitism, antibiotic production, competition for space and nutrients, lytic enzyme excretion, and induction of host resistance (Pal and McSpadden Gardener, 2006).

A hyperparasite directly attacks either resistance spores or living hyphae of the pathogen (Holt and Hochbert, 1998). For example, *Coniothyrium minitans*, a naturally occurring soil fungus, can attack the sclerotia of *Sclerotinia sclerotiorum* and *Sclerotinia minor*, two ubiquitous fungi that cause diseases in a variety of agricultural important crops (Aertsens and Michi, 2004). *Acremonium alternatum*,

Acrodontium crateriforme, *Cladosporium oxysporum*, and *Gliocladium virens* are fungal species known to attack hyphae (Kiss, 2003).

Research has focused on establishing the involvement of antibiotics and enzymes in the biological control process. Antibiotics are microbial metabolites that can be involved in disease suppression when produced in sufficient quantities (Pal and McSpadden Gardener, 2006). The involvement of antibiotics in disease control was repeatedly demonstrated (Howell, 2003; Howell and Stipanovic, 1995). For instance, *Acremonium zeae* was shown to produce two kinds of antibiotics, pyrrocidines A and B, displaying antifungal activity in assays against *Aspergillus flavus* and *Fusarium verticillioides* (Wicklow et al., 2005). *Chaetomium globosum* produces 19 antibiotic substances; among these, chetomin is of particular importance in fungal pathogens biocontrol (Di Pietro et al., 1992). *Chaetomium globosum* is antagonistic to the cereal root pathogen *Fusarium culmorum* (Mačkinaitė, 2001; Knudsen et al., 1995). In addition, diverse microorganisms secrete lytic enzymes that can hydrolyze a wide variety of polymeric compounds such as chitin, proteins, cellulose, hemicellulose, and DNA (Pal and McSpadden Gardener, 2006). The involvement of these enzymes in disease suppression has been extensively demonstrated in the case of *Trichoderma* species. *Trichoderma* spp. are common saprophytic fungi in the rhizosphere, which produce mainly chitinases (N-acetylglucosaminidases, chitobiosidases, and endochitinases), glucanases (endo- and exo-glucanase), and alkaline protease enzymes that enable the partial degradation of pathogens' cell wall (Viterbo et al., 2002). Through the activity of these enzymes, *Trichoderma* species can efficiently antagonize *Pythium*

ultimum, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Alternaria alternata* (Thrane et al., 2000; Baek et al., 1999, El-Katatny et al., 2001; Mora and Earle, 2001).

Soil and living plants are nutrient limited environments and therefore, microbes must effectively compete for nutrient sources. Competing for nutrients such as nitrogen, carbon, macro- and micro elements can result in a reduced rate of pathogen spore germination and in slower germ tube growth (Janisiewicz et al., 2000). *Trichoderma* and non-pathogenic *Fusarium* spp. are examples of antagonists having improved biocontol properties because of their increased ability to compete with pathogens for carbon, nitrogen, and iron at the root surface (Mandeel and Baker, 1991).

Over the past fifty years, research has shown that plants could be protected against infection through induced or acquired resistance to diseases (Fravel, 2005). This phenomenon is based on the early activation of host defense mechanisms preparing the plant to respond rapidly to the threat posed by the later arrival of pathogens (Hammerschmidt, 1999). The most commonly reported examples in the literature involve the use of non-pathogenic fungal strains to induce plant resistance against pathogenic strains. For example, it has been documented that tomatoes can be protected against the pathogenic *Fusarium oxysporum* by dipping the plants' roots in a suspension of non-pathogenic *F. oxisporum* to activate plant defense mechanisms some days before likely exposure to the pathogen (Campbell, 1989). Similarly, the inoculation of mint (*Mentha* spp.) with mildly pathogenic *Verticillium* spp. was proven to greatly reduce the symptoms and death caused by the pathogen *V. dahliae* (Campbell, 1989).

There are many reports in the literature describing the biocontrol of various plant diseases under laboratory or greenhouse conditions. However, the efficacy and survival of the biocontrol organisms under field conditions is influenced by environmental parameters (Sivasithamparam, 2002). Biocontrol researchers need to look forward to define ways to manipulate the environment through cultural practices in order to enhance the efficacy of biocontrol organisms. Several agronomic practices permit to promote biocontrol agents. These include advantageous regimes for crop plants, soil types, crop rotations, soil amendaments (manures and composts), suitable ploughing, and planting dates (Spadaro and Gullino, 2005).

2.3 Molecular tools to study the diversity of *Fusarium* and *Pyrenophora* spp.

In many studies, *Fusarium* and *Pyrenophora* spp. were identified based on micro- and macro-morphological characteristics, such as colony characteristics on specific culture media, and the size, shape and development of sexual and asexual spores. Unfortunately, these morphological characters are limited in number, probably subjected to selection, and their expression is sensitive to environmental changes. Therefore, the assessment of *Fusarium* and *Pyrenophora* diversity in the fields based on morphological characters is difficult and time consuming. Furthermore, culture based methods provide little information on the phylogenetic relationship between species. Molecular techniques for species identification offer rapid tests with high sensitivity and specificity that provide phylogenetic information based on sequence similarities. These techniques are often based on the

polymerase chain reaction (PCR), in which primers are used to amplify DNA fragments. The primers are highly specific, and adhere to corresponding sequences present only in the target DNA.

However, PCR based techniques have limitations and problems too. Firstly, amplification of DNA from soil samples can be unsuccessful due to inhibitory substances such as humic acids co-extracted from soil (Kirk et al., 2004). Secondly, lysis of fungal cells or structures differs among fungal groups. If the method of DNA cell extraction is too harsh, the nucleic acids will be sheared, leading to difficulties in subsequent PCR amplification. In addition, DNA strands that have a lower G+C content are separated more easily in the denaturing step of the PCR and, therefore, could be preferentially amplified (Wintzingerode et al., 1997). Such limitations could result in biased representation of the real diversity of a microbial community.

Once amplified, the PCR amplicons can be analyzed using different approaches, depending on the goal of the research. All methods though have their own limitations and only provide a partial picture of the microbial diversity. Kirk et al. (2004), suggests that a multi-methods approach should be applied when studying microbial populations. The use of several methods would provide a more complete and accurate assessment of a microbial community.

Denaturing gradient gel electrophoresis (DGGE) was initially used to study point mutation in DNA sequences (Kirk et al., 2004). DGGE was then applied to study bacterial genetic diversity (Muyzer et al., 1993), and a few years later, for the study of fungal communities (Kowalchuk et al., 1997). This fingerprinting

technique rapidly became a popular method to study the biodiversity of microbial communities. On the denaturing chemical gradient gel, PCR derived sequences are separated based on their melting domains (Kisand and Wikner, 2003). These melting domains rely on differences in the stability of G-C pairing (3 hydrogen bonds) as opposed to A-T pairing (2 hydrogen bonds). Therefore, DNA fragments rich in GC are more stable and remain double-stranded until they reach a higher denaturant concentration. DNA fragments which are rich in AT are less stable and become larger earlier, as the double-stranded molecule denatures into a partially single-stranded state. These fragments slow down or stop in the gel before fragments richer in GC. Based on the concept described above, molecules with different nucleotide sequences will stop migrating at different position in the gel. Moreover, for the purpose of DGGE, a GC sequence, a so-called GC-clamp, is attached to the 5'-end of the PCR primer. This GC sequence acts as a high melting domain preventing the double-stranded DNA from complete dissociation into single strands (Muyzer and Smalla, 1998). The optimal time for the electrophoresis is that required to obtain a maximum differentiation between the DNA fragments. Then, the bands in DGGE can be visualized using different protocols. Most laboratories are using ethidium bromide, although it is a strong mutagen and fairly toxic compound.

PCR–DGGE is an approach for the detection of both culturable and unculturable microorganisms. It is rapid, relatively inexpensive (Kirk et al., 2004) but has some limitations. Only the organisms accounting for more than 1% of DNA in a sample are displayed in the banding profile (Muyzer et al., 1993; Murray et al.,

1996) and the banding pattern relates more to the relative abundance of the populations than to species richness (Muyzer and Smalla, 1998). PCR-DGGE is useful to detect the most dominant populations. Minor populations often require enrichment, through nested protocols, prior to detection (Jackson et al., 1998).

A limitation of the DGGE technique comes from the fact that sometimes the outer lanes of the gel are difficult to analyze due to a so-called "smiling effect". Brinkhoff and Hannen (2001) explain that in the outer lanes, the migration behavior of the DNA is different due to the fact that urea and formamide are leaking into the buffer during the electrophoresis run and as a result, the concentration of the denaturing substances at the edges of the gel is lower. To solve this problem Brinkhoff and Hannen (2001) have successfully applied silicone grease on the outer edge on the front and back side of the spacers, fact that prevented later the contact of the gel with the surrounding buffer.

DGGE analysis, theoretically allows different sequences to separate into unique positions in the gel. The generated banding pattern is regarded as an "image" of the whole microbial community under study. However, in a study of fungal isolates infecting marram grass, Kowalchuk et al. (1997) showed that co-migrating bands can sometime correspond to distantly related sequences. The same researchers showed that some plant-derived fragments amplified by primers lacking specificity can also be recovered together with fungus-derived fragments from the DGGE gels. Moreover, some artificial bands, so-called heteroduplexes, may sometime be detected in the gels where they complicating the DGGE profile (Ferris and Ward, 1997).

One potential source for gel misinterpretation is the presence of multiple melting domains in the sequence of nucleotides (Wu et al., 1998). Getting single band profiles is important in molecular diversity analysis of microbial communities, but multiple banding patterns are often encountered. One reason for the occurrence of a multiple banding pattern is the presence of PCR fragments with multiple melting domains. Caution should always be taken when analyzing DGGE profiles.

PCR-DGGE analysis is a useful method that has produced substantial amount of information in microbiology. After optimizing the conditions that result in a good resolution of the bands in the gel, community fingerprints can be interpreted by several techniques such as hybridization with oligonucleotide probes (Stephen et al., 1998) and sequence analysis of the excised bands (Ferris et al., 1996). An alternative and much easier procedure to the sequencing of the DGGE bands is the comparison of the bands with some reference patterns called "markers" (Ercolini, 2004). DNA sequences of representative species expected to occur in a DGGE profile can be used as a molecular ladder. This ladder which is run into a DGGE gel along with the unknown samples can facilitate species identification by bands comparison.

The PCR-DGGE technique is useful for the detection and identification of fungal species. So far, most PCR-DGGE studies focusing on fungal diversity have targeted either portions of the 18S gene or the rDNA ITS region (Kowalchuk et al., 1997). Studies using the ITS regions have produced useful phylogenetic data for several fungal species, including *Phytophthora* spp. (Lee and Taylor, 1992) and *Verticillium* spp. (Morton et al., 1995). However, O'Donnell and coworkers (1998)

indicated that these ribosomal regions contain less interspecific variation than the elongation factor 1 alpha gene (EF-1 alpha) for the genus *Fusarium*. Based on this information, Yergeau et al. (2005) designed a *Fusarium*-specific PCR primer pair, targeting a partial region of the EF-1 alpha gene in order to discriminate between different *Fusarium* spp. from asparagus fields with PCR-DGGE. Their study has provided a wealth of new insight into the diversity of *Fusarium* spp. found in infested asparagus plants in Quebec.

Therefore, PCR-DGGE is therefore a molecular tool that is employed to separate DNA fragments according to properties of the nucleotide sequences. The multiple samples that can be analyzed concurrently on DGGE can efficiently provide information regarding changes that take place in the structure and dynamics of a microbial population, thus allowing for better monitoring and understanding of its fluctuations.

3. FUNGAL DIVERSITY ASSOCIATED WITH DURUM WHEAT PRODUCTION CHRONOSEQUENCES, PLANT ORGANS AND PRECEDING CROPS

3.1 Introduction

Geographic location, climate, cultural practices, relationships among microorganisms, and environmental conditions contribute to fungal diversity and distribution in plant ecosystems. Despite the broad occurrence of fungi in agricultural soils and plants, many fungi are restricted to particular plant organs (Arnold, 2005). Some fungi colonize roots and/or aerial plant parts without causing disease. However, numerous fungal species infecting plants cause diseases. Biological control of plant pathogens is accomplished by destroying the existing inoculum, and preventing the formation of inoculum (Gnanamanickam et al. 2002). While the former can be ensured by microorganisms on the basis of antagonistic relationships, the later can be achieved through selected cropping practices. Monoculture increases the incidence and severity of many diseases, whereas the inclusion of nonhost crops in the cropping sequence often decreases disease levels (Ledingham, 1961; Sturtz and Bernier, 1989). It is generally believed that some nonhost crops are more effective in reducing the incidence of disease than other crops. To date, the extent to which crop rotation can impact durum wheat fungal inhabitants, their distribution and prevalence is unclear.

Although there are few fragmentary reports on fungal communities from wheat crops grown in Saskatchewan no comprehensive work has been done on the overall fungal inhabitants on different durum wheat organs, or how plant growth

stages, and crop rotation may affect these inhabitants. In a survey conducted in 1998 and 1999 throughout Saskatchewan, Fernandez and Jefferson (2004) found that *Bipolaris sorokiniana*, *Microdochium bolleyi*, and *Fusarium* spp. were the most prevalent fungi on wheat and durum wheat at the milk to early dough stage. Another study focusing on pathogenic fungal species in Saskatchewan wheat crops revealed that *Pyrenophora tritici-repentis*, *Septoria avenae*, *Septoria tritici*, and *Septoria nodorum* were the principal foliar pathogens, while *Fusarium* spp. and *Bipolaris sorokiniana* were identified as the main root rot pathogens (Bailey et al. 1992).

3.2 Objectives and hypothesis

The goal of this study was to identify fungal pathogens that colonize durum wheat crops, as well as other naturally occurring fungi that could have the potential to decrease the abundance of fungal pathogens. In order to understand fungal diversity in durum production, the distribution of fungal communities through durum phenophases and different plant organs was described and the influence of preceding crops was assessed.

The first hypothesis was that a preceding crop of pea, chickpea, lentil, canola, and durum wheat will have an impact on the pattern of fungal biodiversity associated with durum wheat, and on the relative abundance of the major fungal wheat pathogens. The second hypothesis was that species that could be potential antagonists against pathogenic fungal species were naturally present in durum crops.

3.3 Materials and methods

3.3.1 Site description

The experiment was conducted in the field in 2004 and 2005. The site was at the South Farm of the Semiarid Prairie Agricultural Research Centre, in Swift Current Saskatchewan, Canada (latitude, 50º17 ’N; longitude: 107º41 ’W), Brown Soil zone. The site receives an average (54 years) of 361 mm of annual precipitations and has a yearly mean temperature of 3.6 ºC, with an average minimum of -13.2ºC in January and an average maximum temperature of 18.6ºC in June. Total precipitations were 407 mm in 2004 and 366 mm in 2005. Mean daily temperatures and daily precipitation for the 2004 and 2005 growing seasons are displayed in Figure 3.1. The soil was a Swinton Silt loam (Orthic Brown Chernozem).

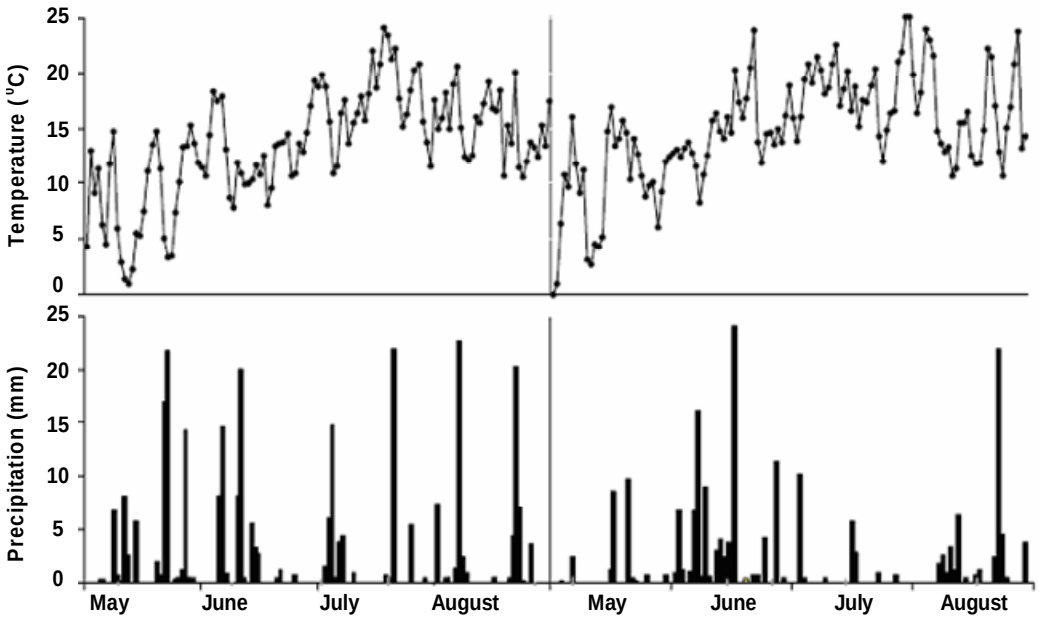

Figure 3.1 Precipitation and mean daily temperature throughout the 2004 and 2005 growing season. Data was recorded at the Environment Canada Swift Current CDA weather station.

