# FITNESS, VIRULENCE AND GENETIC VARIABILITY IN *PYRENOPHORA TRITICI-REPENTIS* ISOLATES CAUSING TAN SPOT OF WHEAT IN OKLAHOMA

By

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FITNESS, VIRULENCE AND GENETIC VARIABILITY

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CAUSING TAN SPOT OF WHEAT IN OKLAHOMA

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is an important cereal crop and is the staple food for nearly 40% of the world's population (Weise 1987). It is an important source of carbohydrate, protein, vitamins and minerals that provides 20% of the world's food calories. Wheat occupies about 20% of the world's cultivated land. In the United States of America, wheat is an important agricultural commodity for domestic use and in international trade. In the southern Great Plains, nearly six million acres of land is under wheat cultivation in the state of Oklahoma, which typically ranks second in hard red winter wheat production across the nation (NASS 2009).

Wheat suffers from many diseases that are major constraints to productivity. About 20% of wheat yield worldwide is lost due to diseases (Weise 1987). One of the major foliar diseases of wheat is tan spot (synonym yellow leaf spot) caused by the ascomycetes fungus *Pyrenophora tritici-repentis* (Died.) Drech. (anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker)) (Ali and Francl 2003, Singh et al. 2006). Tan spot is prevalent in many wheat growing areas in the world and can reduce wheat productivity (Ackermann et al. 1988, Francl et al. 1992, Friesen et al. 2005).

Yield losses in wheat due to tan spot may range from 3 to 50% in the central plains of the United States (Ali and Francl 2003, Hosford 1982, Rees et al. 1982). Nearly a 40% grain yield loss was reported in Kansas due to tan spot (Sone et al. 1994). A lower thousand-kernel weight, reduced number of grain per head, shriveling and discoloration of seeds, and reduced milling quality also were reported as being due to tan spot (Bockus and Classen 1992).

Since first identified in New York in 1940 (Barrus 1942), tan spot has become a major disease in the United States (Engle et al. 2006). Tan spot has been known in the southern Great Plains since the 1970s but has not been a major concern for wheat cultivation in Oklahoma (Hunger and Brown 1987); however, the incidence and severity of tan spot can be of concern in Oklahoma if the proper environmental condition occurs. One factor that has contributed significantly to the increased incidence and severity of tan spot in Oklahoma has been a change in cultivation practices from conventional tillage (clean tillage) to minimum or no-till practices.

Minimum or no-till practices allow for increased residue on the soil surface. The tan spot fungus survives on the wheat residue from the previous year and infects wheat in the following cropping season (Bockus and Shroyer 1998). Sutton and Vyn (1990) found a positive correlation between the severity of tan spot and the amount of infected wheat residue present in the field. Presently nearly 30% of the wheat production area in Oklahoma is under no-till practices, and the trend is increasing (Dr. Jeff Edwards, OSU Department of Plant and Soil Sciences, *personal communication*). Thus, increased levels

of inoculum on wheat residue pose a threat to result in more outbreaks of tan spot. This fact, along with planting susceptible cultivars, contributes greatly to an increased occurrence of tan spot (Baily 1996, De Wolf et al. 1998).

Variability and adaptability of a plant pathogen are major problems to stability of disease management of a crop. Differences between isolates of a pathogen in characters like growth, sporulation, reproduction, virulence, latent period, etc., can be critical for determining disease incidence and severity. Ascospores are the primary inoculum for tan spot initiation, and thus, the ability of the causal fungus to produce pseudothecia (sexual fruiting bodies), number of asci per pseudothecia and mature ascospores per ascus, are important contributing factors to tan spot epidemics. Conidia produced on infected tissue are the secondary inoculum that spread disease in the field. Isolates can also vary in their ability to produce conidia (Hunger and Brown 1987, Rodriguez and Bockus 1996). Virulence is an important character of an isolate, and wide variations in virulence in isolates have been reported by many scientists (Krupinsky 1992, Luz and Hosford 1980, Moreno et al. 2008, Sah and Fehrmann 1992, Schilder and Bergstrom1990). While studying representative isolates of *P. tritici-repentis* from different decades, Kader et al. (2009) found significant variation among isolates in growth, sporulation, pseudothecia formation, ascospore maturity and virulence.

There is also the possibility that less fit isolates are replaced by more virulent and better adapted isolates over time and space. For example, in the wheat-stripe rust pathosystem, Milus et al. (2006) found that older populations of *Puccinia striiformis* f. sp. *tritici* had

been replaced by a newer population that had a 2 day shorter latent period, and a spore germination rate that was double that of older isolates. In Canada, isolates of *Phytophthora infestans* collected in the 1990s were found more virulent on tuber tissue than older isolates (Peters et al. 1999). In a study with a *Cercospora kikuchii* population, the causal fungus of cercospora leaf spot in soybean, recent isolates collected in 2000 and 2001 were found more virulent than older isolates collected in 1979, 1989 and 1994 (Cai and Schneider 2008). Information does not exist for comparing *P. tritici-repentis* isolates collected from wheat over time.

*P. tritici-repentis* is a necrotrophic fungus that produces three host-specific toxins (Ciuffetti and Touri 1999). Thus, *P. tritici-repentis* is considered to be composed of eight races based on their specific reaction (necrosis and/or chlorosis) on wheat differentials (Andrie et al. 2007, Ali and Francl 2003, Lamari and Bernier 1991). Knowing the occurrence of these races in a particular area and the response of germplasm to those races is important to developing wheat cultivars resistant to tan spot. The wheat-*P. tritici-repentis* pathosystem does not follow the classical gene-for-gene system (Flor 1955). Complete resistance is not known in wheat, and thus, susceptible wheat cultivars show a compatible reaction in response to toxins produced by isolates of *P. tritici-repentis* (Strelkov and Lamari 2003). The races present in the *P. tritici-repentis* population in Oklahoma have not been determined, but this knowledge is critical in selecting and using resistant germplasm in a wheat breeding program.

Genetic relationships of isolates based on morphological characters are not usually highly accurate because a phenotypic character is influenced by host-pathogen-environment interaction during disease development. However, there are a number of molecular techniques that can determine the genetic variability and relationship among isolates of a species. These molecular tools can be reliable and accurate approaches for pathogen diagnostics, gene identification, and determining the genetic variability and/or relatedness of a species (McCartney et al. 2003, William et al 1990). Nucleic acid-based techniques are rapid, specific, and highly sensitive and are widely used in applied plant pathology (Vincelli and Tisserat 2008). The genetic relationships of *P. tritici-repentis* isolates present in Oklahoma can be determined by molecular analysis.

The fitness, virulence and genetic variability for *P. tritici-repentis* isolates in Oklahoma have not been investigated, but that information is important to selecting isolates for use in germplasm screening for tan spot resistance and to develop management strategies. Hence, the objective of this research project was to characterize and compare *P. tritici-repentis* isolates collected from Oklahoma over three decades for:

1. Growth, sporulation and pseudothecia production of *P. tritici-repentis* isolates.
2. Virulence of the isolates on winter wheat cultivars.
3. Race structure, fitness and pathogenic and molecular variability among those isolates.

For the reader's information, chapters three to five have been prepared in manuscript format. The references cited for the Introduction and for the Review of Literature are presented in the Reference section.

# CHAPTER II

# REVIEW OF LITERATURE

## History of tan spot in wheat

The foliar disease tan spot of wheat, which is caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker), has been identified as a major limiting factor to wheat production worldwide (Duveillier 1998, Francl et al. 1992). This disease is also known as yellow leaf spot, yellow leaf blotch, leaf blight and eye spot of wheat (Hosford 1971). The sexual stage of the fungus was reported as *Sphaeria trichostoma* Fr. in 1823, *Pyrenophora trichostoma* (Fr.) Fck. in 1870 and *Pyrenophora tritici-repentis* (Died.) Drechs. The asexual stage was named as *Helminthosporium tritici-repentis* (Died.) in 1923, *Drechslera tritici-vulgari*s (Nisikado) Ito in 1930, and *Drechslera tritici-repentis* (Died.) Shoem. in 1959 (Hosford 1982).

The parasitic nature of this fungus was first discovered on a grass (*Agropyron repens*) in Germany in 1902 when it was named *Pleospora tritici-repentis* (Diedicke 1902). The same fungus was reported in the USA in 1923 (Drechsler 1923). In 1928, tan spot was first reported on wheat in Japan (Ito 1930) and in European countries (Anderson 1955).

Tan spot on wheat was identified in India in 1931 (McRae 1932, Mitra 1934), in Canada in 1937 (Conners 1937), in the USA in 1942 (Barrus 1942), in Australia in 1953 (Valder and Shaw 1953), in China in 1959 (Jiang 1959) and in Brazil in 1968 (Costa Neto 1968). During the 1970s, tan spot became a major disease of wheat worldwide, and consequently, CIMMYT (International Wheat and Maize Improvement Center) emphasized tan spot research programs to improve resistance to this disease (Duveiller et al. 1998).

**Disease symptoms and yield loss**

Symptoms of tan spot appear on leaves during the fall and spring in winter wheat. The fungus induces two distinct symptoms, tan necrosis and chlorosis (Gamba et al. 1998, Lamari and Bernier 1989). Wheat cultivars commonly develop either necrosis or chlorosis in response to infection by an isolate, however, both symptoms can be observed in a single cultivar (Lamari et al. 1991). Necrosis is the desiccated tan-colored tissue around the spot while chlorosis refers to yellowing that spreads over the leaf without any well-defined border (Lamari et al. 1995). These two symptoms are produced as a result of toxin secretion by *P. tritici-repentis* (Engle et al. 2006). Lesions on leaves characteristically have a small, tan to brown center, which is surrounded by a yellow circular border. Lesions initially appear as tan-brown flecks and expand into lens-shaped lesions that develop into tan blotches. Large lesions coalesce and develop dark-brown centers surrounded by a chlorotic border. As lesions expand and tissue is killed, a tannish hue develops over the leaves. As plants mature, the fungus infects stems on which it produces black pseudothecia that are a characteristic sign of this fungus (Weise 1987).

Yield losses in wheat due to tan spot may range from 3 to 50% in the central plains of the United States (Ali and Francl 2003, Hosford, 1982). Nearly a 40% grain yield loss was reported in Kansas due to tan spot (Sone et al. 1994). During grain filling, the fungus can infect wheat seed and cause a pink smudge or reddish discoloration (Schilder and Bergstrom 1994). A lower thousand-kernel weight, reduced number of grain per head, shriveling and discoloration of seeds and reduced milling quality have all been reported due to tan spot in wheat (Bockus and Classen 1992).

**Pathogen biology**

*P. tritici-repentis* is a homothallic ascomycetes fungus that propagates sexually by ascospores and asexually by conidia. The fungus develops one-loculed, black, raised fruiting bodies (pseudothecia) on wheat residue during the fall and winter. Inside a pseudothecium, sac-like asci are formed containing sexual spores (ascospores) with eight ascospores per ascus. Asci are bitunicate (double walled) with pseudoparaphysis present between asci. The median cell of an ascospore has a longitudinal septation (Ellis and Waller 1976). Ascospores are the primary inoculum for infection, and lesions found on lower leaves in late winter or early spring resulted from infection by ascospores released from psuedothecia on wheat residue.

Conidia represent the secondary or repeating inoculum for tan spot in a wheat field, and are formed on conidiophores in the lesion on a leaf. Conidiophores are olive-black with a swollen base, and conidia are subhyaline and cylindrically shaped with 4-6 septa. A conically tapered basal cell is diagnostic of conidia of *P. tritici-repentis* (Weise 1987).

Conidia also have been observed on pseudothecia (Shoemaker 1962). The current taxonomy of *P. tritici-repentis* is presented in Table 1.

**Host range**

This fungus can infect and survive on a wide range of grasses, with 26 grasses described as alternative hosts of *P. tritici-repentis* (Krupinsky 1982, Krupinsky 1992). Ali and Francl (2003) recovered races 1, 2 and 4 from volunteer wheat, and races 1 and 5 from durum wheat. In the same study, race 4 was recovered from other grasses and was not pathogenic to wheat. Barley is not a reservoir host of *P. tritici-repentis*, and thus, does not play a significant role in tan spot epidemiology (Ali and Francl 2001). A survey of barley fields from 35 locations in North Dakota during the 1999 growing season found only race 1 from 2-5% of the total samples. Moreover, no disease resulted when 12 barley cultivars were inoculated by races 1 to 5 (Ali and Francl 2001). Race 1 also has been recovered from broomgrass (*Bromus inermis*), which was pathogenic to wheat (Ali and Francl 2003, Krupinsky 1987).

**Infection and host-pathogen interaction**

*P. tritici-repentis* is a necrotrophic fungus which kills host tissue, colonizes the diseased tissue and produces conidia (Friesen et al. 2008). While studying the leaf infection process, Larez et al. (1986) found that conidia produced multiple germtubes and formed appresoria. An infection peg develops from appressoria that penetrates the leaf epidermis and grows intercellularly in mesophyll tissue (Dushnicky et al. 1996). The wheat-*P. tritici-repentis* pathosystem follows a toxin-based gene-for-gene model where a receptor

present in a susceptible host recognizes pathogen effectors (toxins) with the result being a compatible (susceptible) reaction (Strelkov and Lamari 2003).

**Host-specific toxins**

A unique feature of this fungus is the production of host-selective toxins (Ciuffetti et al. 1999, Lamari and Bernier 1989, Strelkov et al. 1998). Initial research provided evidence that toxins are the pathogenicity factors for tan spot development (Tomas and Bockus 1987, Brown and Hunger 1993). *P. tritici-repentis* is one of the few necrotrophic fungi able to produce multiple host-specific toxins (Strelkov et al. 2006).

This fungus secretes toxins into the apoplast that are capable of crossing host plasma membranes (Manning and Ciuffetti 2005). Although the molecular mechanisms are not clearly understood, research has shown that toxins are internalized into mesophyll cells (Manning et al. 2008) where they interact with chloroplast-localized proteins in the chloroplast membrane (Manning et al. 2007, Strelkov et al. 1998). Finally an alteration in the photosynthetic electron transport is induced that leads to accumulation of reactive oxygen species and eventually cell death (Manning et al. 2009). Upon inoculation by toxins, cellular electrolytic leakage was also reported (Kwon et al. 1998).

*P. tritici-repentis* is able to produce multiple host-specific toxins (Strelkov et al. 2006). As of today, three host-specific toxins produced by this fungus have been reported, and different isolates are capable of producing different combinations of toxins (Touri et al. 1995, Hallock et al. 1993). Toxin A (Ptr ToxA) induces necrosis while toxin B and toxin

C (Ptr ToxB and Ptr ToxC, respectively) induce chlorosis (Strelkov et al. 2006). Ptr ToxA, a ribosomally synthesized protein, has a mass of 13.2 kDa. Cloning of the *ToxA* gene revealed a 19.7 kDa protein that undergoes proteolytic cleavage to form the functional 13.2 kDa protein; thus, production of Ptr ToxA requires host metabolism (Ciuffetti et al. 1997). When treated with pure Ptr ToxA, sensitive wheat cultivars produce necrosis 48 h after inoculation (Kwon et al. 1998). Inoculation of susceptible cultivars with 0.2-90 nM of Ptr ToxA induces necrosis (Ballance et al. 1989, Touri et al. 1995, Zhang et al. 1997). When the temperature is >30$^{o}$C, the activity of Ptr ToxA to initiate necrosis is significantly reduced. This could occur because of a failure of the toxin to interact with the putative receptor at the cellular level (Lamari and Bernier 1994).