There were five rotation treatments, all with fallow at stage I and durum at stage III, but with *Pisum sativum* L. (CDC Handel pea), *Lens culinaris* Medik (CDC Sovereign lentil), *Cicer arietinum* L. (Myles chickpea), *Brassica napus* L. (Liberty Link Invigor 2733 canola) or *Triticum turgidum* L. (AC Avonlea durum) at stage II (Table 3.1). Treatments were replicated three times. Plots were 5 x 24 m. Conventional tillage at a depth of 7.5 cm was used during the fallow stage to maintain plots. Crops were differentially fertilized each year based on soil tests to equalize soil fertility among treatments, as determined by soil tests. Durum after pulses and canola received 47 and 61 kg N ha^{-1} in 2004 and 2005, respectively, and durum after durum received 53 and 55 kg N ha^{-1}. Phosphorous (11-51-0) was

routinely applied with seeds at a rate of 45 kg/ha. All fertilizer was side banded 2.5 cm to the side and below the seed. Durum (AC Avonlea) was seeded using a Flexicoil 5000 air drill equipped with Stealth Double Shoot Knives with 22.5 cm spacing. Roundup Transorb (1.25 l ha^{-1}) was applied preseeding (on April 26 2004 and April 22 2005) to control weeds. Post emergence weed control was with Butril M (1.0 l ha^{-1}), applied August 27 in 2004 and with Butril M (1.0 l ha^{-1}) and Horison (2.38 l ha^{-1}) applied on August 10, in 2005.

Table 3.1 Crop varieties used in experiment were common varieties grown in the region.

Rotation [a]	Variety
Fallow - lentil - durum	CDC Sovereign
Fallow - pea - durum	CDC Handel
Fallow - chickpea - durum	Myles
Fallow - durum - durum	AC Avonlea
Fallow - Argentine canola - durum	Liberty Link Invigor [b] 2573/2733

[a] Lentil pea, chickpea, durum and Argentine canola were preceding crops and correspond to the indicated variety. All sampling was completed in the durum phase of the rotation.

[b] The canola variety used in 2004 was Liberty Link Invigor 2573 and in 2005, Liberty Link Invigor 2733.

3.3.2 Soil and plant sampling

Four sampling campaigns were conducted during the growing season to capture the dynamics of the fungal populations as plants developed and the season progressed. Soil and plants were sampled at the following physiological stages of durum growth: two-three leaf, six leaf, flowering, and milk to soft dough stages (Table 3.2). Four plants per plot were taken using a shovel. Roots were detached and placed in plastic bags with the adhering soil, and shoots were placed in paper bags. Under laboratory conditions, the rhizosphere soil was shaken from the roots and shoots were separated into crown, stem, leaf, and awn parts. All samples were held at 4° until analysis.

Table 3.2 Soil and plant samples for AC Avonlea were taken in 2004 and 2005 at four physiological stages

Growth stage	Actual degree day accumulated at sampling time[a]		Sampling date	
	2004	2005	2004	2005
2 - 3 Leaf	-	211.65	-	May - 30
6 Leaf	-	512.45	-	Jun - 20
Flowering	-	874.45	-	Jul - 11
Late milk to soft dough	1205.1	1245.2	Aug - 05	Aug - 02

[a]Degree days are calculated over a period of time by adding up differences between each day's mean daily temperature. Durum development corresponds to the accumulation of degrre day.

3.3.3 Determination of fungal biodiversity

Fungal populations in soil samples were analyzed using the soil dilution plating method. Aliquots of 0.1 ml from $l0^{-4}$ dilutions were spread on PDA (potato dextrose agar) culture media supplemented with antibiotics (100 mg l^{-1} streptomycin sulfate and 15 mg l^{-1} neomycin sulfate, Sigma, St. Louis, Mo, USA). Three Petri plates per sample were prepared and incubated at 22 ºC in the dark for four days. Pure cultures were established by transferring morphologically distinct colonies onto fresh medium.

Plant samples were surface sterilized by submerging the plant fragments into 95% ethanol (10 seconds), sterile distilled water (10 seconds), 5% sodium hypochlorite (2 min), and sterile distilled water (2 min). After sterilization, 1-cm tissue fragments were placed on PDA medium, in Petri plates. The operation was repeated for each plant part: root, crown, stem, leaf and awn. Four plates containing five fragments were made for each plant part of each plot. Plates were incubated at room temperature in the dark for six days. Pure cultures were established using standard microbiological procedures.

Colony forming units (CFUs) were counted and classified into 258 operational taxonomical units (OTUs) based on morphology. Isolates occurring at a frequency of > 1% were morphologically analyzed at the genus level based on characters such as: colony growth rate, pigmentation, and type of spores. To induce sporulation, some isolates were subcultured onto solid V8 juice agar medium (Stevens, 1974) or carnation-leaf agar medium (Fisher et al., 1982) and incubated at room temperature for four days. The fungal isolates of this culture library were

identified through DNA analysis. All isolates are deposited in Dr. V. Vujanovic's Saskatchewan Microbial Collection and Database under SMCD numbers: 2121-2198.

Mycelial mats were collected using a sterile scalpel blade and ground in liquid nitrogen with a mortar and pestle. Genomic DNA was extracted using the UltraClean microbial DNA isolation kit (MoBio, Inc. CA) following the manufacturer's instruction. The internal transcribed spacer (ITS) of the rDNA gene was amplified using fungal specific primers ITS1-F (5'CTTGGTCATTTA GAGGAAGTAA) (Gardes and Bruns 1993) and ITS4 (5'TCCTCCGCTTAT TGATATGC) (White et al. 1990). EF-1 alpha gene, used to discriminate between *Fusarium* spp., was amplified using the EF1 (5'ATGGGTAAGGA RGACAAGAC) and EF2 (5' GGARTGACCAGTSATCATGTT) primer set (O'Donnell et.al., 1998). A negative control (no DNA) was included in the PCR. Amplification with the primer set ITS1-F/ ITS4 was performed in a 25 µl reaction mixture in a Thermal Cycler epgradient S (Eppendorf) through 30 cycles of 94°C for 30 sec, 55 °C for 30 sec, and 72°C for 1 min. The same conditions were used for the EF1/EF2 primer set except the annealing temperature was increased to 57 °C. Aliquots of 5 µl of the PCR products, including the negative control probe, were run on an 0.8 % agarose gel electrophoresis containing ethidium bromide to reveal the presence/absence of the DNA bands. Images were acquired with a Bio Doc-IT Imaging System (UVP Inc. CA). The remaining PCR products were purified with a DNA purification kit (QIAquick PCR Purification Kit, Qiagen Inc., Valencia, CA) and submitted for sequencing at a commercial laboratory (Plant Biotechnology

Institute, Saskatoon, Saskatchewan). Similarity analyses were performed using the BLAST research module in GenBank (http://www.ncbi.nlm.nih.gov).

3.3.4 Statistical analyses

Fungal communities in durum from different crop rotations were compared based on the number of CFUs recorded for each species using cluster analysis. Clustering was performed with the average linkage method and Euclidean distance, in SYSTAT version 10 software (SPSS Inc., 2000).

The species richness (*S*) and Shannon's diversity index (*H'*) in each plant part collected at each growth stage from each plot were calculated. The spatio-temporal distribution of each fungal taxon was described using the persistence index (Vujanovic et al., 2007) defined as $Pi = -[\Sigma\, qi/Q \ln (qi/Q)]/[\ln(x)]$ where *qi* is the CFU value for one of the rotation system, plant part, or growth stage, Q is the sum of all the CFU values obtained in all crop rotations, plant parts, or growth stages for a particular taxon, and x is the number of rotation systems, plant parts, or plant growth stages examined in the study. A value of *Pi* equal to 1 means that the CFU values were constant throughout crop rotations, plant parts, or growth stages, whereas a value of *Pi* equal to 0 means that the fungal taxon was recovered from only one of these spatio-temporal locations.

Differences in fungal species richness and diversity among treatments were determined with ANOVA and least significant difference (LDS) tests. Data sets belonging to four different durum growth stages were summed first over plant

organs and second, over preceding crops. Separate one-way analyses of variance (ANOVA) were also conducted to determine the effects of crop rotation and plant part on pathogenic fungal communities associated with durum wheat. Since the data set was in some cases not normally distributed, transformations (ln) were applied to fulfill the requirement of the test. LSD was used to assess the significance of differences between treatment means at $P = 0.05$. ANOVA was performed using JMP software (SAS Institute Inc., Cary, North Carolina). Principal components analysis (PCA) was carried out to describe the tendency of segregation or coexistence of fungal species. The analysis was performed using SYSTAT version 10 software (SPSS Inc., 2000).

3.4 Results

Most fungi recovered from soil and durum plants were Ascomycota species belonging to the orders Hypocreales (33.33%), Pleosporales (16.66%), Eurotiales (11.90%), Capnodiales (7.14%), Sordariales (7.14%), Xylariales (4.76%), Botryosphaeriales (2.38%), Trichosphaeriales (2.38%), Dothideales (2.38%), Pezizales (2.38%), and undefined taxonomic order "Incertae sedis" (9.52%). A small number of isolates from the Basidiomycota and Zygomycota divisions were also recovered from soil and root tissues respectively. Seventeen fungal species recovered from durum tissues were potential pathogenic species. Of these, 47% belong to Hypocreales, and 29% to Pleosporales. *Fusarium* (Hypocreales) pathogens were *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. sporotrichioides*, *F.*

reticulatum, *F. equiseti*, *F. flocciferum*, and *F. torulosum*. *F. avenaceum*, *F. reticulatum*, *F. tricinctum*, and *F. torulosum* were prevalent accounting for 29.67%, 8.56%, 3.92%, and 2.32% of all recovered pathogenic species. Two of the most important fungal pathogens belonging to Pleosporales were recovered: *Bipolaris sorokiniana*, accounting for 2.32% of the total CFU of pathogenic species, and *Pyrenophora tritici-repentis* (5.56%). The other Pleosporales pathogens recovered, *Phaeosphaeria avenaria*, *Alternaria tenuissima*, and *Epicoccum nigrum* accounted for 4.06%, 16.74%, and 1.93% of the total CFU of pathogenic species. Some other pathogens isolated from durum wheat were *Cladosporium* spp. and *Microdochium bolleyi*. They had a total relative abundance of 13.55%, and 7.93% off all pathogenic species recovered. The recovery of *Arthrinium sacchari* and *Nigrospora oryzae* pathogens from durum tissues was atypical and their prevalence was low (2.12%, and 1.25%).

3.4.1 Fungal biodiversity in soil and plant organs

Fungal species richness (S) and Shannon's diversity index (*H'*) were calculated at all four sampling times (Table 3.3 and 3.4). *S* and *H'* values indicated a higher diversity and species richness in roots of durum plants where *S* ranged from 14.66 to 20.66 and *H'* from 1.55 to 2.44. The distribution of fungal taxa recovered at a frequency of > 1% from soil and plant parts at one specific durum developmental stage is shown in the Figure 3.2. *Penicillium*, *Geomyces*, and *Fusarium* were commonly encountered in soil and all durum plant parts. *Alternaria*,

Acremonium, *Bipolaris*, *Cladosporium*, *Epicoccum*, *Chaetomium*, *Nigrospora*, *Phaeosphaeria*, and *Pyrenophora* were other prevalent species. They were recovered from at least two different plant parts. *Trichoderma* and *Microdochium* were recovered from one plant part and soil. Five *Penicillium* (*P. aurantiogriseum*, *P. commune*, *P. griseofulvum*, *P. solitum*, *P. tricolor*), three *Cladosporium* (*C. minourae*, *C. cladosporioides*, *C. herbarum*), two *Chaetomium* (*C. globosum*, *C. funicola*), and two *Pyrenophora* (*P. tritici – repentis*, *P. teres*) were identified.

Table 3.3 Species richness (*S*) in soil and plant organs of durum wheat, at different plant growth stages

Treatment	Species Richness (*S*)				
	2-3 Leaf	6 Leaf	Flowering	Late milk to soft dough	
	2005[a]	2005	2005	2005	2004
Soil	7.33 b	10.66 b	10.00 b	7.66 c	6.93 c
Roots	20.66 a	15.00 b	17.00 a	14.66 ab	16.33 a
Crowns	12.66 b	–	–	–	–
Stems	–	18.00 ab	9.66 b	17.00 ab	17.66 a
Leaves	–	20.33 a	11.33 b	11.66 bc	11.33 b
Awns	–		9.00 b	19.33 a	14.33 ab
Significance	$P < 0.02$	$P < 0.02$	$P < 0.01$	$P < 0.02$	$P < 0.02$

[a] Means with the same letters in a column are not different according to ANOVA protected LSD test ($P < 0.05$, $N = 3$).

Table 3.4 Shannon's diversity index (*H'*) in soil and plants organs of durum wheat, at different plant growth stages

Treatment	Shannon's diversity index (*H'*)				
	2-3 Leaf	6 Leaf	Flowering	Late milk to soft dough	
	2005[a]	2005	2005	2005	2004
Soil	1.29 c	1.66 b	1.75 b	0.84 c	0.43 c
Roots	2.44 a	2.36 a	2.16 a	1.55 b	2.40 a
Crowns	1.90 b	–	–	–	–
Stems	–	2.58 ab	1.78 b	2.23 a	2.02 ab
Leaves	–	2.58 a	2.02 ab	2.17 a	1.66 b
Awns	–		1.81 b	2.40 a	2.27 a
Significance	$P < 0.02$	$P < 0.02$	$P < 0.04$	$P < 0.02$	$P < 0.02$

[a] Means with the same letters in a column are not different according to ANOVA protected LSD test ($P < 0.05$, $N = 3$).

The persistence index of fungal species was assessed to better understand species distribution on durum plant parts (Table 3.5). Fungal species with $Pi = 0$ were recovered from only one specific plant organ. Therefore, among fungi specific to roots were *Periconia macrospinosa*, *Ophiosphaerella* sp., *Hypocrea* sp., *Ceratobasidium* sp., and *Mortierella hyalina*. *Fusarium sporotrichioides* was found only on stems. *Macrophoma* sp. and *Thielavia hyalocarpa* were recovered exclusively from awns. Other species such as *Fusarium avenaceum*, *Fusarium reticulatum*, *Cladosporium minourae*, *Cladosporium cladosporioides*, *Geomyces pannorum*, *Penicillium aurantiogriseum*, and *Penicillium griseofulvum* colonized all plant parts to a high extent ($Pi \geq 0.80$).

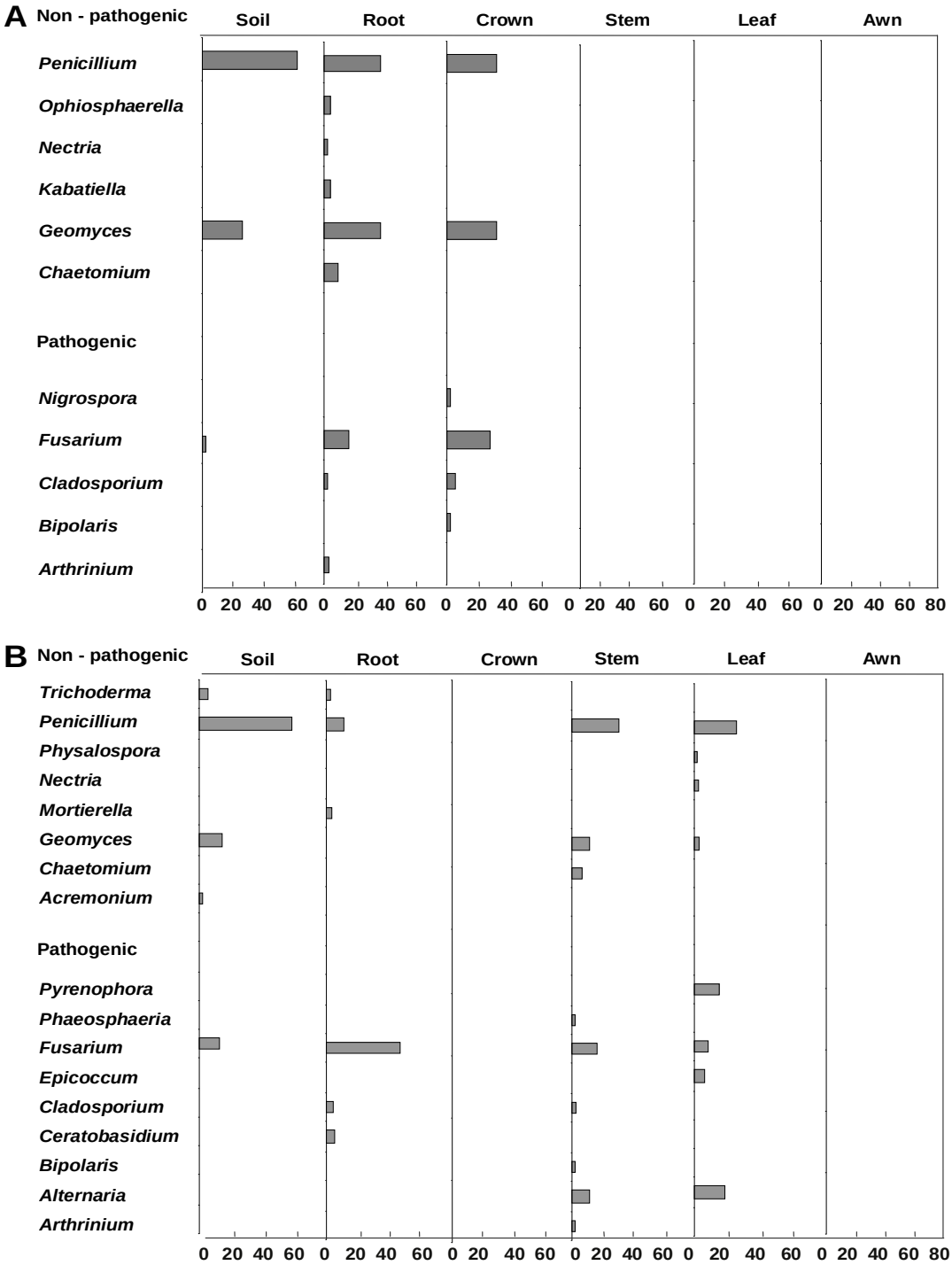

Figure 3.2 Prevalence of fungal taxa in soil and different durum wheat organs: A, 2- 3 leaf stage, B, 6 leaf stage. Units of measurement indicate percentage of CFU.

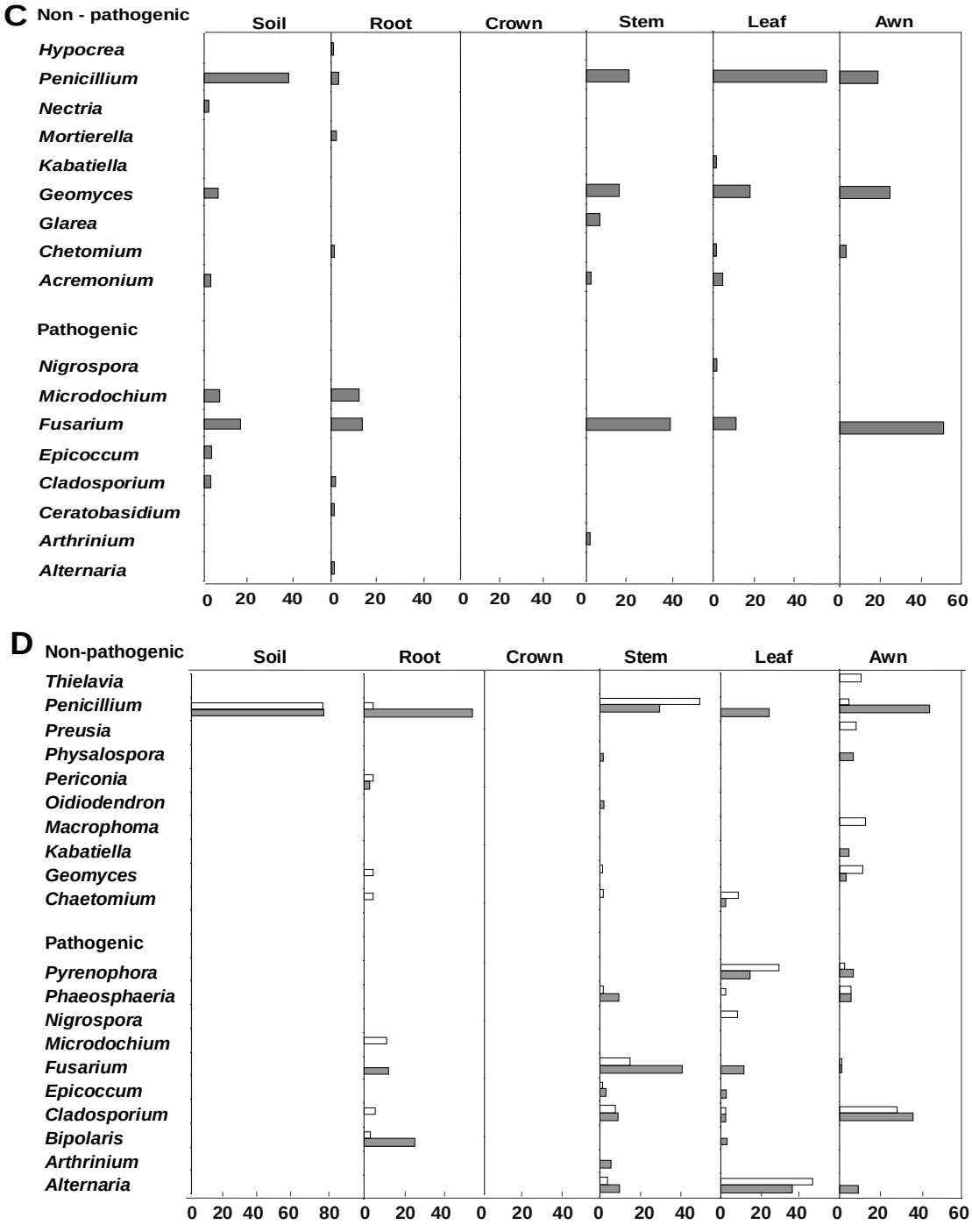

Figure 3.2 Continued. Prevalence of fungal taxa in soil and different durum wheat organs: C, flowering stage, and D, late milk to soft dough stage. Units of measurement indicate percentage of CFU. Filled and empty bars represent fungal taxa in 2004 and 2005 respectively.

Table 3.5 Fungal species recovered from durum crops and their persistence in durum wheat

	Persistence Index (*Pi*)				
Species of Fungi	Plant parts	Previous crops	Growth stages	GenBank Accession No[a]	Similarity
Acremonium sp.	0.69	0.95	0.59	AY625058	97%
Alternaria teniussima	0.49	0.98	0.47	AY154712	99%
Arthrinium sacchari	0.52	0.99	0.81	AF455478	99%
Bipolaris sorokiniana	0.55	0.93	0.32	AF158105	99%
Ceratobasidium sp.	0	0.76	0.40	EF536968	97%
Chaetomium funicola	0.49	0.97	0.71	EF017204	98%
Chaetomium globulosum	0.66	0.95	0.77	DQ518178/ AY429052	100%
Cladosporium cladosporioides	0.83	0.79	0.66	AF538619	98%
Cladosporium herbarum	0.30	0.96	0.44	AF455517	99%
Cladosporium minourae	0.85	0.97	0.72	AF393716	94%
Cordyceps cylindrica	0	0	0.41	EF029230	94%
Epicoccum nigrum	0.43	0.85	0.58	AF455455	99%
Fusarium avenaceum	0.94	0.98	0.73	AJ543522	99%
Fusarium equiseti	0.47	0.74	0.55	AJ543576	96%
Fusarium flocciferum	0.35	0.77	0.64	AJ543574	99%
Fusarium poae	0	0	0	AJ420851	100%
Fusarium reticulatum	0.82	0.97	0.89	DQ29514	97%
Fusarium sporotrichioides	0	0	0	AJ420821	100%
Fusarium torulosum	0.55	0.87	0.88	AJ543612	98%
Fusarium tricinctum	0.44	0.77	0.73	AJ543626	98%
Geomyces pannorum	0.85	0.97	0.73	DQ1892224	99%
Hypocrea sp.	0	0.32	0.41	AY241587	97%
Kabatiella caulivora	0.58	0.86	0.73	EU167576	94%

[a] GenBank Accession No column displays the result of the BLAST search in NCBI.

Table 3.5 Continued

Species of Fungi	Persistence Index (*Pi*)			GenBank Accession No[a]	Similarity
	Plant parts	Previous crops	Growth stages		
Macrophoma sp.	0	0.33	0	DQ100416	96%
Microdochium bolleyi	0.42	0.92	0.65	AJ279454	99%
Mortierella hyalina	0	0.62	0.49	AY157495	99%
Nigrospora oryzae	0.48	0.87	0.68	DQ219433	99%
Nectria sp.	0.53	0.62	0.36	DQ779785	97%
Ophiosphaerella sp.	0	0.25	0	AJ246157	99%
Oidiodendron cerealis	0.23	0	0	AF062788	100%
Penicillium auranteogriseum	0.82	0.98	0.81	AJ005488/AY380455	99%
Penicillium commune	0	0	0	AF2366103	98%
Penicillium griseofulvum	0.80	0.97	0.86	DQ339557	99%
Penicillium solitum	0	0.42	0	AY373932	99%
Penicillium tricolor	0.72	0.73	0.57	AY373935	99%
Periconia macrospinosa	0	0.31	0	AJ246158	99%
Phaeosphaeria avenaria	0.69	0.87	0.60	U77359	99%
Physalospora vaccinii	0.34	0.61	0.10	AY075113	91%
Preussia africana	0.62	0.62	0.72	DQ865095	98%
Preussia minima	0	0	0.27	AY943054	97%
Pyrenomataceae sp.	0.28	0.41	0.20	DQ317369	97%
Pyrenophora teres	0.26	0.76	0.62	AF400895	100%
Pyrenophora tritici-repentis	0.18	0.99	0.46	AY004808	99%
Pyronema domesticum	0	0.43	0.45	DQ491517	98%
Thielavia hyalocarpa	0	0	0	AJ271583	98%
Trichoderma harzianum	0	0	0	AJ230664	99%

[a] GenBank Accession No column displays the result of the BLAST search in NCBI.

Although *Fusarium avenaceum* was abundant in all plant parts, root and awn tissues were colonized to the highest degree (6.5 and 5.94 CFU mean values) (Table 3.6). The lowest mean (4.45 CFU) was in leaf tissues. *Fusarium reticulatum* and *Fusarium tricinctum* colonized roots preferentially (5.52 and 5.15 CFU mean values). *Bipolaris sorokiniana* was the pathogenic Pleosporales recovered most frequently from durum roots and crowns but was also present in stem and leaf tissues. *Pyrenophora tritici-repentis* was isolated exclusively from leaf and awn tissues. *Pyrenophora tritici-repentis* and *Bipolaris sorokiniana* were evenly distributed throughout the plant organs they colonized.

Table 3.6 Abundance of CFU of known durum pathogens isolated from different plant parts

Treatment	*Fusarium avenaceum*	*Fusarium reticulatum*	*Fusarium tricinctum*	*Fusarium torulosum*	*Bipolaris sorokiniana*	*Pyrenophora tritici-repentis*
canola	5.35	4.40	5.55	3.26 b	5.01 a	3.25
chickpea	5.60	5.07	4.30	4.10 ab	4.94 a	4.20
lentil	5.70	4.89	4.46	5.28 a	4.46 a	4.10
pea	5.21	4.49	4.09	5.09 a	3.22 b	4.11
wheat	6.10	4.23	0	4.10 ab	4.48 a	4.05
Significance	ns	ns	ns	$P < 0.01$	$P < 0.05$	ns

[a] Means with the same letters are not different according to ANOVA protected LSD test ($P < 0.05$, $N = 3$)

3.4.2 Fungal biodiversity as related to durum phenophases

The greatest biodiversity of fungi was found in durum roots at the two-three leaf growth stage (Table 3.4). Stem and leaf tissues had generally high *S* and *H'* values at the six leaf growth stage. At the flowering stage a decrease in biodiversity and species richness was observed in all aboveground plant tissues (Table 3.3). At flowering and just before plant maturity stems and awns had the highest S and H' values (Table 3.3 and 3.4).

The persistence of fungal species through wheat growth stages varied widely (Table 3.5). Most of the *Fusarium* species recovered from durum tissues had a high persistence index as related to durum phenophases. *Fusarium avenaceum*, and *Fusarium tricinctum* (*Pi* = 0.73) were more abundant at the flowering stage, while *Fusarium torulosum* (*Pi* = 0.88) and *Fusarium reticulatum* (*Pi* = 0.89) appeared to be proportionally distributed throughout the four growth stages sampled. Other fungi with a $Pi \geq 0.80$, such as *Penicillium griseofulvum* and *Penicillium aurantiogriseum* had a nearly constant distribution through all durum growth stages. Some fungal species such as *Fusarium poae* and *Trichoderma harzianum* were recovered only in the six leaf growth stage, while *Fusarium sporotrichioides*, *Oidiodendron cerealis*, *Macrophoma* sp., *Penicillium commune*, and *Thielavia hyalocarpa* were recovered only in the late milk to soft dough stage (*Pi* = 0).

3.4.3 Fungal biodiversity as related to preceding crops

Cluster analysis shows the most similar fungal communities in durum following durum and durum following lentil. The community in durum grown after canola was the most distinct (Figure 3.3).

Twenty seven fungal species had a $Pi \geq 0.7$ having nearly a similar pattern of distribution on durum plants regardless of the preceding crop (Table 3.5). *Cordyceps cylindrica*, *Thielavia hyalocarpa*, and *Fusarium sporotrichioides* were specific to the preceding crop ($Pi = 0$). They were recovered only from durum after durum. *Oidiodendron cerealis* was only isolated from durum following lentil, *Penicillium commune* was found on durum following pea, and *Preussia minima* was recovered only from durum following canola crops.

Two fungal species pathogenic on durum wheat were found to be significantly influenced by the effect of crop rotation. *Fusarium torulosum* was less abundant in durum following canola and more abundant following lentil (3.26 and 5.28 CFU mean values, $P = 0.01$). The other species was *Bipolaris sorokiniana* which was less abundant in durum following pea and more abundant in durum following canola crops (3.22 and 5.01 CFU mean values $P = 0.05$).

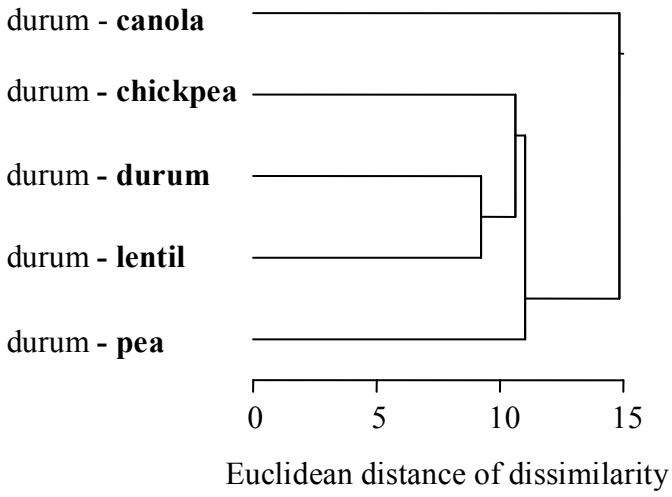

Figure 3.3 Cluster dendrogram of the five crop rotations evaluated based on their fungal colonization. The shorter linkage distances demonstrate the greater degree of similarity. Crops in bold were preceding crops of interest. All sampling was completed in the durum phase of the rotation.

3.4.4 Relationships among fungi

Principal component analysis was done taking into consideration all species that counted at least 20 CFUs in soil and durum tissues. The analysis revealed the coexistence of *Fusarium tricinctum* with *F. reticulatum* and *F. torulosum*, and their tendency to segregate from *Acremonium*, *Epicoccum*, and *Chaetomium*. *Fusarium avenaceum* was unrelated to species such as *Alternaria tenuissima*, *Cheatomium globosum*, and *Epicoccum nigrum* (Figure 3.4A and 3.4B).

Alternaria tenuissima, *Pyrenophora tritici-repentis* and *Nygrospora oryzae* co-occured on durum plants. These colonizers appeared to be negatively related to *Penicillium aurantiogriseum*, *Fusarium avenaceum, Kabatiella caulivora*, and *Cladosporium herbarum* (Figure 3.4C). The root and crown rot pathogen *Bipolaris*

sorokiniana was negatively related to *Chaetomium* and *Cladosporium* species (Figure 3.4D). *Phaeosphaeria avenaria*, *Arthrinium sacchari, and Fusarium flocciferum* seemed to co-occur. *Arthrinium sacchari* and *Fusarium flocciferum* were negatively related to *Cladosporium cladosporioides*, and *Phaeosphaeria avenaria* was negatively related to *Penicilium* species (Figure 3.4E).

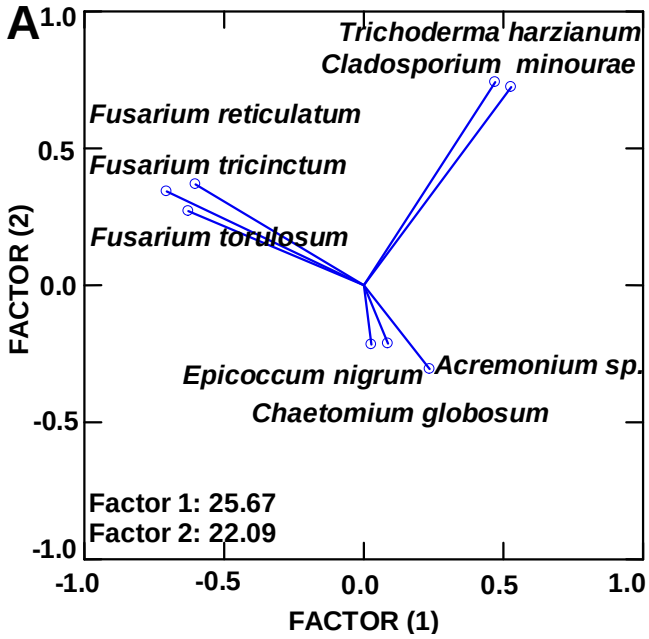

Figure 3.4 Ordination biplots from PCA analysis showing negative, neutral, and positive relationships between fungal species. The variance explained by Factor 1 and 2 is given in the left corner of each biplot. A, relationships with *Fusarium reticulatum*, B, relationships with *Fusarium avenaceum*, C, relationships with *Pyrenophora tritici-repentis*, D, relationships with *Bipolaris sorokiniana*, and E, relationships with *Fusarium flocciferum*.