Ptr ToxB is a small protein with a mass of 6.6 kDa that induces extensive chlorosis (Lamari and Bernier 1989). Ptr ToxB is stable to heat up to 55$^{o}$C (Orolaza et al. 1995). A minimum of 14 nM of purified Ptr ToxB was reported to produce chlorosis on susceptible cultivars (Strelkov et al. 1999).

Although not fully characterized, another low molecular weight (approx. 1.0 kDa) chlorosis-inducing toxin, Ptr toxin C (Ptr ToxC), also has been discovered (Gamba et al. 1998, Orolaza et al. 1995). Ptr ToxC is not proteinaceous but a nonionic, polar molecule (Effertz et al. 2002).

**Genetics of pathogen and disease resistance**

A single-copy of a gene was discovered for the necrosis-inducing Ptr ToxA (Ciuffetti et al. 1997) while multiple-gene copies were identified for chlorosis-inducing Ptr ToxB (Martinez et al. 2001, Strelkov 2002). When an avirulent isolate was transformed to express toxin genes, it became pathogenic (Ciuffetti et al. 1997). The toxins produced by *P. tritici-repentis* are only pathogenicity factors and do not seem to influence other biological functions of the fungus. The absence of toxin production did not affect the survival and reproduction of *P. tritici-repentis* (Strelkov and Lamari 2003).

Although *P. tritici-repentis* has existed in nature for a long time (Hosford 1982), the fungus has only recently been thought to have become pathogenic on wheat via acquisition of a toxin gene (*ToxA*) from the fungus *Phaeospora nodorum* (anamorph: *Stagonaspora nodorum*), the causal agent of wheat glume blotch (Friesen et al. 2006). Since both fungi live in the same niche, the toxin gene appears to have been transferred from *P. nodorum* to *P. tritici-repentis* by horizontal gene transfer. Oliver et al. (2008) also supported the hypothesis of horizontal transfer of the *ToxA* gene because only after 1970 did tan spot become evident on wheat cultivars grown in Australia since 1911. The formation of conidial anastomosis tubes between conidia of *P. tritici-repentis* and *Phaeospora nodorum* in the same niche could facilitate horizontal gene transfer (Roca et al. 2005).

Complete resistance to tan spot has not been found but low to moderate resistance has been reported in wheat lines (Singh et al. 2008). Wheat resistance to tan spot is

characterized by small, dark brown lesions that do not increase in size (Singh and Hughes 2005), and resistance has been reported as qualitative (Lamari et al. 1991, Duguid et al. 2001, Duguid et al. 2001, Gamba et al. 1998) or quantitative (Elias et al. 1989, Faris et al. 1997, Nagle et al. 1982). Many researchers have found wheat resistance to tan spot necrosis to be governed by a single recessive gene (Anderson et al. 1991, Ciuffetti et al. 1997, Singh et al. 2005, Singh et al. 2006), whereas resistance to chlorosis is governed by a single dominant gene (Lamari and Bernier 1991) or can be quantitative (Faris et al. 1997).

**Races of *Pyrenophora tritici-repentis***

*P. tritici-repentis* is a highly specific pathogen, and physiological variation or specific reaction to a host is due to the production of host-specific toxins on a set of wheat differentials. Populations of *P. tritici-repentis* are classified into eight races based on their ability to induce necrosis and/or chlorosis on wheat differentials (Ali and Francl 2003). Andrie et al. (2007) described race identification based on the specific reactions (symptoms) on four wheat differentials (Appendix 2). Thus, Lamari et al. (2003) proposed that races 2, 3 and 5 carry one virulence factor (toxin), races 1, 6 and 7 carry two, and race 8 carries three virulence factors (toxins), respectively. The avirulent race 4 does not carry any of the virulence factors.

All eight races have been discovered in nature (Lamari and Bernier 1989a, 1989b, Lamari et al. 1995, 2003, Strelkov and Lamari 2003). Races 1 and 2 are present in all wheat growing areas and are the most prevalent races in the USA and Canada. Races 3 and 4

also are found occasionally in the USA and Canada (Freisen et al. 2003, Engle et al. 2006). In a study with 270 isolates collected from bread wheat, durum wheat and non-cereal grasses in the Great Plains, Ali and Francl (2003) recovered races 1, 2, 4 on bread wheat, race 1 and 5 on durum wheat, and race 1 and 4 on non-cereal grasses. In this study, 107 isolates were collected from bread wheat in both the northern and southern Great Plains, 93%, 2% and 5% of them were identified as race 1, 2 and 4, respectively. Race 4 was predominant (96%) in 22 isolates collected from non-cereal grasses in the southern Great Plains (Kansas and Oklahoma) except one (4%), which was identified as race 1 (Ali and Francl 2003). An increased number (50%) of race 2 was observed in western Canada (Lamari et al. 1998). Races 5 and 6 are common in Africa (Lamari 1995); race 5 has also been reported in the USA and Canada (Ali and Francl 2003, Strelkov et al. 2002), and in Azerbaijan (Lamari et al. 2003). Algeria, and the Caucasus and Fertile Crescent regions are the center of origin and diversity for wheat (Vavilov 1951), and following a long-term host-pathogen coevolution, almost all the races (1, 2, 3, 5, 6, 7 and 8) were found in those areas (Strelkov et al. 2002, Lamari et al. 2003, Lamari et al. 2005).

**Genetic diversity of *Pyrenophora tritici-repentis***

*P. tritici-repentis* reproduces by both sexual and asexual processes, and thus, genetic diversity likely contributes to tan spot epidemics. Considerable variation in growth, sporulation, virulence and fungicide response has been reported among isolates of *P. tritici-repentis* (Hunger and Brown 1987, Krupinsky 1992b). Recently, Kader et al. (2009) reported significant variation in isolates collected from winter wheat in Oklahoma

in 1983, 1996 and 2006 for growth, sporulation and virulence. Schilder and Bergstrom (1990) studied virulence of 17 isolates of *P. tritrici-repentis* on 12 wheat cultivars and found significant differences among the isolates for virulence, host susceptibility, and isolate X cultivar interactions. Pathogenic variation is observed among isolates of *P. tritici-repentis*, which are able to induce two distinct symptoms (necrosis and/or chlorosis) on wheat leaves (Lamari and Bernier 1991), however, occurrence of both symptoms on the same cultivar is also observed. Moreno et al. (2008) has reported pathogenic variability on wheat cultivars and molecular variability among the isolates.

Phenotypic variation is often difficult to explain as it can be greatly influenced by the complex interaction of the host, pathogen and environment. Thus, employment of molecular tools is useful to determine genetic variability in the population of a species (McCartney et al. 2003; Williams et al. 1990). Researchers have used different molecular markers to study variability in *P. tritici-repentis* such as rapid amplified polymorphic DNA (RAPD) (Mironenko et al. 2007, Santos et al. 2002, Singh and Hughes 2006), simple sequence repeats (SSR) (Cambell et al. 1999 and Johnson et al. 2000), inter simple sequence repeats (ISSR) (Moreno et al. 2008), and restriction fragment length polymorphism (RFLP) (Faris et al. 1997).

AFLP, a PCR-based marker, is being used to estimate genetic relationship in different species (Vos et al. 1996). It is a reliable, reproducible and robust technique which is unaffected by small variations in amplification parameters like PCR cycle profiles or template concentration (Zhong and Steffenson 2001). AFLP has been employed to study

the genetic relationship of many fungi (Garzon et al. 2005, Hielmann et al. 2006, Majer et al. 1996, O'Neill et al. 1997, Serenius et al. 2007, Zhan et al. 2006). Using AFLP analysis along with sequence data from the internal transcribed spacer region of the ribosomal DNA, Friesen et al. (2005) found variability in the population genetic structure of *P. tritici-repentis* isolates belonging to different races and geographic locations. AFLP markers were successfully used to study genetic differentiation in other *Pyrenophora* species (Leisova et al. 2005, Serenius et al. 2007).