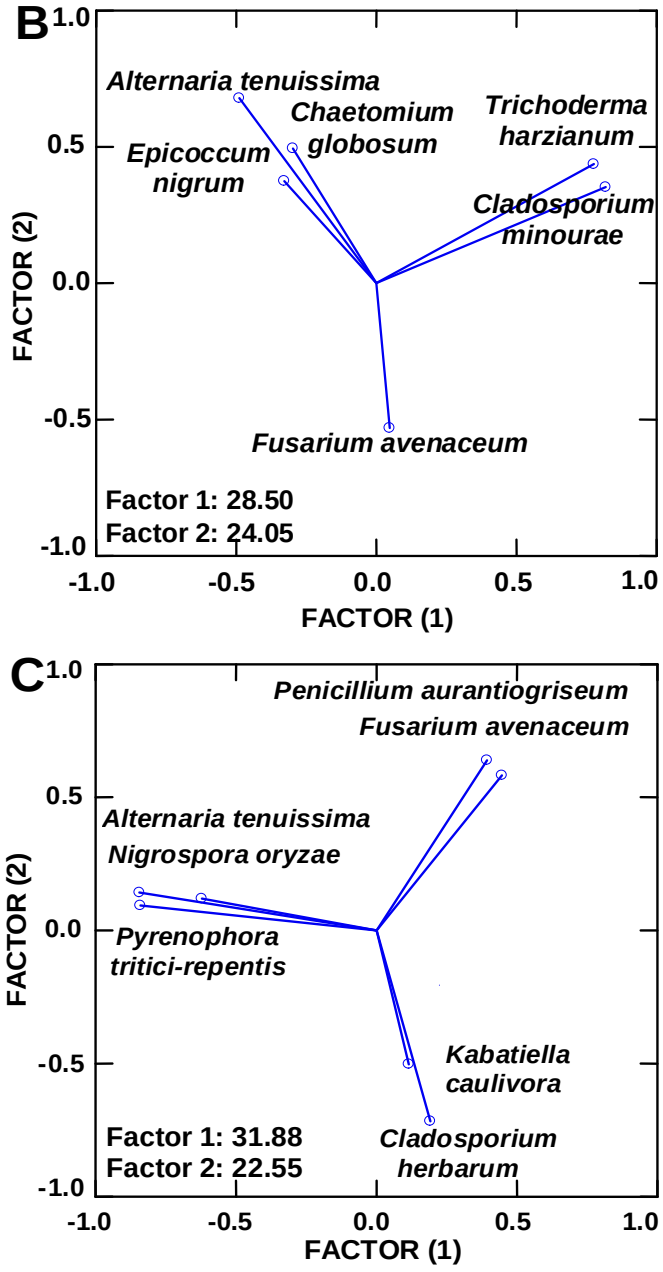

Figure 3.4 Continued

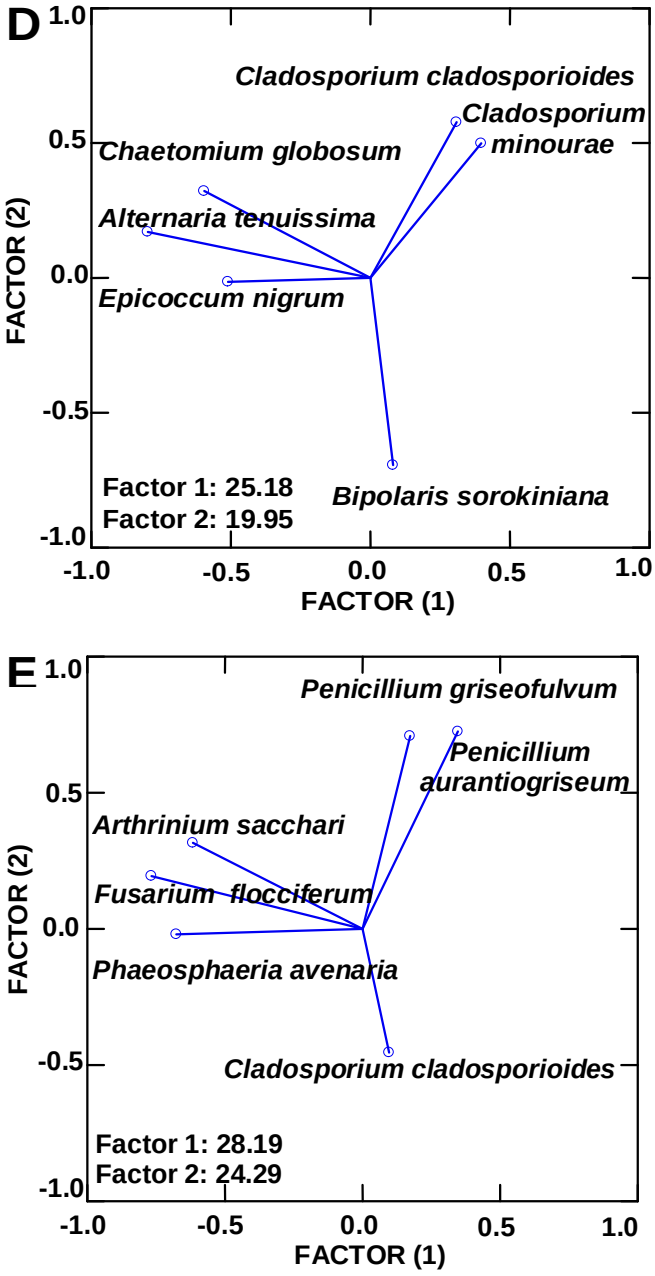

Figure 3.4 Continued

3.5 Discussion

3.5.1 Interactions among fungi

PCA analysis showed relationships among fungal communities in durum plants. The main mechanisms by which fungal antagonists may exert activity against pathogens have been attributed to effects such as hyperparasitism, antibiosis, competition for nutrients and space, and indirect effects such as plant induced resistance (Benhamou et al., 2002). It is imperative though to say that fungal species that do not colonize the same plant organ at the same developmental stage might not be antagonistic.

Fusarium tricinctum, *Fusarium torulosum*, and *Fusarium reticulatum* showed a tendency of spatial and temporal co-occurence. These species were found mostly in soil and durum roots organs and their persistence through the plant growth stages was high in all cases. *Fusarium tricinctum* and *Fusarium reticulatum* occurred together in soil samples at the six-leaf and flowering stages of durum growth. *Fusarium tricinctum*, *Fusarium reticulatum* and *Fusarium torulosum* co-occurred in roots at the six-leaf, flowering, and late milk to soft dough stages. It is possible that these populations coexist through the avoidance of competition. According to Lockwood (1981), the consumption of resources by one population may not restrict the access of other populations to the same resource pool if these resources are unlikely to become scarce. The combined action of fungal pathogens is known to reduce plants' capacity for response to diseases and contribute to the slow growth of crops (Einhellig, 1999). It would be therefore expected that together, these pathogenic *Fusarium* species increase root damage. This group of

Fusarium spp. showed a negative relationship with fungi such as *Epicoccum nigrum*, *Acremonium* sp. and *Chaetomium globosum*. Among these species *Epicoccum nigrum* and *Acremonium* sp. were isolated from soil, stems, and leaves parts, while *Chaetomium globosum* was found in roots, stems, leaves and awns. These fungi were found in soil and plant tissues when *Fusarium* spp. were also present. It is possible that these species are involved in antagonistic interactions with pathogenic *Fusarium* spp.

Fusarium avenaceum, generally known as the main cause of Fusarium head blight (FHB) and Fusarium damaged kernels (FDK) in the Saskatchewan cereal crop districts, was one of the most abundant species recovered from soil and durum plants at almost all growth stages. *Fusarium avenaceum* was prevalent in root and awn tissues but was also present in stem, crown and leaf tissues. Durum wheat is a good habitat for this fungus. The high persistence of this species throughout durum phenophases was associated with an early invasion of durum tissues. A plant tissue, once occupied, often becomes highly resistant to subsequent invasions by other fungi, providing a protected niche in which the primary colonizer has an increased capability to resist replacement (Lockwood, 1981).

According to our results, *Fusarium avenaceum* appeared inversely related to *Alternaria tenuissima*, *Chaetomium globosum*, and *Epicoccum nigrum*. *Fusarium reticulatum*, *Fusarium tricinctum*, and *Fusarium torulosum* were inversely related to *Epicoccum nigrum*, *Acremonium* sp., and *Chaetomium globosum*. It is important to mention that species of *Epicoccum* and *Alternaria* are known pathogenic fungi associated with discolored and black-pointed wheat heads (Wiese, 1987) and

therefore, species of these genera can not be regarded as potential biocontrol agents. Some fungal species can restrict the spread of other species by producing antibiotics. The involvement of antibiotics in disease control has been demonstrated repeatedly (Howell, 2003; Howell and Stipanovic, 1995). *Acremonium zeae* is known to produce pyrrocidines A and B, two antibiotics that were proved to have antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* (Wicklow et al., 2005). *Chaetomium globosum* produces 19 antibiotic substances. Among these, chetomin is considered of a great importance in fungal pathogens biocontrol (Di Pietro et al., 1992). *Chaetomium globosum* is antagonistic against the cereal root pathogen *Fusarium culmorum* (Mačkinaitė, 2001; Knudsen et al., 1995). Even if the abundance of the *Acremonium* and *Chaetomium* spp. recovered in this study was low when compared to *Fusarium* spp., they may have good antagonistic properties and should be further investigated for their potential as biocontrol agents against *Fusarium* pathogens colonizing durum wheat in the semiarid Saskatchewan.

Bipolaris sorokiniana, the causal agent of wheat common root rot, spot blotch, and crown rot, was frequently encountered in roots, crowns, stems, and leaves of durum wheat. This species was negatively related to *Cladosporium* and *Chaetomium* spp. An interaction between *Bipolaris sorokiniana* and *Cladosporium* spp. is unlikely to exist since *Bipolaris sorokiniana* and *Cladosporium* spp. colonized durum wheat at different plant developmental stages. A more confident interaction may exist between *Bipolaris sorokiniana* and *Chaetomium* species. Aggarval and co-workers (2004) already showed that under *in vitro* conditions four strains of *Chaetomium globosum* produced inhibition zones in *Bipolaris*

sorokiniana cultures, while under *in vivo* conditions two strains of *Chaetomium globosum* significantly reduced the infection level of *Bipolaris sorokiniana*. The production of antifungal compounds by the *Chaetomium* isolates tested was positively correlated with the antagonism against *Bipolaris sorokiniana* on wheat plants.

Pyrenophora tritici-repentis, *Alternaria tenuissima* and *Nigrospora oryzae* are leaf pathogens that seemingly co-occur on durum leaves. The pathogens *Arthrinium sacchari*, *Phaeosphaeria avenaria* and *Fusarium flocciferum* co-existed on durum stems. These fungal species displayed a degree of plant organ specificity. The specificity for particular plant organs colonization is poorly understood. Binding mechanisms to plant surfaces, types of penetration structures, thickness and composition of plant organ cuticle, and chemical signal at the plant surface may all be involved in the specific colonization of a plant part by a fungus (Schäfer, 1994; Kolattukudy et al., 1995). Fungal taxa such as *Penicillium* and *Cladosporium* appeared negatively related to durum leaf and stem pathogens. Vujanovic et al. (2007) suggested that *Penicillium* could be effective antagonist against pathogenic species on other plant hosts; however, direct evidence through laboratory testing on wheat is still required. *Penicillium* spp. were found to produce chitin and glucan-degrading enzymes, and other antifungal metabolites (patulin, citrinin, palitantin, and arthrographol) when grown in the presence of some pathogenic fungi (Yamaji et al., 1999; Roberti et al., 2002).

3.5.2 Impact of preceding crops on pathogenic fungal species

Cropping systems influence the abundance of plant pathogens (Ball et al., 2005). In our study, two pathogenic fungi *Fusarium torulosum* and *Bipolaris sorokiniana* were found to be significantly impacted by crop rotation: *Bipolaris sorokiniana* is one the most important root rot pathogens in Saskatchewan and a serious concern to wheat production. Disease is caused by conidia produced by the anamorph *Bipolaris sorokiniana*. In the cropping system tested, durum following a pea crop resulted in a significantly lower abundance of *Bipolaris sorokiniana*. It has been reported that usually sporulation of *Bipolaris sorokiniana* is highest in cereal residues and lower in oilseed and pulse crops (Duckzek et al., 1996). The data reported here agree with the above findings only partially since durum following canola resulted in the highest prevalence of *Bipolaris sorokiniana*. Our results suggest however that pea residue can reduce the prevalence of *Bipolaris sorokiniana* in a subsequent durum wheat crop. These results agree with those of Bailey et al. (2001), who found that wheat grown after pea has reduced incidence of root rot caused by *Bipolaris sorokiniana*.

3.5.3 Fungal biodiversity

Most fungal species colonizing durum wheat grown in monoculture or after pulses and canola crops were ubiquitous, but their abundance often varied with cropping systems. Similarity analysis revealed that composition of the fungal community inhabiting durum grown after canola was dissimilar to that in durum

after pulses (Figure 3.3). This may be partly explained by the unique allelochemicals (principally isothiocyanates (ITCs)) released during the decomposition of canola crop residues. The trends regarding the diversity and abundance of fungal communities as influenced by allelopathic compounds released through decomposition of crop residues are not completely understood. Allelopathy however, remains a possible explanation for the greater dissimilarity in fungal community after canola than after the other four rotation crops. Crop species can also have impacts on soil quality, nitrogen and water availability in a subsequent crop. It was reported that soil following canola was more porous than soil after pulses, and had less free nitrogen and less available water (Chan and Heenan, 1996; Roper and Gupta, 1995; Larney and Lindwell, 1995). Despite the differential fertilization of durum that was used to minimize the N effect of pulses, it remains that differential soil quality might affect the microbial community composition in durum wheat following canola crops.

Fungal communities varied with plant parts. Species richness (S) and Shannon's diversity index (*H'*) were higher in roots than elsewhere in durum plant parts. According to Garbeva et al. (2004) the main sources of easily accessible substrate are root tips. It may be that wheat roots release a wide variety of compounds into the surrounding and create a nutrient rich environment favouring fungal survival and coexistence in roots organs. Besides the richness of nutrients available in roots, an enhanced species richness and diversity in root organs could be attributed to the buffering capacity of soil against environmental conditions.

Roots might be considered as a relatively stable environment adequate for many species.

With a few exceptions most fungal species were able to colonize more than one plant organ. *Fusarium* species were among the most ubiquitous and abundant in durum tissues. According to Prescott et al. (1986) *Fusarium* species colonize nearly all tissues of wheat plants and cause diseases such as: common root rot, crown rot, leaf blotch, head blight, seedling blight, and black point. The high recovery of *Fusarium avenaceum* was expected. Several studies have indicated that this species accounts for a large proportion of the *Fusarium* population in wheat, at least in Saskatchewan (Gordon, 1952; Bailey et al., 2000; Fernandez et al., 2001). Less prevalent *Fusarium* species were *Fusarium sporotrichioides* and *Fusarium poae*. Their low rate of recovery could be attributed to the fact that they are less addapted to the dry conditions prevailing in Saskatchewan. *Fusarium* species recovered from agricultural ecosystems have distinct climatic preferences, and climate and local variations in weather can limit the range of species and influence their relative frequency.

The recovery of *Arthrinium sacchari* in durum was not expected. This species, known as a pathogen of sugarcane crops, had a low relative prevalence when compared to other fungi. This species could pose a threat to durum wheat production if environmental conditions were conducive. It was shown that durum wheat is susceptible to damping off disease caused by *Arthrinium sacchari* (see chapter 6). Moreover, this fungus is an important mycotoxigenic species that can cause acute food poisoning (Wei et al., 1994).

The functional borders between endophytic, pathogenic and saprotrophic fungal communities overlap and are not well established (Arnold, 2007). Moreover, it was repeatedly demonstrated that the functional aspects regarding the fungal inhabitants of plants differ with the fungus life cycle, strain, environmental conditions, and plant host (Freeman and Rodriques, 1993; Carroll, 1988, Stanosz et al., 2001). The classification of the identified fungi (Table 3.5) in one of the functional groups mentioned above is difficult to address because of the limited information available in the literature on the functional or ecological specificity of these fungi in durum hosts. Fungal endophytes are defined functionally by occurrence within asymptomatic tissues of plants (Arnold, 2007). A review of the literature revealed that genera *Acremonium*, *Chaetomium* and *Preussia* species are considered as endophytes on *Triticum* species (Arenal et al., 2007; Noor et al., 2006, Marshall et al., 1999). Still, the majority of fungal species identified in this study are regarded as *Triticum* pathogens. Species such as *Alternaria tenuissima*, *Nigrospora oryzae*, *Bipolaris sorokiniana*, *Cladosporium* spp., *Epicoccum nigrum*, *Ophiospaerella* sp., *Ceratobasidium* sp., *Microdochium bolleyi*, *Nectria* sp., and *Pyrenophora* spp.were reported as pathogens of common and durum wheat (Wiese, 1987; Murray et al., 1998). Fungi such as *Mortierella*, *Hypocrea*, *Trichoderma*, *Geomyces*, and *Penicillium* are generally regarded as saprophytic fungal communities (Kalicka, 2004; Arnold, 2007; Frankland, 1998).

Little is it known about the relationship between *Periconia macrospinosa*, *Kabatiella caulivora*, *Cordyceps cylindrica*, *Pyronema domesticum*, *Thielavia hyalocarpa*, *Physalospora vaccinii*, *Macrophoma sp*., *Oidiodendron cerealis* and

durum wheat. Although these species have been previously recovered from a large variety of hosts (Farr et al. 2008) further research is warranted to explore the functional interaction between these species and durum plants. Also, some known nonpathogenic fungal species, in our laboratory, were proved to be pathogenic on durum plants (personal communication). Therefore, all fungal species identified in this study should be further tested in order to confirm their functional status in durum hosts.

In conclusion, this study identified fungal species that may be important in the biological control of durum wheat fungal pathogens, and provided a baseline for expected fungal species associated with durum wheat under five rotation systems throughout the growing season. An understanding of this biodiversity is crucial for the development of biocontrol strategies for the suppression of fungal diseases.

4. DIVERSITY OF *FUSARIUM* SPECIES AND THE EFFECT OF PRECEDING CROPS OF PULSES AND CANOLA ON THEIR PREVALENCE IN DURUM WHEAT

4.1 Introduction

Generally, pathogens attack a specific plant species or a family of plants and diseases can be avoided through crop rotation with nonhost crops (Ledingham, 1961; Sturtz and Bernier, 1989). In Western Canada, wheat is grown in rotation with noncereal crops (Fernandez and Zenter, 2005) as it is seen as the most efficient way of decreasing the inoculum levels of wheat pathogens that persist in crop residues and soil. But reports on the effect of crop rotation on the prevalence of *Fusarium* species in wheat fields are sometimes conflicting. While some reports suggested that inclusion of noncereal crops in the cropping sequence reduces *Fusarium* carryover on crop residue or in the soil (Sturz and Bernier, 1987; Conner et al., 1996) other reports show absence of rotation effect on the density of pathogens after cropping to noncereal species (Bailey et al., 2000; Fernandez et al., 2007). Whether or not crop rotation with noncereal crops can reduce inoculum potential of *Fusarium* pathogens below critical levels is unclear.