**Disease cycle and epidemiology**

*P. tritici-repentis* oversummers and overwinters on wheat stubble by forming sexual fruiting bodies (pseudothecia). An increased occurrence of tan spot has been reported with the retention of wheat residue in no-till farming practices (Hosford 1982, Rees and Platz 1980, Schuh 1990, Watkins et al. 1978). In winter, the fungus lives on the stubble and passes through the cold period. These fruiting bodies produce and release ascospores during spring rains. Ascospores are the primary inoculum for tan spot epidemics, and a positive correlation exists between tan spot severity and the level of primary inoculum in the field (Adee and Pfender1989, Wright and Sutton 1990).

Disease spread by ascospores is limited to short distances, while long-distance dispersion largely depends on wind-borne conidia (Schilder and Bergstrom 1992). Conidia, which are produced on diseased tissue and serve as secondary inoculum, facilitate tan spot epidemics in the field (Shabeer and Bockus 1988). In a cropping year, repeated cycles of conidial production can occur, thus, tan spot is a polycyclic disease (Ronis and

Samaskiene 2006). Isolates of *P. tritici-repentis* might differ in conidial production; however, number of conidia produced is not always correlated with disease severity (Rodriguez and Bockus 1996).

Abiotic factors including temperature, moisture, nutrients, and light can play an important role in disease onset and inoculum production (Fernandes et al. 1991, Hosford et al. 1987, Luz and Bergstrom 1986, Khan 1971). Tan spot development in wheat is temperature-sensitive. Lamari and Bernier (1994) observed the effect of temperature on tan spot development on eight susceptible wheat cultivars. Tan spot development was constant over a range of 10-25$^{o}$C, but the severity was drastically reduced when temperature was more than 27$^{o}$C. The cultivar 'Glenlea' did not produce any necrotic symptom at 30$^{o}$C.

The length and degree of coldness in a particular area could influence the production of pseudothecia and ascospores by isolates of *P. tritici-repentis*. Although the maturation of pseudothecia and ascospores can occur over a range of 5-20$^{o}$C, the optimum temperature of 15-18$^{o}$C has been reported (Ronis and Semaskiene 2006). Summerwell and Burgess (1988) reported ascospore maturation in pseudothecia on wheat straw required 18 days at 15$^{o}$C.

Temperature and the availability of free moisture greatly influence germination of conidia. The optimum temperature for conidial production on V8 juice medium was 21$^{o}$C, although conidiogenesis took place over a range of temperatures from 10-31$^{o}$C (Platt et al. 1977). A certain combination of temperature and wet period is critical for

conidial germination. For example, 95% conidial germination was observed at 20$^{o}$C with a 6-h period of wetness (Larez et al. 1986). However, 6-12 h of wet period is required for successful infection, but increased wet period (96 h) at 23$^{o}$C produced maximum disease (Hosford et al. 1987). An incubation period of 2 days and a latency period of 6-7 days were observed by Wolf and Hoffman (1993) at 23$^{o}$C with 95% relative humidity.

*P. tritici-repentis* is a diurnal fungus that produces conidiophores following exposure to light and conidia following exposure to dark (Khan 1971). The greatest number of conidia is produced using a 12/12 h light/dark cycle. Although the fungus produces conidia when exposed to a range of 1 to 21 h of light per day, no conidial development occurred when exposed to continuous light or darkness (Khan 1971). This light-dependency was further confirmed by Francl (1998).

Plant age, cultivars and leaf position can influence tan spot development. In a greenhouse experiment, plants were most susceptible to tan spot on a scale of 0-5 at the boot (Feekes' scale 10, disease severity 3.4) and flowering (Feekes' scale 10.5.1, disease severity 3.9) stages and yield losses of about 50% were recorded when plants were infected at the boot stage (Shabeer and Bockus 1988). Perello et al. (2003) found a significantly higher severity of tan spot at heading stage when compared to tillering and first hollow stem. Resistance to tan spot differed among the wheat genotypes following artificial inoculation, and more disease was recorded in older (lower) leaves than in younger (upper) leaves (Hosford et al. 1990).

Tan spot epidemics also depend on the aggressiveness of isolates and resistance in host genotypes. In an isolate X cultivar experiment, isolates with a high level of aggressiveness produced more disease in comparison to isolates with a low-level of aggressiveness, and the severity of tan spot significantly differed among cultivars with different level of resistance (Krupinsky 1992).

Antagonistic relationships have been observed between *P. tritici-repentis* and other fungi when co-inoculated on wheat. For example, following a mixed infection with *Cochliobolus sativus*, the causal agent of wheat spot blotch, *C. sativus* dominated over *P. tritici-repentis*. In mixed inoculations, only a 20% component of *C. sativus* conidia was needed to significantly reduce germination, germ tube development and appressoria formation by conidia of *P. tritici-repentis* (Luz and Bergstrom 1987). However, a neutralism was observed between *P. tritici-repentis* and *Phaeosphaeria nodorum* (Luz 1986).

Nutrient availability can affect the survival and reproduction of fungi (Hall 1971, Ross and Bremner 1971). Thus, nutrients in wheat straw can influence the reproduction of *P. tritici-repentis*. Pseudothecia production and ascospore maturity of *P. tritici-repentis* differed significantly on nutrient-amended media (Pfender and Wootke 1987), and pseudothecia and ascospore production was proportional to the level of nitrogen (9-900 ppm) supplied in the medium. The greatest number of pseudothecia was observed when nitrogen was applied at 900 ppm in media, with this number reduced to 60% at 90 ppm. A maximum number of fertile pseudothecia were produced on media at 900 ppm

nitrogen, but phosphorus and potassium at a rate of 50 and 20 ppm, respectively, also were required. In another experiment (James et al. 1991), nutrient-amended wheat straw produced more pseudothecia and mature ascospores than straw without amendment, but the difference was insignificant, indicating that *P. tritici-repentis* is able to produce pseudothecia on straw without additional nutrients.

**Management of tan spot in wheat**

Resistant cultivars, tillage methods and application of fungicides are useful in controlling tan spot of wheat (Jorgensen and Olsen 2007). Genetic resistance is the most safe, economical and sustainable method for tan spot management (Rees and Platz 1990, Reide et al. 2003). A 50-75% reduction in tan spot severity was reported by using moderately resistant cultivars (Singh et al. 2008). In some areas use of multilines and cultivar mixture also has been used to control tan spot (Cox et al. 2004).

Cultural practices including residue management, crop rotation, fertilizer application and deep plowing also are useful in controlling this disease. Maintaining low levels of residue in a field is a simple but effective way to minimize tan spot incidence. Sone et al. (1994) reported about a 90% reduction in tan spot when the source of inoculum (ascospores) was located 3.6-5.4 m away from wheat. Bockus and Classen (1992) reported that rotation with sorghum, which allowed a 15 month period between two wheat crops, significantly reduced tan spot. Ascocarp production on straw was greatly reduced where previous wheat residue was disked before wheat planting (Zhang and Pfender 1992). Huber et al. (1987) reported a reduction in tan spot severity of wheat

when nitrogen was applied in ammonium form. In contrast, Bockus and Davis (1993) did not find any consistent affect of nitrogen fertilizers on tan spot severity in wheat. Burning of stubble can effectively control tan spot by reducing infected residue.