Several surveys indicated that *Fusarium* species make up a large proportion of the fungal community in Saskatchewan fields. In 1998-1999, Fernandez et al. (2001) found that about 20% of the fungal species in durum wheat crops belonged to *Fusarium* taxa. The isolates were identified as: *F. avenaceum*, *F. equiseti*, *F. culmorum*, *F. acuminatum*, *F. oxysporum*. Recently, *F. avenaceum* was found to be

the most abundant and evenly distributed species in the province of Saskatchewan (Clear et al., 2005). Other species such as *F. graminearum*, *F. poae*, *F. sporotrichioides*, and *F. culmorum* were also associated with durum wheat but only in specific districts (Clear et al., 2005).

Fusarium species were identified based on micro- and macro-morphological characteristics such as colony characteristics on specific culture media, and the size and shape of asexual spores. Culture-dependent techniques are easily biased by the selectivity of the media chosen and storage conditions (Hagn et al., 2003b; Kale and Bennet, 1992). Culture-independent approaches are increasingly used in studies of *Fusarium* biodiversity (Nicholson, 2001; Yergeau et al., 2005). Molecular approaches can also be biased by several factors such as DNA derived from non-living microbes, the preferential PCR amplification of the DNA of some species while others remain undetected, and suboptimal gel separation resolution (Hagn et al., 2003a; van Elsas et al., 2000).

4.2 Objectives and hypothesis

The objective of this study was to use a combined approach of culture-dependent and culture-independent techniques to assess *Fusarium* biodiversity in durum wheat and to evaluate the impact of preceding crops of pulses and canola on *Fusarium* prevalence in durum wheat.

I hypothesized that *Fusarium* diversity will be large in durum wheat and that a preceding crop of pea, chickpea, lentil, canola, and durum wheat will have an impact on the *Fusarium* spp. prevalence in a subsequent durum wheat crop.

4.3 Materials and methods

4.3.1 Experimental design and sampling

We used plots of a study initiated in Swift Current in1998 to test the carry over effect of pulse rotation crops on the soil environment and the productivity of a subsequent crop of durum wheat. Our investigation was conducted in 2005 and 2006. The site was at the South Farm of the Semiarid Prairie Agricultural Research Centre, in Swift Current Saskatchewan, Canada (latitude, 50º17'N; longitude: 107º 41'W), in the Brown Soil zone. The site receives an average (54 years) of 361 mm of annual precipitations and has a yearly mean temperature of 3.6ºC, with an average minimum of -13.2 ºC in January and an average maximum temperature of 18.6ºC in June (Environment Canada Swift Current CDA weather station). The soil was a Swinton Silt loam (Orthic Brown Chernozem).

Treatments were five rotations, all with fallow at stage I and durum at stage III, but with *Pisum sativum* L. (CDC Handel pea), *Lens culinaris* Medik (CDC Sovereign lentil), *Cicer arietinum* L. (Myles chickpea), *Brassica napus* L. (Liberty Link Invigor 2733 canola) or *Triticum turgidum* L. (AC Avonlea durum) at stage II. Treatments were replicated three times. Plots were 5 x 24 m. Conventional tillage at a depth of 7.5 cm was used during the fallow stage to maintain plots. Crops were differentially fertilized each year to equalize soil fertility among treatments, as

determined by soil tests. For durum plots the total N level was equalized to 73 kg ha^{-1} with urea (46-0-0). This was calculated as 73 minus the amount of N in top 60 cm as determined by fall soil sampling. Ammonium phosphate (11-51-0) was routinely applied with seeds at 45 kg ha^{-1}. All fertilizer was banded 2.5 cm to the side and below the seed. Durum (AC Avonlea) was seeded using a Flexicoil 5000 air drill equipped with Stealth Double Shoot Knives with 22.5 cm spacing. Seeding rate was 100 kg ha^{-1} and seeding depth 5 cm.

Soil and plants were sampled at the following physiological stages of durum growth: two to three leaf, six leaf, flowering, and milk to soft dough stages (Table 4.1). Four plants per plot were consistently removed using a shovel. Roots were detached and placed in plastic bags with the adhering soil, and shoots were placed in paper bags. Under laboratory conditions, the rhizosphere soil was shaken from the roots and shoots were separated into crown, stem, leaf, and awn parts. All samples were held at 4° until analysis.

Table 4.1 Sampling dates and growth stages of durum at sampling, in 2005 and 2006

Growth Stages	Sampling Date	
	2005	2006
2-3 Leaf	May-30	
6 Leaf	Jun-20	Jun-18
Flowering	Jul-11	
Late milk to soft dough	Aug-02	

4.3.2 Determining the biodiversity of *Fusarium* using a PCR-DGGE procedure

Environmental plant material collected in 2005 and 2006 was used for the assessment of *Fusarium* diversity using the PCR-DGGE method. Plant roots, leaves, stems and awns were ground individually in liquid nitrogen with a mortar and pestle, and total DNA was subsequently extracted from 100-mg tissue subsamples with a DNAeasy plant mini kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Following DNA extraction, a polymerase chain reaction (PCR) was performed with the EF1-EF2 primer set. Because PCR products were 600-700 bp and the optimal length for DGGE analysis is about 500 bp, the amplicons were 100X diluted and reused as templates for a second PCR amplification with the primer Alfie1-CG (5'CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGTCGTCATCGGCCA and Alfie2 (5' CCTTACCGA GCTCRGCGGCTTC) (Yergeau et al., 2005). PCR amplifications were carried out in 25-µl of reaction mix using a Taq PCR Core Kit (Qiagen, Valencia, CA) and performed in a Thermal Cycler epgradient S (Eppendorf) through 40 cycles of 94°C for 1 min, 69°C for 1 min, and 72°C for 1 min. Analyses of the PCR amplicons were performed using a DGGE system model 2001 manufactured by C.B.S. Scientific. Electrophoreses were run for 18. 20 hours at 80V on a 7.5 acrylamide/ bis-acrylamide (37.5:1) gel with a 30-70% denaturant gradient. A gradient for the gel was obtained by using the two well gradient former (model GM-40 C.B.S. Scientific). Electrophoresis gels were stained with ethidium bromide and images were acquired with a Bio Doc-IT Imaging System (UVP Inc, CA).

An alternative and much easier procedure to the sequencing of the DGGE bands is the comparison of the bands with some reference patterns. Therefore, prior to DGGE analysis, eleven *Fusarium* species expected to occur in the DGGE profile were run and optimized for a better discrimination on DGGE gels in order to construct a molecular ladder. The molecular markers (*F. avenaceum*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. sporotrichioides*, *F. poae*, *F. tricinctum*, *F. torulosum*, *F. reticulatum*, *F. flocciferum*, *F. equiseti*) were loaded each time on one side of the gel to facilitate species identification by bands comparisons. Bands migrating to an unknown position were excised from the gel with a sterile scalpel blade, mixed with 50 µl of Tris-HCL (10mM, pH 8.0), incubated at 50°C for 10 min, crushed with a pipette tip, and centrifuged at 10,000 X g for 1 min. The resulting supernatant was amplified using Alfie1/Alfie2 primer pair and commercially sequenced (Plant Biotechnology Institute, Saskatoon, Saskatchewan). The presence or absence of bands in the DGGE gels at the levels of markers was recorded as species presence or absence in the material analyzed.

4.3.3 Determining the biodiversity of *Fusarium* using culture-dependent, polymerase chain reaction, and sequencing methods

Aliquots of 0.1 ml from 10^{-4} dilutions were spread on PDA (potato dextrose agar) culture media supplemented with antibiotics (100 mg l^{-1} streptomycin sulfate and 15 mg l^{-1} neomycin sulfate, Sigma, St. Louis, Mo, USA). Three plates per treatment were prepared and plates were incubated at 22ºC in the dark for four days.

Pure cultures were established by transferring morphologically distinct colonies onto fresh medium plates.

Fungi isolated from plant samples were estimated using surface sterilization procedures followed by plating of 1 cm plant tissue fragments onto PDA (potato dextrose agar) culture media supplemented with antibiotics (100 mg l^{-1} streptomycin sulfate and 15 mg l^{-1} neomycin sulfate, Sigma, St. Louis, Mo, USA). The process of surface sterilization consisted in submerging the plant fragments into 95% ethanol (10 seconds), sterile distilled water (10 seconds), 5% sodium hypochlorite (2 min), and sterile distilled water (2 min). Surface sterilized tissue fragments were placed on PDA culture media. The operation was repeated for each plant part: root, crown, stem, leaf and awn. Four plates containing five fragments were made for each plant part of each plot. Plates were incubated at room temperature in the dark for 6 days. Pure cultures were established using standard microbiological procedures.

All recovered pure cultures were visually separated into operational taxonomical units (OTUs) and quantified as colony-forming units (CFUs). *Fusarium* isolates were morphologically analyzed based on characters such as: colony pigmentation and type of spores. To induce sporulation in some *Fusarium*, isolates we subcultured onto solid carnation-leaf agar media (Fisher et al., 1982) and incubated at room temperature for four days.

Further, *Fusarium* isolates were analyzed by PCR amplification and sequencing of the elongation factor-1 alpha gene (EF-1 alpha). Mycellial mats collected using a sterile scalpel blade were ground in liquid nitrogen. Genomic DNA was extracted using the UltraClean microbial DNA isolation kit (MoBio,

Inc.CA) following the manufacturer's instruction. The EF-1 alpha gene was amplified using the EF1 (5'ATGGGTAAGGARGACAAGAC) and EF2 (5' GGARTGACCAGTSATCATGTT) primer set (O'Donnell et.al., 1998). The EF-1 alpha regions were amplified using a Taq PCR Core Kit (Qiagen, Valencia, CA). Amplification was performed in a Thermal Cycler epgradient S (Eppendorf) through 30 cycles of 94°C for 30 sec, 57 °C for 30 sec, and 72°C for 1 min. PCR products (5 µl) were run on agarose gel (1 %) containing ethidium bromide to reveal the presence/absence of DNA bands. The remaining PCR products were purified with a DNA purification kit (QIAquick PCR Purification Kit, Qiagen Inc., Valencia, CA) and commercially sequenced (Plant Biotechnology Institute, Saskatoon, Saskatchewan. Similarity analyses were performed using the BLAST research module in GenBank (http://www.ncbi.nlm.nih.gov/).

4.3.4 Statistical analyses

One-way analysis of variance (ANOVA) was conducted to determine the effect of crop rotation on the prevalence of *Fusarium* species associated with durum wheat, as determined by the culture-dependent approach. Since the data set was not always normally distributed, transformations (ln) were applied when appropriate to fulfill the requirement of the test (Limpert et al., 2001). ANOVA was performed using JMP software (SAS Institute Inc., Cary, North Carolina). The least significant difference (LSD) test was used for mean comparison at $P = 0.05$. Microbial communities found in different rotations with the culture-dependent approach were compared using cluster analysis. Clustering was performed with the average linkage

method and Euclidean distance, a measure of dissimilarity, in SYSTAT version 10 software (SPSS Inc., 2000).

DNA fingerprinting from DGGE banding pattern on the gel were analyzed using correspondence analysis in SYSTAT version 10. Cluster analysis was carried out on DGGE banding patterns by scoring each band in the profiles as present (1) or absent (0). Unweighted Pair Group Method with Arithmetic mean (UPGMA) analysis was performed on the NTSYS pc version 2.2 statistical package, using Jaccard's coefficient of similarity.

Canonical correspondance analysis with Monte Carlo test was conducted using PC_ORD v.4 (Edinburg UK) to test the corrlation between the data obtained by cultural method and DGGE. Rare species data were not included in the analysis to circomvent the zero-truncation problem (Legendre and Legendre 1998). The analysis was thus conducted on *F. avenaceum*, *F. reticulatum*, *F. tricinctum* and *F. flocciferum*.

4.4 Results

4.4.1 Impact of preceding crops

Preceding crops significantly affected the abundance of *Fusarium torulosum*, which was reduced in durum following canola crops (Table 4.2). There was no significant effect of preceding crops on the abundance of other *Fusarium* species considered in isolation. However, similarity analysis of the composition of the *Fusarium* populations associated with durum grown after five different

preceding crops showed that the *Fusarium* population associated with durum monoculture was dissimilar to that of diversified rotations. Populations after canola and pea were most distinct from that after durum (Figure 4.1A and 4.1B). The DGGE band profiles analysis revealed that most *Fusarium* species (*F. avenaceum*, *F. reticulatum*, *F. sporotrichioides*, *F. proliferatum*, *F. tricinctum*, *F. oxysporum* and *F. graminearum*) were associated with durum following durum. *F. flocciferum* was the only *Fusarium* more rarely found in durum following durum. This species seemed associated with durum following a crop of canola or pea (Figure 4.2A).

Table 4.2 Effect of preceding crops on the prevalence of *Fusarium* spp. recovered from durum wheat using the culture-dependent approach

Treatment	*Fusarium avenaceum*	*Fusarium reticulatum*	*Fusarium tricinctu*	*Fusarium torulosum*	*Fusarium flocciferum*	*Fusarium equiseti*	*Fusarium poae*	*Fusarium sporotrichioides*
Canola	5.35	4.40	5.55	3.26 b[a]	4.64	1.47	0	0
Chickpea	5.60	5.07	4.30	4.10 ab	3.39	1.91	0.33	0
Lentil	5.70	4.89	4.46	5.28 a	3.39	2.40	0	0
Pea	5.21	4.49	4.09	5.09 a	2.17	2.10	0	0
Wheat	6.10	4.23	0	4.10 ab	4.61	1.47	0	1
Significance	ns	ns	ns	$P = 0.01$	ns	ns	ns	ns

[a] Means with the same letters are not different according to ANOVA protected LSD test ($P < 0.05$, $N = 3$).

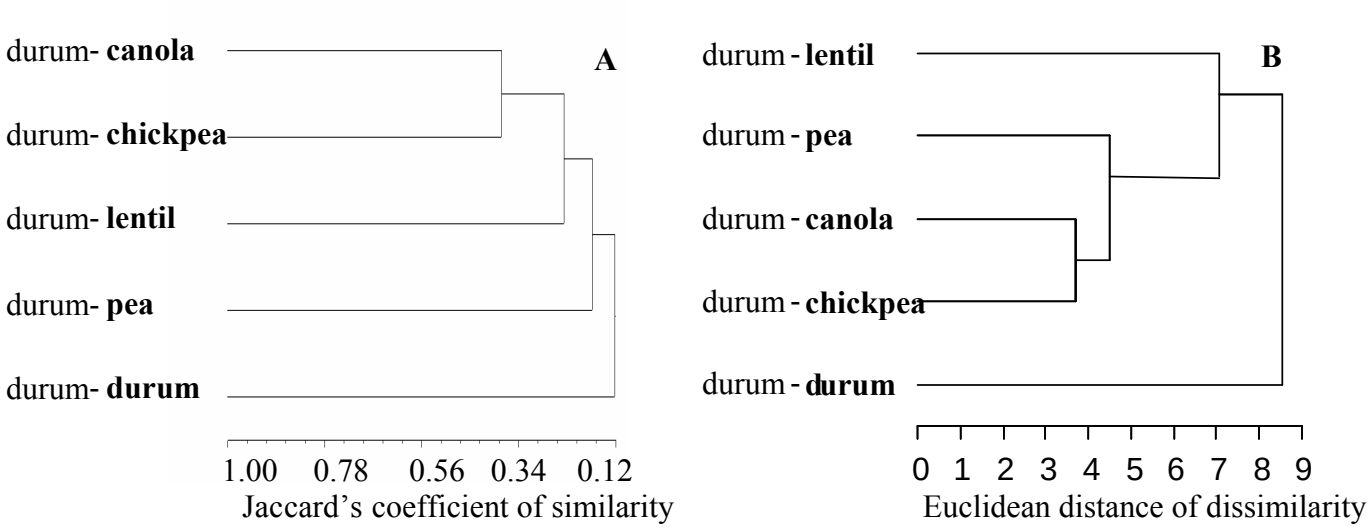

Figure 4.1 Cluster dendrograms classifying the *Fusarium* communities colonizing durum wheat in five different crop rotations. (A) Cluster diagram based on numerical abundance data of *Fusarium* spp recovered using cultivation methods. (B) Cluster diagram based on presence – absence data of *Fusarium* spp. identified using DGGE. Crops in bold were preceding the crop of durum examined. All sampling was completed in the durum phase of the rotation.

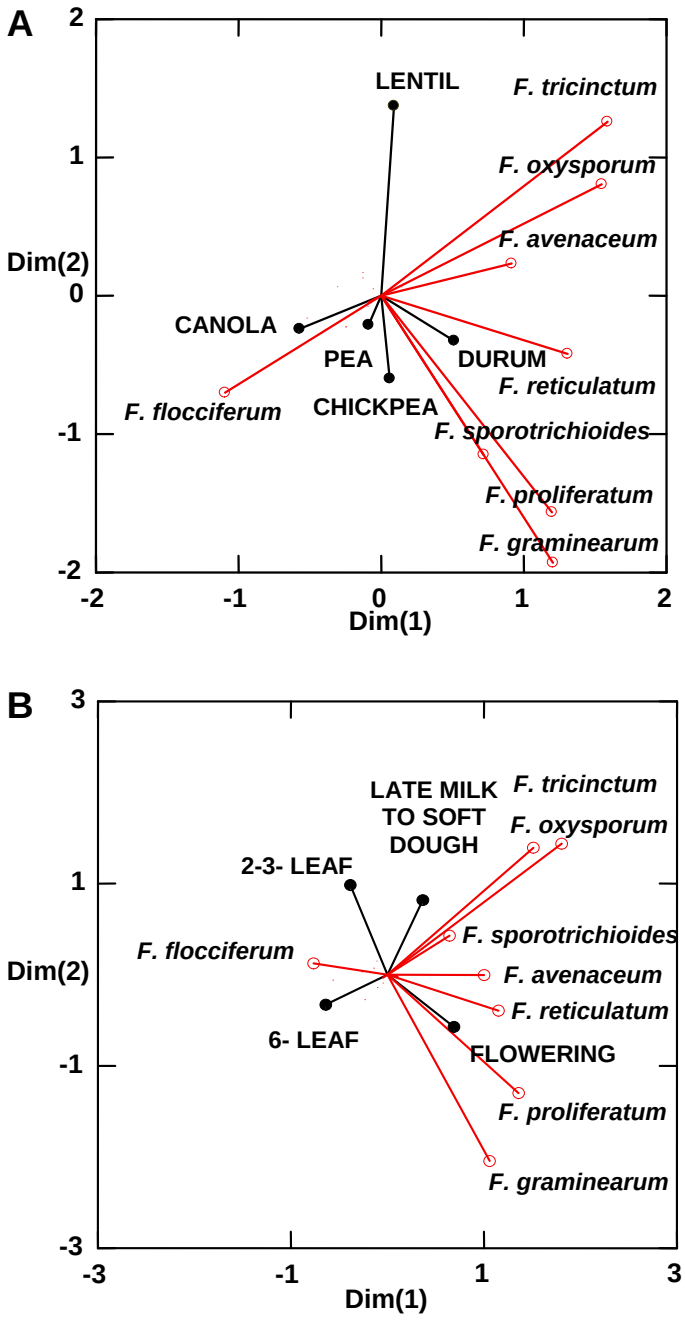

Figure 4.2 Correspondence analysis biplots showing the relationships between detected *Fusarium* species and different crop rotations (A) and durum growth stages (B).

4.4.2 *Fusarium* biodiversity in the culture-dependent approach

Eight *Fusarium* species were recovered from 2005 durum samples. *F. avenaceum* was the most abundant followed by *F. reticulatum*, *F. torulosum*, *F. tricinctum*, *F. flocciferum*, *F. equiseti*, *F. sporotrichioides*, and *F. poae* (Figure 4.3). *Fusarium* isolates were recovered at all growth stages considered but more isolates were recovered at the flowering and late milk to soft dough stages than at 2-3 leaf and 6 leaf stages (384 and 163 isolates versus 68 and 130 isolates). The *Fusarium* most abundant in soil samples was *F. avenaceum* (87%) while *F. tricinctum* and *F. reticulatum* were less frequent (8.01% and 4.19%). Stem tissues were colonized by all *Fusarium* species mentioned above while roots were colonized by six *Fusarium* species only. Nevertheless, *Fusarium* species abundance in roots was higher than in any other plant part. In roots, *F. avenaceum*, *F. reticulatum* and, *F. tricinctum* were the most abundant *Fusarium* species, accounting for 40.51%, 25.81 %, and 13.12% of the total CFU of *Fusarium* species. In crowns and awns, *F. avenaceum*, *F. reticulatum*, *F. tricinctum*, and *F. torulosum* were frequent, while in leaves, *F. equiseti* substituted for *F. tricinctum*.

4.4.3 *Fusarium* biodiversity in the culture-independent approach

Twelve *Fusarium* species were recovered from 2005 durum wheat tissues using DGGE. *F. avenaceum* was the most frequent species followed by *F. reticulatum*, *F. tricinctum*, *F. flocciferum*, *F. proliferatum*, *F. sporotrichioides*, *F. graminearum*, *F. oxysporum*, *F. torulosum*, *F. poae*, *F. redolens*, and *F. equiseti*

(Figure 4.3). Stem tissues hosted the largest *Fusarium* diversity where *F. avenaceum*, *F. flocciferum*, and *F. tricinctum* were the dominant species of a population of 12 species, accounting for 33.3%, 14.8% and 11.1% of total bands of *Fusarium* recovered, respectively. Awns were colonized by eight *Fusarium* species (*F. avenaceum*, *F. reticulatum*, *F. proliferatum*, *F. tricinctum*, *F. poae*, *F. flocciferum*, *F. graminearum*, and *F. oxysporum*). In awns, *F. avenaceum*, *F. reticulatum*, and *F. proliferatum* were dominant accounting for 22.3%, 22.7%, and 13.6% of the recovered DGGE bands. *Fusarium* species were more frequent in stems and awns than in roots, leaves, and crowns. *F. avenaceum*, *F. reticulatum*, *F. flocciferum*, and *F. tricinctum* were commonly encountered in durum leaf and crown tissues, and *F. avenaceum*, *F. reticulatum*, *F. proliferatum*, and *F. graminearum* were common in roots.

The results of the PCR-DGGE approach are in good accordance to the ones of the culture-dependent approach showing *F. avenaceum*, *F. reticulatum*, and *F. tricinctum* to be the most prevalent *Fusarium* in durum wheat (Figure 4.3). Canonical correspondance analysis indicated that the data otained with the cultural and DGGE methods were correlated ($P = 0.02$).

Infection at the six leaf stage is usually associated with yield losses. At this growth stage plants require an increase amount of energy for the initiation of the reproductive stage. The biodiversity of *Fusarium* species at this developmental stage of durum plants was analyzed for both 2005 and 2006 years. *F. avenaceum*, *F. reticulatum* and *F. flocciferum* were identified in both years. *F. torulosum*, *F.*

tricinctum, *F. sporotrichioides*, and *F. proliferatum* where detected only in 2005, and *F. equiseti*, and *F. graminearum* were detected only in 2006.

Frequency of recovery peaked at the flowering and late milk to soft dough growth stages of durum for most *Fusarium* species. *F. flocciferum* was the only *Fusarium* to be infrequent at these stages. This species seemed to be positively associated with the 2-3 leaf and 6-leaf durum growth stages (Figure 4.2B).

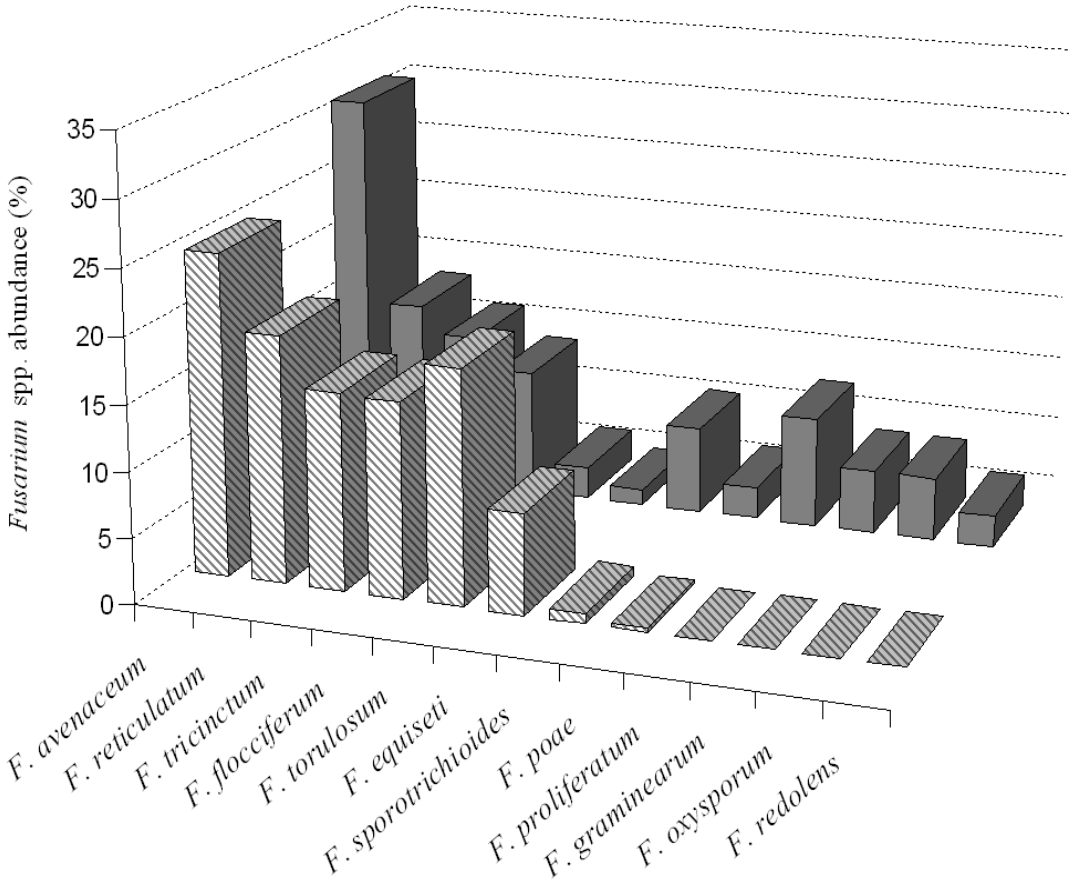

Figure 4.3 Abundance of *Fusarium* spp. in 2005 durum samples: (■) as determined by the number of CFUs on culture medium; (▧) as recovered by DGGE (percentage of all bands detected).

4.5 Discussion

Twelve *Fusarium* species were recovered from durum wheat crops. Of these, *F. avenaceum*, *F. graminearum*, *F. tricinctum*, and *F. poae* are considered pathogens causing severe damage to wheat production (Parry et al., 1995; Uhlig et al., 2007). *F. proliferatum*, *F. sporotrichioides*, *F. oxysporum*, *F. equiseti* and *F. torulosum* are regarded as less aggressive or opportunistic pathogenic species (Bottalico and Prerrone, 2002; Akinsanmi et al., 2004). Recently, *F. reticulatum* was identified as part of the pathogenic *Fusarium* complex, attacking wheat and barley roots (Strausbaugh et al., 2005). Two of the *Fusarium* species identified in our study are regarded as non pathogenic. *F. flocciferum* is known as a non pathogenic endophyte of wheat (Bateman et al., 1998). Also, no previous record in the literature refers to *F. redolens* as a wheat pathogen. To date, *F. redolens* is only known as a pathogen of asparagus and tomato crops (Baayen et al., 2000; Wilcox et al., 1970).

F. avenaceum, *F. reticulatum*, and *F. tricinctum* were the dominant *Fusarium* species in our durum wheat crops (Figure 4.3). The high abundance of *F. avenaceum* was expected and observed. Several studies have indicated that this species accounts for a large proportion of *Fusarium* population in wheat growing in Saskatchewan (Gordon, 1952; Bailey et al., 2000; Fernandez and Jefferson, 2004, Clear et al., 2005). Unexpectedly, *F. reticulatum* was the second most abundant *Fusarium*. This species, common in United States, China, Australia, and New Zealand, is uncommon in Canada where it was only reported in association with *Pinus montana* (Farr et al., 2008). Considering that *F. reticulatum* was never

detected in Saskatchewan before and it is capable of producing severe root infection in wheat, further study should establish the risk it represents for cereal crops in this region.

F. tricinctum was among the most prevalent *Fusarium* in durum wheat tissues. We failed to detect *F. tricinctum* in durum grown after durum treatment, using the culture-dependent approach, but it was detected by PCR-DGGE. A problem in *Fusarium* identification remains the fact that different plants can be infected with different *Fusarium* strains of the same species (Leslie and Summerell, 2006). It could be that a morphologically different *F. tricinctum* strain was selected by a preceding durum crop. Preceding crops have been previously shown to select for strains' aggressiveness. In a recent study, the *Fusarium* isolates obtained from wheat following wheat were significantly more aggressive and leading to higher disease levels than those from wheat following a nonceral crop (Akinsanmi et al., 2004). It is therefore possible that *F. tricinctum* detected by the PCR-DGGE in durum plants grown after durum belonged not only to a morphologically distinct strain, but also to a more virulent strain.

F. graminearum, the most important species associated with FHB of wheat in North America (Ward et al., 2008; McMullen and Gallenberg, 1997; Parry et al., 1995), was rarely detected in our study. *F. graminearum* is prevalent in the east side of Saskatchewan but is rare in the dryer and warmer part of the province (Anonymous, 2007), and its rare occurrence in Swift Current was expected.

The dominance of *Fusarium* species in a particular plant host from a specific geographic area is mainly influenced by climatic factors (Doohan et al., 2003). For instance, several studies have established that *F. graminearum* tend to dominate cooler to moderately warm climates, while *F. proliferatum* and *F. moniliforme* are dominant under warm conditions (Sutton, 1982; Miller et al., 1995). The dominance of *F. avenaceum*, *F. reticulatum*, and *F. tricinctum* in durum wheat could be attributed to several factors. It could be that these species are better adapted to the dry and warm climate of Swift Current and can better compete for space and nutrients than other *Fusarium* species in this environment. The competitive abilities of *Fusarium* species can be influenced by temperature. Marin et al. (1998) suggested that *F. graminearum* has a competitive advantage over *F. moniliforme* and *F. proliferatum* at 15°C, this advantage decreasing with increased temperatures. Moreover, environmental moisture and temperature influenced the *Fusarium* inoculum dispersal and germination mechanisms (Paulitz et al., 2002, Doohan et al., 2003). *F. avenaceum*, *F. reticulatum*, and *F. tricinctum* may be better adapted to semiaridity and better able to dominate over other *Fusaria* in the semi-arid climate of Swift Current. While a great deal of literature describes the influence of climatic factors on the germination, survival, growth, and competitive abilities of *F. graminearum* and *F. culmorum* (Hope et al., 2005; Pettitt et al., 1996; Bateman and Murray, 2001), little is known about how climate influences other *Fusarium* species.

Because of the frequent association of *Fusarium* species with roots of plants these fungi are often regarded as soilborne fungi. However, many of *Fusarium* have

dispersal mechanisms that help them colonize aerial plant parts where infection may result in disease. While crop residues are an ideal substrate for the fruiting bodies of *Fusarium* that forms perithecia, in the soil many *Fusarium* form thick-walled chlamydospores that can infect wheat crops as soon as they are sown in the infected seedbed (Leslie and Summerell, 2006). Further, the lesisons produced on plants provide *Fuarium* species with ideal condition for conidiation. Most of the *Fusarium* species can infect hosts at different growth stages but their infection rate increases at warm temperatures (Doohan et al., 2003; Sutton et al., 1982). Warm temperatures recorded for July and August (data not shown) could explain the frequent detection of *Fusarium* species at the flowering and late milk to soft dough stages of durum wheat (Figure 4.2B). The flowering stage is also recognized as the critical stage for FHB infection (Parry et al., 1995; Sutton 1982). Although it is not clear if chemical compounds such as choline and betaine found in anthers stimulate the *Fusarium* infection, it is believed that they may stimulate *Fusarium* germination and growth (Schisler et al., 2006; Bai and Shaner, 1994a). If this is the case, then the colonization of newly developed anthers and an increased germination rate would be expected to contribute to the increased abundance of *Fusarium* species observed at the flowering stage.

According to Krupinsky et al., (2002) crop rotation can be less effective in decreasing the prevalence of some organisms that have a wide host range and good survival mechanisms. It is possible that the high frequency of isolation of *Fusarium* species from all five cropping systems studied is due to the ability of these species to survive in pulses and canola grown prior to durum crops. *F. avenaceum*, *F.*

equiseti and *F. tricinctum* have been previously colonizing crops of canola, lentil, pea and chickpea (Fernandez et al., 2003; Mazur et al., 2002; Arshad et al., 2005). Different other *Fusarium* species have been found to occur as opportunistic colonizers in diverse vegetables crops (Logrieco et at., 2003). The ability of these fungi to survive in a wide range of plant hosts can be expected to reduce the efficacy of rotation systems in decreasing *Fusarium* inoculum levels. Another possible reason for the high percent isolation of *Fusarium* species from all cropping systems involved could be attributed to the fact that *Fusarium* species may spread easily within experimental plots. It was shown that *F. graminearum* inoculum can escape the crop canopy and spread over kilometers to tens of kilometers (Francl et al., 1999). If widely dispersed inoculum is a significant factor in *Fusarium* contamination, then sources of inoculum in a nearby experimental plot could have contributed to the recovery of *Fusarium* species in all experimental plots tested. Still, the clustering of the rotation treatments showed that *Fusarium* community in durum following durum was the most dissimilar when compared to other preceding crops (Figure 4.1A and 4.1B), and the analysis of the DGGE bands profile showed that the highest diversity of the *Fusarium* species was found in durum grown in monoculture (Figure 4.2A). This indicates that *Fusarium* species diversity and prevalence varied with different residue types.

Crop residues are different in their chemical nature and decomposition rate, and they can directly affect the composition of decomposer microorganisms (Lupwayi and Kennedy, 2007; Krupinski et al., 2007). The rate at which host tissues decomposes can influence the survival of *Fusarium* species, which is better

on tissues that breakdown slowly (Inch and Gilbert, 2003). Since wheat residues decomposes more slowly than canola or pulses residues (Lupwayi et al., 2004), it is possible that *Fusarium* inoculum survived longer in durum wheat residue, this improved survival leading to a most dissimilar and a highest diversity of *Fusarium* community in a subsequent year of durum crop.

The results of the current study suggest that *Fusarium* species are ubiquitous in durum wheat and the noncereal preceding crops tested contribute to the maintenance of *Fusarium* inoculum in the field. The pathogenicity of *Fusarium* species in durum wheat grown in rotation with pulses and canola was not investigated in our study. However, since previous research showed that variation in aggressiveness is well established within individual *Fusarium* species, and less pathogenic *Fusarium* strains can be selected by non-cereal preceding crops (Akinsanmi et al., 2004), it may be that the *Fusarium* species aggressiveness recovered varied with the preceding crop.