Following fungicide treatment in winter wheat in Denmark, up to a 90% reduction in tan spot was observed, which increased grain yield from 0.8-1.7 t/ha over a non-treated plot (Jorgensen and Olsen 2007). In another field experiment in Australia, application of propiconazole at 62 ml a.i./ha after flag leaf emergence resulted a 59% increase of grain yield compared to the untreated control (Colson et al. 2003). Although not practiced widely, microbial competition and antagonism may significantly reduce ascocarp formation and ascospore production of *P. tritici-repentis* in warm and low-humidity environments (Pfender 1988).

Table 1. Taxonomy of the fungus *Pyrenophora tritici-repentis*
(Agrios 2005, Webster and Weber 2007)

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Superkingdom: Eukaryota

Kingdom: Fungi ---- Produce mycelium, cell wall consists of chitin, lack of chloroplast

Subkingdom: Dikaryota ---- cell contain two genetically distinct nuclei

Phylum : Ascomycota ---- Have sexual stage (teleomorph) and asexual stage (anamorph), Sexual spore called ascospore, 8 ascosopres in asci, produce asexual spores (conidia) on free hypha

Subphylum: Pezizomycotina---- Filamentous ascomycetes; hyphae

Class: Dothideomycetes---- Fruiting body is ascostromata, produce asci within locule preformed in stroma, ascus have double wall (bitunicate), ascostroma is monolocular called pseudothecium

Order: Pleosporales ----- Asci surrounded by pseudoparaphysis inside pseudothecia

Family: Pleosporaceae

Genus: *Pyrenophora* ---- anamorph is *Drechslera*, causing leaf spots in cereals and grasses

Species: *Pyrenophora tritici-repentis*, cause of tan spot in wheat.

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# CHAPTER III

## GROWTH, SPORULATION AND PSEUDOTHECIA PRODUCTION OF *PYRENOPHORA TRITICI-REPENTIS* ISOLATES COLLECTED OVER THREE DECADES IN OKLAHOMA


### ABSTRACT

Variability in fitness characters like growth, sporulation and reproduction can significantly affect disease onset, incidence and severity. Hence, variability in these characters was determined for isolates of *Pyrenophora tritici-repentis* collected from Oklahoma over three decades. Seventeen isolates were studied for growth on commercial potato dextrose agar (CPDA), PDA made from fresh potatoes (RPDA), and clarified V8 juice agar (CV8). Conidia production was determined on CV8 and on wheat leaves of wheat cultivars Deliver (moderately susceptible), TAM 105 (susceptible) and Red Chief (resistant). Isolates were also evaluated for pseudothecia production and ascospore maturity formed on wheat straw. Radial growth for all isolates was significantly higher on RPDA and CV8 than on commercial PDA. Isolates differed significantly ($P$ <0.05) for conidia production on CV8 and on leaves. The isolate designated as Cherokee produced the highest number of conidia on CV8 while isolate OKD4 did not produce conidia on CV8.

The isolate Kiowa produced the highest number of conidia on leaves of all three cultivars. A weak correlation ($r$ = 0.362 - 0.586) was found between conidial production on CV8 and on cultivars; however, higher correlations ($r$ = 0.71 - 0.85) were observed within cultivars. Isolates also varied significantly in pseudothecia production and ascospore maturity on wheat straw. Although isolate OKD2 produced the highest number of pseudothecia, no mature ascospores were found after 23 days of incubation. Based on these fitness characters the most recently collected isolates from the 2000s appeared more fit than isolates collected in the 1980s and 1990s. Variability in these characters that contribute to fitness in the field should be taken into account when selecting isolates for use in studies screening wheat germplasm and studying the etiology and epidemiology of tan spot.

## INTRODUCTION

Tan spot caused by the ascomycetes fungus *Pyrenophora tritici-repentis* (Died.) Drech. (anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker) is a major disease of wheat worldwide (Singh et al. 2006, Ali and Francl 2003). Yield losses in wheat due to tan spot may range from 3 to 50% in the central plains of the United States (Ali and Francl 2003, Hosford, 1982), and a near 40% grain yield loss due to tan spot was reported in an experimental study in Kansas (Sone et al. 1994). Lower thousand-kernel weight, reduced number of grain per head, shriveling and discoloration of seeds, and reduced milling quality have all been attributed to tan spot (Bockus and Classen, 1992).

In Oklahoma in the southern Great Plains, winter wheat typically is grown on 5-6 million acres annually (NASS 2009). Tan spot has been present in Oklahoma since the 1970s (Hunger and Brown 1987), but generally has not been considered a major disease concern until recently when cultivation practices have changed from clean to minimum or no-till practices that leave increased residue on the soil surface. As a result, an increase in the incidence and severity of tan spot has been noticed.

Presently no-till wheat is employed on nearly 30% of the acres cultivated with wheat in Oklahoma (Dr. Jeff Edwards, OSU Plant and Soil Sciences Department, *personal communication*). The tan spot fungus survives on wheat residue from the previous year and infects wheat in the following spring (Bockus and Shroyer 1998). Ascospores produced in pseudothecia are the primary inoculum that initiates tan spot in the spring. Sutton and Vyn (1990) found a positive correlation between the severity of tan spot and the amount of infected wheat residue present in the field. This fact along with planting susceptible cultivars can contribute greatly to an increased occurrence of tan spot (Baily 1996, De Wolf et al. 1998).

Characters such as growth, sporulation and fruiting body formation are important in disease onset and epidemics because these characters help define the fitness of the pathogen. *P. tritici-repentis* is a necrotrophic fungus, which kills tissue by secreting host-specific toxins and then colonizes the dead tissue. Mycelia growing on the dead tissue produce conidia that are the secondary inoculum for spreading tan spot in the field. Hunger and Brown (1987) found significant variation in growth and sporulation on media

among isolates of *P. tritici-repentis* collected from winter wheat in Oklahoma during the 1980s. Furthermore, sporulation on host tissue is not only a fitness character of the pathogen but also provides a means to evaluate host resistance, which is an important parameter in breeding for resistance to tan spot (Riaz et al. 1991). In the corn-*Bipolaris maydis* pathosystem, Nelson and Tung (1973) found a positive relationship between sporulation on the host and disease severity. Variation in pseudothecia formation and ascospore development can also affect wheat tan spot epidemics in Oklahoma. Less fit isolates might be replaced by more aggressive and better adapted isolates over time. For example, in the wheat-stripe rust pathosystem, Milus et al. (2006) found that older populations of *Puccinia striiformis* f. sp. *tritici* have been replaced by a newer population that has a 2 day shorter latent period, and a spore germination rate that is double that of older isolates. Little is known about *P. tritici-repentis* isolates collected from wheat in different time periods. In a preliminary study, Kader et al. (2009) found significant difference in fitness characters among the isolates such as radial growth, sporulation, pseudothecia formation and their maturity. However, a more thorough investigation is necessary, and the objective of this study was to compare the fitness characters such as growth, sporulation and pseudothecia production of *P. tritici-repentis* isolates collected in Oklahoma over three decades.

## MATERIALS AND METHODS

### Isolate collection and storage

Isolates of *P. tritici-repentis* originally collected from single ascospores or conidia in the 1980s, 1990s and 2000s were included in this study (Table 1). Single ascospore isolates

were obtained by washing wheat straw on which pseudothecia had formed in 10% bleach for one min, rinsing thoroughly in sterile water, drying and placing the straw on 15% water agar (WA) in 90 mm petri plates. After 3 days of incubation at 21$^{o}$C, plates were examined using a dissecting microscope, and single ascospores ejected from pseudothecia were transferred to potato dextrose agar (PDA) using a sterile transfer needle. Conidial isolates were obtained from leaves. Leaves having typical tan spot symptoms were collected and surface sterilized as described above, and incubated in 12 hr light at 23$^{o}$C and 12 hr dark at 16$^{o}$C for 3 days. After identifying conidia using a microscope, single conidia were taken aseptically and placed on PDA. Isolates were maintained on PDA while being used in experiments but were maintained in liquid nitrogen for long term storage.