5. A PCR-DENATURING GEL ELECTOPHORESIS TO ASSESS *PYRENOPHORA* DIVERSITY

5.1 Introduction

Wheat is one of the world's most important crops. Yet, wheat fields are frequently attacked by many groups of fungi that reduce considerably grain yield and quality. Leaf spots and leaf blights contribute to the decline in wheat productivity. Leaf diseases are mainly caused by *Pyrenophora* fungi some of which have anamorphs in *Drechslera*. *Pyrenophora tritici-repentis* is most commonly recovered from wheat leaf tissues, but as much as 10 species of *Pyrenophora* have been isolated from wheat fields worldwide (Sivanesan, 1987). *P. tritici-repentis*, known mainly as a leaf pathogen, is also a pathogen of wheat kernels. *Pyrenophora* infected wheat kernels have a negative impact on seedling emergence and plant growth (Fernandez et al., 1998). In contrast to the extensive information on the wheat infection by *P. tritici-repentis*, little is known about the other *Pyrenophora* species. These species might impact wheat production, as pathogens or exerting activities against pathogens through mycoparasitism, antibiosis or competition for nutrients and space. A need exists for a better understanding on the dynamics of these infrequent but potentially important species, if we want to develop biological control strategies.

It is difficult to use morphological features to establish the identity of *Pyrenophora* fungi (Stevens et al., 1997). Since morphological characteristics of fungi are limited in number, subjected to selection, and sensitive to environmental influence, numerous molecular techniques have been employed in the detection and

identification of fungal species. Fragments of 18S ribosomal DNA, 28S rDNA, internal transcribed spacer (ITS) regions, and elongation factor 1 alpha gene (EF-1 alpha) have been used as effective markers in the analysis of fungal communities when combined with denaturing gradient gel electrophoresis (DGGE) (Vainio and Hantula, 2000; Zuccaro et al., 2003; Anderson et al., 2003; Yergeau et al., 2005). Denaturing gradient gel electrophoresis (DGGE) separates PCR amplicons according to their nucleotides composition and reveals microbial community dynamics in both environmental and pure cultures population studies (Muyzer et al., 1993; Muyzer and Smalla, 1998).

The ITS regions are good targets for molecular identification of fungi because they are noncoding, conserved within species, but can vary sufficiently to allow the construction of taxon-specific primer sequence (Nazar et al., 1991). Taxon-selective amplification of the ITS region has already become an approach in molecular identification of pathogenic *Verticillium* (Nazar et al., 1991), *Pythium* (Wang et al., 2003) and *Phytophthora* (Tsai et al., 2006). The correct identification of the causal agent of a disease is important for the development of efficient management strategies.

5.2 Objectives and hypothesis

The objectives of this study were: (1) to design a *Pyrenophora*-specific primer set targeting a partial region of the ITS rDNA region, (2) to evaluate the sensitivity of these primers in discriminating between different wheat infecting *Pyrenophora* isolates using the PCR-DGGE technique, and (3) to detect and

identify *Pyrenophora* species directly from wheat tissues that have been infected *in vitro* with different *Pyrenophora* isolates.

I hypothesized that the ITS rDNA sequences of *Pyrenophora* species contain conserved regions that can generate a *Pyrenophora* - specific primer set able to amplify DNA fragments containing enough variability in the succession of the base pairs to distinguish species of *Pyrenophora*. Variability is the most important factor that makes these DNA fragments suitable as markers in the DGGE molecular approach. Based on the sequences' variability DNA fragments migrate to unique positions in the DGGE gels.

5.3 Materials and methods

5.3.1 Fungal material

Pyrenophora isolates belonging to six species, and other fungi belonging to 15 different species were previously recovered from durum wheat, obtained from culture collections or received from other researchers (Table 5.1). Fungal isolates, grown on potato dextrose agar (PDA) were incubated at 22ºC in the dark for six days.

Table 5.1 Pyrenophora isolates and other fungi used in this study.

Taxon	Isolate	Host/Origin	Source[a]
Pyrenophora			
P. tritici-repentis	PTR18	wheat (Emerson, MB)	AAFC Winnipeg
P. teres	1049 WRS	barley (Winnipeg, MB)	AAFC Winnipeg
P. avenae	2130 WRS	oats (Carman, MB)	AAFC Winnipeg
P. graminea	2015 WRS	barley (Glenlea, MB)	AAFC Winnipeg
P. semeniperda	RMC 23	wheat (Portage La Prairie, MB)	CCFC Ottawa
P. japonica	RMC 69	wheat (Winnipeg, MB)	CCFC Ottawa
Other fungi			
Fusarium avenaceum	FBC 27	wheat (Swift Current, SK)	U of S
Fusarium graminearum	AB 3	wheat (Alberta, ON)	U of S
Eurotium sp.	FBC 4D	wheat (Swift Current, SK)	U of S
Alternaria tenuissima	FBC 4	wheat (Swift Current, SK)	U of S
Cladosporium minourae	FBC 6	wheat (Swift Current, SK)	U of S
Cochliobolus sativus	FBC 273	wheat (Swift Current, SK)	U of S
Ceratobasidium sp.	FBC 39	wheat (Swift Current, SK)	U of S
Laccaria sp.	FBC 3D	wheat (Swift Current, SK)	U of S
Physalospora vaccinii	FBC 26	wheat (Swift Current, SK)	U of S
Trichoderma harzianum	FBC 41	wheat (Swift Current, SK)	U of S
Penicillium aurantiogriseum	FBC 12	wheat (Swift Current, SK)	U of S
Mortierella hyalina	FBC 35	wheat (Swift Current, SK)	U of S
Epicoccum nigrum	FBC 8	wheat (Swift Current, SK)	U of S
Geomyces pannorum	FBC 109	wheat (Swift Current, SK)	U of S
Oidiodendron cerealis	FBC 69b	wheat (Swift Current, SK)	U of S

[a] AAFC: Agriculture and Agri-Food Canada, Winnipeg, Manitoba: Dr. A. Tekauz. CCFC: Canadian Collection of Fungal Cultures, Ottawa, Ontario, curator Carolyn Babcock. U of S: University of Saskatchewan, Saskatoon, Saskatchewan, Dr. V. Vujanovic fungal collection.

5.3.2 Primer design

Internal transcribed spacer (ITS rDNA) sequences of nine different *Pyrenophora* species (known *Pyrenophor*a telemorphs), three of different *Drechslera* species (known *Pyrenophora* anamorphs), and seven closely related fungal genera were recovered from GenBank and aligned using Custal W multiple sequence alignment program version 1.8 (Thompson et al., 1994). All selected *Pyrenophora* (*Drechslera*) sequences belonged to species that occured in wheat fields. Sequences were visually checked for regions with homologies among *Pyrenophora* and *Dreschlera* species, but no other fungi. The optimal sequences for the *Pyrenophora* forward primer (Pyr1; 5'-CCATATTCACCCATGTCTTT-3') and the reverse primer (Pyr2; 5'- GWTTTTAAGGCGAGTCTYG-3') were determined, and a GC clamp (5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGG-3') was attached to the 5' end of the reverse primer, for enhanced separation during DGGE analysis (Myers et al., 1985). The taxon specific primers designed in this study border an approximate 300-bp corresponding to conserved and variable sequences in the ITS rDNA. Primers Pyr1 and Pyr2-GC were commercially synthesized (Invitrogen, CA, USA). The specificity was assessed by submitting their sequence to the GenBank BLAST algorithm. Primers' feasibility was also tested by PCR amplification of DNA extracted from 21 fungal isolates belonging to Ascomycota, Basidiomycota, and Zygomycota.

5.3.3 Seed inoculation

Durum wheat seeds (variety AC Avonlea), provided by the Semiarid Prairie Agricultural Research Centre, in Swift Current (Saskatchewan, Canada) were inoculated with the fungi listed in Table 5.1. Prior to inoculation seeds were surface sterilized by submersion into 95% ethanol (10 seconds), distilled autoclaved water (10 seconds), 5% sodium hypochlorite (2 min), and autoclaved distilled water (2 min). Six PDA plates were prepared (plate A to F) by placing into each plate ten durum seeds. An *in vitro* assay was performed by placing four 1-cm^2 agar plugs containing mycelia of different fungal species, onto surface-sterilized durum seeds. The fungal species inoculated in each plate is shown in Table 5.2. After 14 days of growth, infected seedlings were collected, surface sterilized as described above and stored at - 80°C prior to DNA extraction.

5.3.4 DNA isolation and PCR amplification

Mycelial mats of pure cultures were collected using a sterile scalpel blade and ground in liquid nitrogen with a mortar and pestle. Fungal genomic DNA was extracted using the UltraClean microbial DNA isolation kit (MoBio, Inc. CA) following the manufacturer's instruction. Also, plant material was ground in liquid nitrogen with a mortar and pestle. Total genomic DNA was extracted from 100 mg of subsample using the DNAeasy plant mini kit (Qiagen, Valencia, CA) following manufacturer's instruction. The DNA obtained was stored at -20 °C until PCR amplification.

PCR were carried out in 25 µl reaction mixtures using a Taq PCR Core Kit (Qiagen, Valencia, CA) and containing 12.87 µl deionized water, 2.5µl of 10X PCR buffer, 5 µl of Q solution, 0.12 µl of Mg Cl_2, 0.5 µl of dNTPs, 1µl of each of the primer (50 µM), 0.125 µl of Taq DNA polymerase, and 2 µl of DNA extract per reaction. A negative control (no DNA) was included in the PCR. Amplification was performed in a Thermal Cycler epgradient S (eppendorf).

For DNA amplification from pure fungal cultures with ITS1-F (5'CTTG GTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (5'TCCTCCG CTTATTGATATGC) (White et al. 1990) the following protocol was used: one cycle of initial denaturation (94°C for 3 min), 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 1 min), followed by a final cycle of extension (72°C for 10 min). The amplicons were subsequently reamplified using a nested approach with Pyr1 and Pyr2-GC. The following protocol was used: one cycle of initial denaturation (94°C for 3 min), 30 cycles of denaturation (94°C for 1 min), annealing (57°C for 30 s) and extension (72°C for 30 s), followed by a final cycle of extension (72°C for 10 min).

For DNA amplification from infected plant material, the same PCR procedures were applied, using ITS1-F - ITS4 and then Pyr1 - Pyr2-GC primers. Amplified DNA fragments were visualized by 1 % agarose gel electrophoresis containing 0.5 µg/ml ethidium bromide. When necessary, products were stored at −20°C before they were used for DGGE analysis.

Table 5.2 Fungal inoculants and their distribution in the experimental plates.

Plate A	Plate B	Plate C
Pyrenophora teres	*Pyrenophora tritici-repentis*	*Pyrenophora avenae*
Fusarium avenaceum	*Fusarium graminearum*	*Ceratobasidium sp.*
Eurotium sp.	*Alternaria tenuissima*	*Laccaria sp.*
Cladosporium minourae	*Cochliobolus sativus*	*Physalospora*
Plate D	**Plate E**	**Plate F**
Pyrenophora semeniperda	*Pyrenophora graminea*	*Pyrenophora japonica*
Epicoccum nigrum	*Trichoderma harzianum*	*Cladosporium minourae*
Geomyces pannorum	*Penicillium aurantiogriseum*	*Alternaria tenuissima*
Oidiodendron cerealis	*Mortierella hyalina*	*Penicillium aurantiogriseum*

5.3.5 DGGE analysis

Analyses of the PCR amplicons were performed using a DGGE system model 2001 manufactured by C.B.S. Scientific. Electrophoreses were run for 17h 30 min at 80V on a 7.5 % acrylamide/ bis-acrylamide (37.5:1) gel with a 30-70% denaturant gradient. A gradient for the gel was obtained by using the 2-well gradient former (model GM - 40 C.B.S. Scientific). Electrophoresis gels were stained with ethidium bromide (10μg ml^{-1}) and images were acquired with a Bio Doc-IT Imaging System (UVP Inc, CA). A molecular marker composed of known *Pyrenophora* species (*P. tritici-repentis*, *P. teres*, *P. graminea*, *P. semeniperda*, *P. avenae*, and *P. japonica*) was loaded on one side of the gel to facilitate band-to-band comparisons.

5.4 Results

5.4.1 Primers specificity

A comparison of ITS rDNA sequences of nine *Pyrenophora* species, three *Drechslera* species and seven non-*Pyrenophora*, but belonging to the same fungal family (*Pleosporaceae*) obtained from GenBank, revealed one region at positions 108 to 127 that was potentially specific for all *Pyrenophora* sequences analyzed. Searches for sequence similarity were then performed in GenBank nucleic acid databases using the BLAST program. The search for the Pyr1 binding site resulted in 191 significant hits. Eighty two percent of these hits were known *Pyrenophora* species or known *Drechslera* anamorphs, and of those, 95 % showed 100 %

similarity. The BLAST also showed that 3 % of the hits showing 100 % similarity belonged to unculturable or as-yet-unidentified fungal isolates. All other hits were ITS rDNA regions from a variety of organisms having imperfect similarity with the primer sequence. The sequences' alignment revealed a consensus region at positions 458 to 476 that was used as reverse primer. This region was found only in the genus *Pyrenophora* and was present in all *Pyrenophora* species despite some variations in the second and eighteenth bases at the 3' end (G to A, and T to A conversions). The BLAST report revealed that sequences containing the nucleotides G and T at positions 459 and 475 were widespread in *Pyrenophora* species, occurring in 104 (63. 41 %) of the 164 *Pyrenophora* ITS rDNA sequences which were available in the database and contained that region. Sequences belonging to other fungal genera such as *Stemphylium* and *Arcyria* were also shown to have a perfect similarity with the primer sequence. Those sequences were not detected in the BLAST output obtained with the forward primer indicating that the specificity of the primer system was good.

Fungi, belonging to Ascomycota, Basidiomycota, and Zygomycota were successfully amplified with the ITS1-F and ITS4 universal primers, but no amplification was observed for non-*Pyrenophora* species when the PCR amplicons were subjected to reamplification using Pyr1 and Pyr2-GC primers. The six *Pyrenophora* species tested (*P. tritici – repentis*, *P. teres*, *P. semeniperda*, *P. japonica*, *P. graminea* and *P. avenae*), resulted in products of approximately 350 bp when reamplified.

5.4.2 PCR-DGGE analyses of pure *Pyrenophora* isolates

PCR-DGGE analyses of the partial ITS rDNA sequences showed that there were sufficient differences in the migration of the amplicons to discriminate among the six *Pyrenophora* species mentioned above. Amplicons obtained from the *Pyrenophora* species using the Pyr1/Pyr2-GC, displayed different electrophoretic mobility (Figure 5.1). These amplicons' migration positions, once optimized, were further used in this study as a molecular ladder. The use of a degenerate reverse primer did not result into multiple banding patterns for any of the analyzed species. All PCR amplifications resulted in single band amplicons.

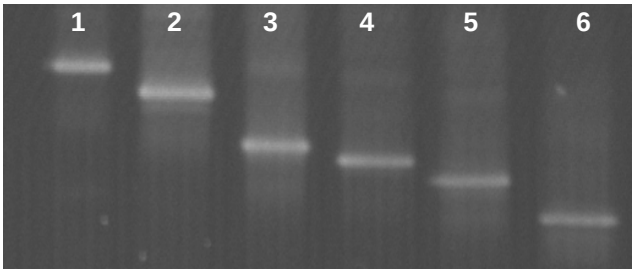

Figure 5.1 DGGE separation of amplicons of a portion of the ITS rDNA region for six *Pyrenophora* isolates that were used as genetic markers. Lanes 1 to 6: 1. *P. semeniperda*, 2. *P. teres*, 3. *P. graminea*, 4. *P. avenae*, 5 *P. japonica*, 6. *P. tritici-repentis*.

5.4.3 PCR-DGGE analyses of infected durum seeds

The DNA extracted from infected plant material (plates A to F) was amplified with the Pyr1/Pyr2-GC primer set and amplicons were run on the DGGE gel. The DNA of all the *Pyrenophora* species inoculated onto plants was detected

by the method. They were identified by comparison to the ladder constructed with DNA extracted from pure cultures (Figure 5.2). No multiple banding patterns were present in the gel.

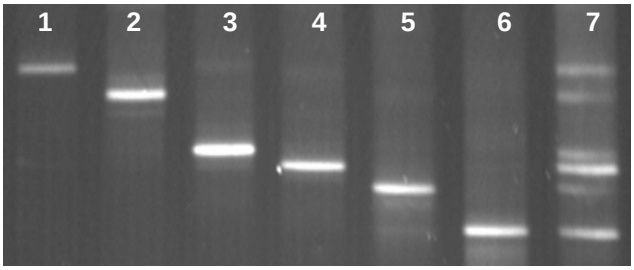

Figure 5.2 DGGE separation of *Pyrenophora* species recovered from infected plant material and their migration position on DGGE gel. Lane 7 molecular ladder composed of (from top to bottom) *P. semeniperda*, *P. teres*, *P. graminea*, *P. avenae*, *P. japonica*, and *P. tritici-repentis*. Lane 1 to 6: 1. *P. semeniperda*, 2. *P. teres*, 3. *P. graminea*, 4. *P. avenae*, 5. *P. japonica*, 6. *P. tritici-repentis*.

5.5 Discussion

The purpose of this study was to develop molecular tools for the identification of *Pyrenophora* fungi from infected wheat material. Identification of plant fungal inhabitants usually depends on the accurate interpretation of their morphological characters. Conidia are not always obtained in culture, and colonial morphology can easily be altered during subculturing or storage conditions (Kale and Benett, 1992; Manoharachary et al., 2005). This study introduces a molecular-based approach that permits the rapid and precise identification of different *Pyrenophora* species in plant tissues, with no need for isolation and cultivation.

Still, some difficulties for an accurate identification of P*yrenophora* fungi can arise from the fact that species boundaries are not well defined in this genus.

The question of whether *P. teres* and *P. graminea* are two forms of the same species is still open and, on the molecular side, results are controversial. These fungi, classified as separates species, can be crossed under laboratory conditions to produce fertile progeny (Smedegaard-Petersen, 1983). Moreover, relationships between *P. teres* and *P. graminea*, based on analysis of the internal transcribed spacer (ITS1 and ITS2) and rDNA 18S, revealed a low level of interspecific variation suggesting that *P. graminea* and *P. teres* may be forms of the same species (Stevens et al. 1997; Zhang and Berbee, 2001). On the other hand, the amplified fragment length polymorphism (AFLP) based on PCR markers genotyping technique discriminated between these species and revealed the existence of overall relevant DNA differentiation among these taxa (Leisova et al. 2005). In our study, the electrophoretic mobility of *P. graminea* sequence varied from that of *P. teres* on the DGGE gel. In fact, all tested *Pyrenophora* isolates migrated to unique positions and they could be easily distinguished from one another. The species-specific motifs in the ITS rDNA proved therefore to be useful in the identification of *Pyrenophora* species when combined with DGGE.

The method described here was highly effective in distinguishing and identifying *Pyrenophora* isolates in pure culture, as well as to assess *Pyrenophora* species in infected plant tissues. An alternative procedure, much easier than sequencing the DGGE bands, was the comparison of bands with the reference patterns of a constructed molecular ladder. The ladder was made of six different *Pyrenophora* species and loaded on one side of the gel to facilitate species identification. This approach permits to identify rapidly and at a low cost unknown

samples. Generally, the recovery of *P. tritici-repentis* from durum wheat or wheat samples is expected. This species is the most commonly recovered from leaf tissues in wheat growing regions all over the world. On the other hand, other *Pyrenophora* species are restricted to specific geographic regions. For example, *P. semeniperda*, is known to be highly dependent on temperature for germination and infection of leaves. Its current world distribution is in cereals growing regions of Argentina, Australia, Canada, Egypt, New Zealand, South Africa, and the United States (Medd, 1992). By using a molecular approach such as PCR-DGGE, we believe that *Pyrenophora* species colonizing wheat worldwide can be detected and identified.

In this study we surveyed *Pyrenophora* isolates belonging to six species but other species might also occur in wheat fields. The species used in this study showed different migration patterns on the DGGE gels but, despite the high sensitivity of this technique, it is possible that some non-tested *Pyrenophora* species end up having similar band migration pattern. Cases of co-migration of distinct species have been previously reported in other PCR-DGGE studies (Yergeau et al., 2005; Salles at al., 2002). DGGE theoretically, can detect down to 1 bp sequence variation (Muyzer et al., 1993) but separation of DNA fragments is influenced not only by nucleotide differences in DNA fragments but also by the fragments' length (Yergeau et al., 2005). To overcome this limitation, if a band migrating position is suspected to be common to more than one species, it should be excised, cloned into vectors and sequenced.

Studies have revealed that the microbial diversity is far greater than formerly anticipated and that culture-dependent methods are sometime insufficient

to explore the biodiversity (Muyzer et al., 1993). This study provides a molecular tool for the study of *Pyrenophora* species composition in samples where they are expected to be present. Because the method is rapid and easy, PCR amplification of ITS rDNA fragments combined with DGGE will facilitate the study of *Pyrenophora* biodiversity in wheat, or to confirm the identification of *Pyrenophora* species in infected tissues. We believe that the constructed primer set (Pyr1/Pyr2-GC) will be useful to detect and monitor *Pyrenophora* populations directly in plant tissues and environmental samples.

6. SUSCEPTIBILITY OF DURUM WHEAT TO DAMPING OFF DISEASE CAUSED BY *ARTHRINIUM SACCHARI* AND *NIGROSPORA ORYZAE*

6.1 Introduction

It is generally known that damping-off disease of seeds and seedlings is a widely distributed problem throughout the world. Since durum wheat is an important crop in western Canada in this study we investigated the damping-off potential of two fungal species, *Arthinium sacchari* and *Nigrospora oryzae* recovered during a biodiversity study of durum fungal inhabitants during 2004-2005.

A. sacchari and *N. oryzae* are ascomycete pathogens in the class Sordariomycetes, subphylum Pezizomycotina (Farr et al. 2008). *A. sacchari* was reported in South America and eastern Asia where it is known as an important mycotoxigenic species causing an acute fatal food poisoning known as "deteriorated sugarcane poisoning" (DSP) (Xingjie et al. 1992). 3-Nitropropionic acid, the toxin produced by *A. sacchari*, attacks the central nervous system in humans (Wei et al. 1994). The presence of this fungal species in Canada and its association with durum wheat has never been reported in the literature.

It is known that *N. oryzae* is a pathogen of a wide range of plants, but its pathogenicity on durum wheat has never been tested. This fungus was previously reported to colonize red spring wheat and durum wheat seeds in Manitoba, Saskatchewan, and Alberta provinces, the mean seed infection being the highest in Saskatchewan province (Clear et al., 2005).

6.2 Objectives and hypothesis

The objective of this study was to investigate the susceptibility of durum wheat to damping off disease caused by *Arthrinium sacchari* and *Nigrospora oryzae* fungal species.

I hypothesized that infection of durum wheat seeds with *Arthrinium sacchari* and *Nigrospora oryzae* respectively, will result into a high incidence of pre-emergence and post-emergence damping-off disease.

6.3 Materials and methods

Arthrinium sacchari and *Nigrospora oryzae* were recovered during a biodiversity study of fungal inhabitants of durum wheat (see chapter 3). To confirm the identity of the fungal isolates, DNA was extracted using the UltraClean microbial DNA isolation kit (MoBio, Inc. CA) from 2-week old *A. sacchari* cultures (FBC.3, FBC.45 and FBC.143) and *N. oryzae* (FBC.49 and FBC.43) grown on Potato Dextrose Agar (PDA). The internal transcribed spacer (ITS) of the rDNA was amplified from each isolate and sequenced (Plant Biotechnology Institute, Saskatoon, SK). The primer set used for the PCR amplification was ITS1-F / ITS4 (Gardes et al.,1993, White et al.,1990), and similarity analyses were performed using the BLAST search algorithm in GenBank.

Further, a first *in vitro* assay was performed by placing 1-cm^2 agar plugs containing mycelia of *A. sacchari* isolates FBC.3, FBC.45 and FBC.143 and *N. oryzae* isolates FBC.49 and FBC.43 onto surface-sterlized durum seeds placed on

PDA media. Surface-sterilized seeds inoculated in the same way with *Fusarium graminearum* or *F. avenaceum* were used as negative controls and non-inoculated surface-sterilized seeds were used as a positive control. A second *in vitro* assay involved inoculating the same isolates onto seeds placed in Petri plates containing sterilized sandy soil. In both assays, 10 seeds per Petri plate and three plates per treatment were used. Plates were incubated at 21^{o}C for one week in darkness. All experiments were repeted twice. To verify the pathogen status of the pathogens they were re-isolated only from symptomatic plants, satisfying Koch's postulates.

6.4 Results

On PDA, pre-emergence damping-off ranged from 62.5% to 58%, the most pathogenic fungi being the *N. oryzae* isolates (Figure 6.1). In sterilized soil, the incidence of pre-emergence damping–off ranged from 43% to 30%, the most pathogenic being *N. oryzae* FBC.49 and *A. sacchari* FBC.45. However, subsequent incubation over a period of three weeks resulted in 100% post-emergence damping-off in *A. sacchari* FBC.45 and FBC.3, *N. oryzae* FBC.49 and FBC.43, as well as in both *Fusarium* inoculated controls. A value of 60 % post-emergence damping-off was found in *A. sacchari* FBC.143, and no damping-off was found in the non-inoculated control (Fig 6.2). The infected seedlings became soft, showing dark brown or violet lesions in their tissues.

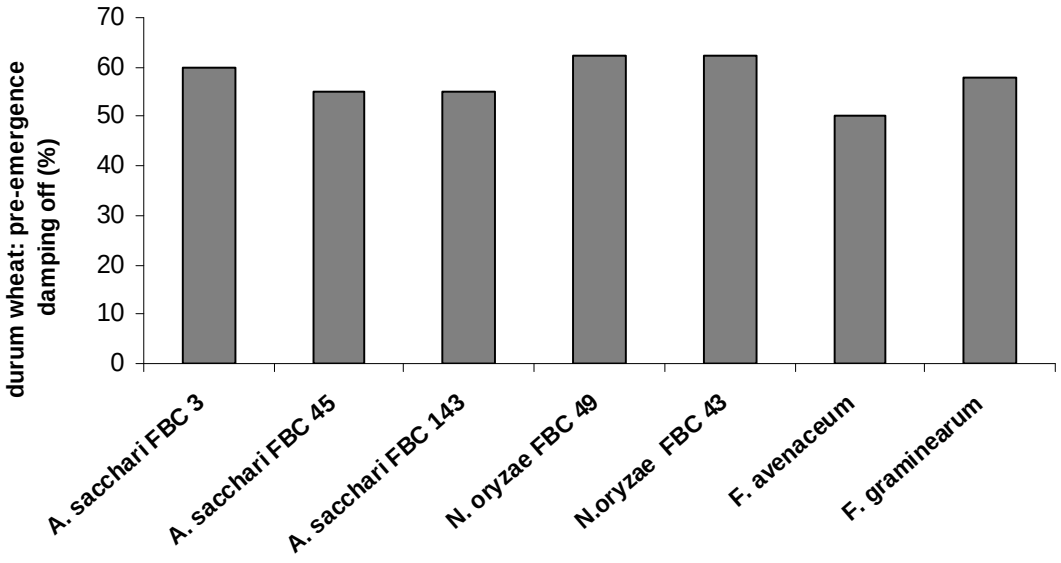

Figure 6.1 Incidence of pre-emergence damping off of durum seeds caused by *A. sacchari* (FBC.3, FBC.45 and FBC.143), *N. oryzae* (FBC.49 and FBC.43), *F. aveanaceum*, and *F. graminearum*.

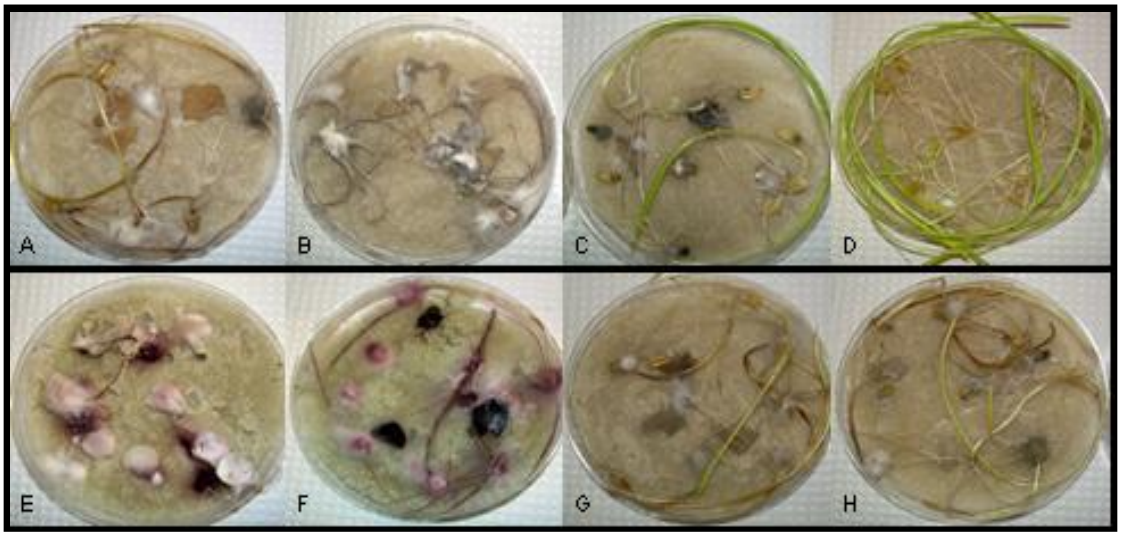

Figure 6.2 Durum seedlings non-inoculated or inoculated with:A. *A. sacchari* FBC.3., B. *A. sacchari* FBC.45., C. *A. sacchari* FBC.143., D. negative control, E. *F. avenaceum*, G. *F. graminearum*, G. *N. oryzae* FBC.49., H. *N. oryzae* FBC.43, after three weeks of incubation.

6.5 Discussion

This is the first report of the presence of *A. sacchari* in North America, and to demonstrate the damping-off potential of *A. sacchari* and *N. oryzae* in durum wheat.

To date, the life history and ecology of *A. sacchari* is unclear. This species needs to be examined to evaluate the risk it poses to cereal crops and durum health. Little is also known about the potential effect of *N. oryzae* on wheat crops. *Nigrospora* fungi, known to survive in soil and plant debris from one year to the next, were previously classified as pathogens of cotton, soybean and maize crops (Dhingra et al., 2002; Worf et al., 1985). A recent study investigating the biological control of pathogenic fungi surviving in soil and soybean stubbles revealed that under field conditions, the applied *Chaetomium globosum* inoculants can successfully compete with *Nigrospora* fungi in stubbles of above and under the soil surface (Dhingra et al., 2002). Although *C. globosum* has shown promising results for the control of *Nigrospora* pathogens in soybean crops, during our study on the biodiversity of durum wheat fungal inhabitants, *C. globosum* was not identified as a potential antagonist of *N. oryzae* (see chapter 3). It could be that in semiarid Saskatchewan durum fields, the low natural abundance of *C. globosum* allows for limited and specific antagonistic interactions.

The prevalence of *A. sacchari* and *N. oryzae* in durum crops was relatively low (2.12% and 1.25% respectively) (see chapter 3). However, in both in vitro assays performed in this study, the death of seeds or emerged seedlings,

demonstrated that durum wheat is highly susceptible to damping-off by *A. sacchari* and *N. oryzae*. If environmental conditions become conductive to increased levels of these pathogens in durum wheat, it is possible that *N. oryzae* and *A. sacchari* will become a real threat to durum wheat production. Further research is needed to understand the extent of these fungi distribution under field conditions where disease development depends on the environmental conditions to which plants and their endophytic fungal communities are exposed (Matthews and Clay, 2001; Colhoun 1973).

7. GENERAL SUMMARY AND CONCLUSIONS

Fungal biodiversity is an important natural bioresource offering new avenues towards a more sustainable agricultural production. In this project, the fungal diversity in durum wheat grown in rotation with pulses and canola crops at the South Farm of the Semiarid Prairie Agricultural Research Centre, in Swift Current Saskatchewan was analyzed. Forty seven fungal species occurred in durum wheat tissues at a frequency of > 1%. Seventeen species belonging to *Fusarium*, *Bipolaris*, *Pyrenophora*, *Alternaria*, *Epicoccum*, *Cladosporium*, *Microdochium*, *Arthrinium Nigrospora*, and *Phaeosphaeria* fungal taxa were potential pathogens.

Preceding crops had a limited impact on the prevalence of fungal pathogens in durum wheat. Only two pathogenic species were significantly influenced by rotation crops: *Fusarium torulosum* was less abundant in durum following canola while *Bipolaris sorokiniana* was less abundant in durum following pea. These results suggest that a preceding crop of pea, chickpea, lentil, canola, and wheat can help to maintain or increase pathogens' inoculum in crop residues, and contribute to disease development in subsequently cereal crops. Further research is warranted to explore the impact of increased crop diversity and for a longer term rotation in reducing pathogens inoculum in durum wheat. An alternative to the agricultural crops used in this study in rotation with durum wheat there are several other crops that occur in the prairies. Among these, mustard, sunflower, flax and potato crops are considered to be well suited for growth in the short season areas of the prairies. Rotating durum wheat with one of these alternative crops might contribute to a

better decline of pathogen populations in durum wheat then the preceding crops tested in this project. Important consideration should also be given to cultivar selection. Resistant cultivars could be used together with a diversified cropping system to reduce disease risk for several pathogens. The variety of durum wheat used in this study was AC Avonlea but there are seventeen more durum wheat varieties developed and registered for production in Canada. These cultivars are not resistant to Fusarium head blight disease but many of them are considered to be moderate or highly resistant to a wide range of stem and leaf diseases (Prairie recommending committee for wheat, rye and triticale, 2006). If resistance is available it should be used together with other management practices to minimize the opportunity of these pathogenic fungi to infest agricultural important durum crops.

The importance of *Fusarium* species in common and durum wheat crops has been recognized for many decades but these contaminants remain a severe problem because no resistance has been yet discovered. Biological control may offer a promising approach to enhance *Fusarium* diseases suppression by microorganisms. Some naturally occurring fungi such as *Acremonium* and *Chaetomium* were identified as potential biocontrol candidates against *Fusarium* pathogens. Based on the available literature it is suspected that these fungi may be antagonistic to *Fusarium* spp. due to their ability to produce antifungal compounds.

During this project it was also shown that durum wheat can be colonized by fungal species that were not associated with durum before. The recovery of *Arthrinium sacchary* from durum tissues increases our knowledge with regard to

durum fungal diversity and at the same time opens a question about the immensity of the undiscovered yet fungal species in durum crops. Much of what is known today about the fungal diversity in common and durum wheat crops resulted from field studies in which fungal species recovered from plant tissues were identified based on their morphological description or genetic similarities with other fungi. In this study, several fungal taxa isolated from durum wheat are still not completely identified and need to be further characterized. Indeed, current identification methods have numerous limitations that can lead to the underestimation of the overall fungal biodiversity. In this regard, further research should be oriented towards developing multiple approaches that can overcome problems associated with the recovery and validation of fungal isolates.

The on-going durum field experiments examined provided data on the seasonal succession of fungal species associated with durum wheat grown in five rotation systems. This investigation proved that durum wheat is inhabited by many pathogenic species that could affect durum wheat production. Some of the pathogenic species recovered can be extremely damaging whereas others can cause little damage. Some of the non-pathogenic species colonizing durum wheat were identified as potential antagonists and should be further examined for their potential as biocontrol agents against the pathogenic species recovered. On-going surveys of fungi inhabiting durum would be useful in determining trends in pathogen populations and would allow the development of biological control strategies for the suppression of fungal diseases.

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