**Growth and sporulation on media**

Growth and sporulation of isolates were determined using the procedure as described by Hunger and Brown (1987). A 5-mm diameter mycelial plug, excised with a sterilized cork borer from the edge of an actively growing isolate, was removed and placed on commercial PDA (CPDA) and PDA made from fresh potatoes (RPDA) (200 g potato, dextrose 20 g, agar 15 g in l L) and clarified V8 (CV8) juice agar (150 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 850 ml water) in 90 mm petri plates. Plates were maintained in an incubator (Percival model I-36LL, Boone, IA) at 23$^{o}$C in dark for 5 days. Radial growth was measured by averaging the length of two opposite diameters and subtracting 5 mm from each reading. A two-factor randomized complete block design was followed with four replications. Each plate was a replication for a combination of isolate and medium,

and plates were arranged randomly within a replication. The experiment was repeated twice.

To determine conidial production of each isolate on CV8, about 10 drops of sterile water was added and mycelia were matted down using a sterile bent glass rod. Plates were then kept in the incubator for 12 hr at 23$^{o}$C with under cool-white fluorescent tubes (40W, 30 $\mu Es^{-1}m^{-1}$) to produce conidiophores. This was followed by 12 hr dark at 16$^{o}$C to induce conidia production. Conidia were washed from the plate into a beaker using a stream of sterile water. One ml of conidial suspension was pipetted onto a segmented petri plate (40 mm), and the number of conidia per ml was determined with a stereomicroscope. Area of mycelial colony ($\pi r^2$; r = radius of colony) was calculated, and the number of conidia per colony unit area was calculated. A randomized complete block design with four replications was followed and the experiment was conducted twice.

**Sporulation on leaves**

To determine sporulation on leaves, the moderately susceptible wheat cultivar 'Deliver' was used. Cultivar TAM105 (susceptible) and Red Chief (resistant) were included as checks. Conidia of each isolate were produced and harvested as described above and adjusted to 2000 conidia per ml. Seedlings were raised in commercial 'Ready-Earth' soil (Sun Gro Co., Bellevue, WA) in 6-inch x 1.5 in. dia plastic containers. Seedlings with three leaves fully expanded were inoculated with the conidial suspension (approximately 1.5 ml per plant) of each isolate using an atomizer (DeVilbiss Co., Somerset, PA) following the procedure of Rodriguez and Bockus (1996). Inoculated plants were

allowed to dry for 30 min so conidia would adhere to leaves, and then were placed in a mist chamber. After 48 hr, inoculated plants were removed from the mist chamber and placed in a greenhouse at 21$^{o}$C following a cycle of 14 hr light (510 $\mu Es^{-1}m^{-1}$) and 10 hr dark. After 5 days, the 2$^{nd}$ and 3$^{rd}$ leaves from the bottom were cut into 5-7 cm segments and placed on to filter paper moistened by 2.5 ml of sterile water in a 90-mm petri plate. Petri plates, sealed by parafilm to retain moisture, were incubated in the light (30 $\mu Es^{-1}m^{-1}$) for 12 hr at 23$^{o}$C followed by 12 hr dark at 16$^{o}$C for 5 days. Leaf segments were again cut into 1-cm pieces and placed in a sterile test tube containing 25 ml sterile water. The tubes were vortexed at maximum speed for 30 sec. One ml of conidial suspension was transferred into a segmented petri plate and conidia were counted using a stereomicroscope. The average number of conidia produced per leaf was calculated. This experiment was done twice in a randomized complete design with four replications (five plants per replication).

**Pseudothecia production**

To determine pseudothecia production by each isolate, the procedure of James et al. (1991) was basically followed but without adding nutrients to the wheat straw. Wheat straw was cut into pieces (2-in long), boiled for 10 minutes at 80$^{o}$C and autoclaved. About 2.5 ml of sterile water was added to filter paper in a petri plate and four pieces of straw were placed parallel to each other on the moistened filter paper. A 5-mm dia mycelial plug was placed in between each piece of straw. Petri plates were sealed with parafilm and were kept in dark at 21$^{o}$C for 14 days during which time pseudothecia

formed. Plates were then incubated at 15$^{o}$C in a 12 hr light (30 $\mu Es^{-1}m^{-1}$) and 12 hr dark cycle for 23 days.

The total number of pseudothecia and mature pseudothecia per straw were counted. A pseudothecium was considered mature only if at least one clearly mature ascospore was found as indicated by the presence of pigmentation and clear septation under a compound microscope (Friesen et al. 2003). The experiment was conducted twice in a randomized complete block design with four replications. Each petri plate with four pieces of straw was a replication for each isolate.

**Statistical analysis**

All statistical analyses were done using SAS 9.1 software (SAS Institute, Inc., Cary, NC). Data were transformed if necessary. Data were analyzed using the PROC MIXED module for radial growth on media and sporulation on leaf of wheat cultivars. Treatment means were separated by protected *t*-test using pair-wise difference option (PDIFF) at 5% level of significance. An ANOVA was performed using the GLM procedure for sporulation on CV8, total pseudothecia production on straw and pseudothecial maturity. Means were separated by Fisher's least significance difference test ($\alpha = 0.05$). The relationships (Pearson correlation coefficients) between the ability of sporulation on CV8 and the three cultivars for all isolates were determined using the PROC CORR option ($\alpha = 0.05$).

RESULTS

**Radial growth**

Isolates varied significantly ($P < 0.0001$) for radial growth on three media (Table 2). The mean radial growth was least on CPDA while it was greater on RPDA and CV8 (Table 3). Statistically, the greatest mean radial growth was observed for isolate RBB6 and OK-06-3. GYA3 and Cherokee showed the lowest radial growth. The average mycelial growth (41.8 mm) of the recent isolates from the 2000s was more than that (38.5 mm) of older isolates collected during the 1980s and 1990s.

**Sporulation on CV8 and on leaves**

Isolates differed significantly ($P <0.0001$) for conidia production on CV8 or on leaves of three wheat cultivars (Table 4). OKD1, Guymon, Cherokee and Atoka produced the greatest number of conidia on CV8 while the fewest were produced by OKD3, GYA3, OKD2, OKD5, OK-06-2 and El Reno (Table 5). OKD4 did not produce conidia on CV8. Kiowa produced the greatest number of conidia on wheat leaves, and the fewest were produced by GYA3, OK-06-2 and OKD3. The mean sporulation for the recent and the older isolates was 1023 and 416, respectively on wheat leaves. Lower correlation coefficients ($r = 0.585$, 0.557 and 0.363) were found between conidial production for the sixteen isolates on CV8 and on the leaves of the three cultivars (Table 6). However, higher correlation coefficients ($r = 0.854$, 0.758 and 0.709) were found for conidial production on leaves for all isolates within the three cultivars (Table 6).

**Pseudothecia production**

Isolates differed significantly ($P < 0.05$) in production of pseudothecia and formation of mature ascospores within pseudothecia (Table 7). OKD2 and OK-06-1 produced the highest number of pseudothecia while the least pseudothecia were produced by OKD4, GYA3 and El Reno. The percent of pseudothecia containing mature ascospores was highest for Guymon, while OKD3, OKD4, El Reno and OK-06-2 did not produce mature pseudothecia when then experiment was terminated. The recent isolates produced 47 pseudothecia on wheat straw of which 20% were mature while the older isolates produced 42 psedothecia of which 11% were mature (Table 7).

DISCUSSION

Growth is an important fitness character that enables the fungus to utilize nutrients from infected tissue. For example, Loughman and Deverall (1986) reported that in a study of 3-4 days after inoculation, rapid intercellular hyphal growth of *P. tritici-rep*entis was observed in a susceptible wheat cultivar which formed significantly larger mycelia as compared to growth and mycelia formed in a resistant cultivar. In our experiments, fungal radial growth on artificial media, which lacks host influence, was studied. Isolates grew well on RPDA and CV8 while less growth was observed on CPDA. Agar made using fresh potatoes likely provides more vitamins and minerals that could have contributed to fungal growth.

The number of conidia produced by an isolate is an important fitness character in disease epidemiology, and variation in conidial production both on media and on wheat cultivars

has been reported for isolates of *P.tritici-repentis* (Hunger and Brown 1987, Rodriguez and Bockus 1996). Studying sporulation *in vitro* lacks the effect of host physiology and interaction, and does not take into account the complex affects from wheat genotype, leaf position, fungal isolate, wet period etc. (Cox and Hosford 1987, Hosford et al. 1990). Sporulation on host tissue is a fitness character of a pathogen that can be influenced by host resistance (Rotem et al. 1978). Conidial production was highest on the susceptible cultivar TAM 105 as compared to production on the resistant cultivar Red Chief. In a wheat producing area, Riaz et al. (1991), reported that an isolate of *P. tritici-repentis* produced significantly greater conidia on a susceptible cultivar compared to a resistant cultivars. Thus, selection of wheat germplasm resistant to tan spot and utilizing them in a breeding program is important.

In this study, correlation between conidial production of all isolates on CV8 and on wheat leaves of three cultivars was low; however, correlations between the cultivars were high. Sporulation on CV8 lacks host influence, thus, the relationship was not able to explain a large amount of variability of the experiment (Rotem et al. 1978). For example, the isolate Cherokee, in comparison to other isolates, produced the highest number of conidia on CV8 while its sporulation was much less on all three cultivars (Table 5). On the other hand, the correlation coefficients for sporulation on cultivars were high, which explains a large amount of variability due to the influence of the host. Although studying sporulation on the host is difficult due to tedious experimental requirements, the difficulty of counting spores, and high experimental errors, etc. (Rotem et al. 1978), we concluded that sporulation of *P. tritici-repentis* isolates should be studied on wheat leaves

(*in vivo*) rather than on media in order to obtain the most accurate results. However, environment conditions or competition with other pathogens in the field may also affect sporulation (Luz and Bergstrom 1986). Studying sporulation *in vivo* is also important to help predict tan spot epidemics. Tan spot is a polycyclic disease because it occurs repeatedly in the field by air-borne conidia (Rodriguez and Bockus 1996). Thus, conidia, produced on the host, facilitate secondary infection that leads to severe disease in the field (Mundt 2009).

Tan spot is a disease favored in wheat produced by conservation tillage because *P. tritici-repentis* completes its life cycle on wheat residue. The production of pseudothecia, number of asci per pseudothecia and number of mature ascospores per ascus are important to tan spot epidemics. In this study, production of pseudothecia and their maturity were independent. For example, OKD2, OKD3, OKD5, OK-06-2, OK-06-3 produced a high number of pseuthothecia but few or no mature ascospores. Perhaps isolates required additional nutrients as it has been shown that this fungus differed significantly in pseudothecia production and ascospore maturity in nutrient-amended media (Pfender and Wootke 1987). However, the lack of effect of additional nutrients also has been demonstrated (James et al. 1991). We did not add any nutrient in our study because *P. tritici-repentis* survives naturally on residue left in the field (Odvody et al. 1982). Our experiment was terminated after 23 days of incubation because this is the accepted procedure as developed by James et al. (1991). Ascospores likely continued to mature after 23 days, but early maturation of ascospores would be an important competitive character of an isolate for tan spot initiation and establishment.

Based on parameters that contribute to fitness such as mycelial growth, sporulation and pseudothecia development, the isolates collected most recently during the 2000s appeared to be better fit than isolates collected in the 1990s or 1980s. Wheat production in Oklahoma under no-till cultivation is increasing, and the recently collected isolates are well adapted for completing their life cycle under this production system. *P. tritici-repentis* survives saprophytically during much of its life cycle, and a higher sporulation and rate of pseudothecial development would increase its survival capacity.

Long term storage or continuous subculturing may affect isolate physiology. Hosford (1971) reported that a *P. tritici-repentis* isolate maintained on PDA changed in color and radial growth rate. This phenomenon was also observed by Hunger and Brown (1987). However, *P. tritici-repentis* isolates used in our studies were maintained in liquid nitrogen, which is an accepted means to keep cultures over long periods of time while maintaining stability (Dhamen et al. 1983). Variability in *P. tritici-repentis* isolates for fitness characters can be further studied with other parameters. For example, determining the relationships of its fitness to isolate virulence, latent period, fungicide sensitivity etc. will facilitate understanding wheat tan spot epidemics and management.

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Table 1. *Pyrenophora tritici-repentis* isolates collected from winter wheat in Oklahoma over three decades that were used in this study

| Isolate | Collection year | County | Propagule of initial isolation |
|---|---|---|---|
| OKA1 | 1983 | Garfield | Ascospore |
| OKA2 | 1983 | Garfield | Ascospore |
| OKD1 | 1983 | Blaine | Ascospore |
| OKD2 | 1983 | Blaine | Ascospore |
| OKD3 | 1983 | Blaine | Ascospore |
| OKD4 | 1983 | Blaine | Ascospore |
| OKD5 | 1983 | Blaine | Ascospore |
| RBB6 | 1996 | Kay | Ascospore |
| GYA3 | 1996 | Texas | Ascospore |
| El Reno | 2005 | Canadian | Conidia |
| Guymon | 2006 | Texas | Conidia |
| Cherokee | 2006 | Cherokee | Conidia |
| OK-06-1 | 2006 | Payne | Ascospore |
| OK-06-2 | 2006 | Payne | Ascospore |
| OK-06-3 | 2006 | Payne | Ascospore |
| Atoka | 2007 | Atoka | Conidia |
| Kiowa | 2007 | Pittsburg | Conidia |

Table 2. Analysis of variance of radial growth of seventeen isolates of *Pyrenophora tritici-repentis* collected from winter wheat in Oklahoma on three media

| Source | DF | Type III SS | Mean squares | *F* value | *P* value |
|---|---|---|---|---|---|
| Isolate | 16 | 8592.30 | 537.01 | 29.31 | <0.0001 |
| Media | 2 | 26032.82 | 13016.41 | 710.49 | <0.0001 |
| Isolate*Media | 32 | 4324.38 | 135.14 | 7.38 | <0.0001 |

Table 3. Mean radial growth of *Pyrenophora tritici-repentis* isolates on commercial Potato dextrose agar (CPDA), PDA made from fresh potatoes (RPDA) and clarified V8 (CV8) juice agar

| | Radial growth (mm) on media (Mean ± SE)[a] | | | |
|---|---|---|---|---|
| Isolates | CPDA | RPDA | CV8 | Mean |
| *1980s* | | | | |
| OKA1 | 37.8[b] ± 2.8 A,b | 45.3 ± 1.0 C,a | 49.9 ± 1.1 CD,a | 44.3 |
| OKA2 | 29.8 ± 2.3 BC,b | 48.5 ± 0.9 BC,a | 52.3 ± 0.6 CD,a | 43.5 |
| OKD1 | 17.8 ± 0.8 EFG,b | 49.5 ± 0.6 BC,a | 48.9 ± 1.4 CD,a | 38.7 |
| OKD2 | 20.5 ± 1.5 DEF,b | 48.3 ± 0.8 BC,a | 48.1 ± 0.8 CD,a | 38.9 |
| OKD3 | 22.0 ± 1.4 DE,c | 46.0 ± 0.7 C,a | 32.8 ± 4.3 E,b | 33.6 |
| OKD4 | 24.3 ± 1.1 CD,c | 53.8 ± 0.6 AB,a | 38.0 ± 2.3 E,b | 38.7 |
| OKD5 | 23.3 ± 1.8 DE,b | 45.8 ± 0.7 C,a | 48.8 ± 1.9 CD,a | 39.3 |
| *1990s* | | | | |
| RBB6 | 37.0 ± 5.6 A,b | 58.4 ± 0.4 A,a | 53.5 ± 0.6 BC,a | 49.6 |
| GYA3 | 12.5 ± 1.5 G,c | 38.8 ± 0.5 D,a | 24.0 ± 1.3 F,b | 25.1 |
| *2000s* | | | | |
| El Reno | 23.8 ± 1.9 D,b | 49.3 ± 0.6 BC,a | 51.3 ± 0.5 CD,a | 41.4 |
| Guymon | 25.3 ± 4.1 C,c | 47.5 ± 0.9 C,b | 52.5 ± 0.9 CD,a | 41.8 |
| Cherokee | 15.3 ± 1.3 FG,b | 31.3 ± 0.6 E,a | 34.4 ± 3.2 E,a | 27.0 |
| OK-06-1 | 19.5 ± 2.1 DEF,b | 57.6 ± 0.3 A,a | 58.8 ± 1.3 AB,a | 45.3 |
| OK-06-2 | 25.3 ± 4.5 CD,c | 49.3 ± 2.2 BC,b | 60.5 ± 0.6 A,a | 45.0 |
| OK-06-3 | 23.3 ± 7.2 DE,b | 59.5 ± 0.5 A,a | 63.3 ± 0.8 A,a | 48.7 |
| Atoka | 24.0 ± 1.9 CD,b | 49.9 ± 0.3 BC,a | 47.0 ± 1.4 D,a | 40.3 |
| Kiowa | 33.3 ± 1.3 AB,c | 47.5 ± 0.6 C,b | 53.5 ± 0.6 BC,a | 44.8 |
| Mean | 24.4 | 48.1 | 48.6 | |

[a] Means in a column (upper case) and row (lower case) having same letter did not differ significantly by pair-wise difference option (PDIFF) in PROC MIXED ($\alpha = 0.05$); SE = Standard error.

[b] Average of four replications.

Table 4. Analysis of variance for conidia production of sixteen isolates of *Pyrenophora tritici-repentis* on leaves of wheat cultivars Deliver, TAM 105 and Red Chief

| Source | DF | Mean squares | *F* value | *P* value |
|---|---|---|---|---|
| Isolate | 15 | 3540650.39 | 55.22 | <0.0001 |
| Cultivar | 2 | 7286962.22 | 167.80 | <0.0001 |
| Isolate*Cultivar | 30 | 398027.72 | 9.17 | <0.0001 |

Table 5. Sporulation (mean ± standard error) of sixteen isolates of *Pyrenophora tritici-repentis* on clarified V8 (CV8) juice agar and on leaves of wheat cultivar Deliver, TAM 105 and Red Chief

| Isolates | Sporulation on CV8[c] | Sporulation on wheat leaf[a] | | |
|---|---|---|---|---|
| | | Deliver | TAM 105 | Red Chief |
| *1980s* | | | | |
| OKA1 | 30[b] ± 3.0 DE | 502[b] ± 16 EFG, ab | 765 ± 36 D, a | 198 ± 44 DEFG, b |
| OKA2 | 48 ± 2.1 BC | 272 ± 21 GH, b | 780 ± 12 D, a | 89 ± 9 FG, b |
| OKD1 | 62 ± 8.7 AB | 779 ± 21 CDE, a | 860 ± 267 D, a | 178 ± 14 EFG, b |
| OKD2 | 24 ±3.2 DEF | 621 ± 16 DEF,a | 850 ± 175 D, a | 79 ± 8 G, b |
| OKD3 | 11 ± 1.3 F | 153 ± 24 H, a | 320 ± 30 E, a | 101 ± 25 FG, a |
| OKD4 | -[d] | - | - | - |
| OKD5 | 16 ± 2.1 EF | 370 ± 32 FGH, b | 932 ± 72 CD, a | 248 ± 19 DEFG, b |
| *1990s* | | | | |
| RBB6 | 34 ± 5.6 CD | 528 ± 28 EFG, a | 764 ± 33 D, a | 399 ± 45 CDEF, a |
| GYA3 | 15 ± 1.6 EF | 80 ± 7 H, a | 157 ± 30 E, a | 87 ± 9 FG, a |
| *2000s* | | | | |
| El Reno | 17 ± 1.5 EF | 665 ± 17 DEF, a | 109 ± 21 E, b | 240 ± 32 DEFG, b |
| Guymon | 61 ± 9.2 AB | 1693 ± 75 AB, b | 2923 ± 90 A, a | 687 ± 51 BC, c |
| Cherokee | 68 ± 8.6 A | 855 ± 87 CD, a | 848 ± 53 D, a | 478 ± 31 CDE, b |
| OK-06-1 | 50 ± 2.1 B | 876 ± 90 CD, a | 1174 ± 107 C, a | 493 ± 31 CD, b |
| OK-06-2 | 20 ± 2.2 DEF | 268 ± 37 GH, a | 205 ± 38 E, a | 83 ± 25 G, a |
| OK-06-3 | 29 ± 2.3 DE | 1020 ± 57 C, b | 1575 ± 250 B, a | 815 ± 115 B, b |
| Atoka | 62 ± 8.3 AB | 1575 ± 221 B, a | 1740 ± 202 B, a | 440 ± 72 CDE, b |
| Kiowa | 50 ± 7.1 B | 1910 ±315 A, b | 2629 ±285 A, a | 1271 ± 224 A, c |
| Mean | 37 | 760 | 1039 | 368 |

[a] Sporulation means on wheat leaf following the same letter in a column (upper case) and row (lower case) did not differ significantly by pair-wise difference (PDIFF) option in PROC MIXED using a protected *t*-test ($\alpha = 0.05$).
[b] Average of four replications.
[c] Conidia calculated per $cm^2$ mycelial colony area; means within this column having same letter did not differ significantly by Fisher's least significance test ($\alpha = 0.05$).
[d] Did not produce conidia on CV8, and was excluded from analysis.

Table 6. Pearson correlation coefficients (*r*) between the conidiation of sixteen isolates of *Pyrenophora tritici-repentis* on clarified V8 (CV8) juice agar and on leaves of three wheat cultivars

| | | Wheat cultivars | | |
|---|---|---|---|---|
| | CV8 | Deliver | TAM105 | Red Chief |
| CV8 | - | 0.585 (<0.0001)[a] | 0.557 (<0.0001) | 0.362 (0.0032) |
| Deliver | - | - | 0.854 (<0.0001) | 0.758 (<0.0001) |
| TAM105 | - | - | - | 0.709 (<0.0001) |

[a] Value in the parenthesis indicates probability level.

Table 7. Total number of pseudothecia and percent mature pseudothecia produced by the isolates of *Pyrenophora tritici-repentis* collected from winter wheat in Oklahoma

| Isolates | Total pseudothecia [a,c] | Mature pseudothecia (%) [a,b] |
|---|---|---|
| *1980s* | | |
| OKA1 | 51.0 ± 12.8 bcd | 41.6 ± 11.0 ab |
| OKA2 | 49.7 ± 5.4 bcd | 21.6 ± 4.2 def |
| OKD1 | 41.5 ± 3.4 cd | 7.4 ± 1.8 gh |
| OKD2 | 84.5 ± 9.1 a | 0.0 ± 0.0 i |
| OKD3 | 49.2 ± 3.9 bcd | 5.0 ± 2.1 h |
| OKD4 | 12.3 ± 3.1 e | 0.0 ± 0.0 i |
| OKD5 | 36.1 ± 1.4 d | 3.4 ± 2.3 hi |
| *1990s* | | |
| RBB6 | 44.7 ± 6.7 bcd | 12.9 ± 1.6 efg |
| GYA3 | 12.0 ± 1.7 e | 0.8 ± 0.7 i |
| *2000s* | | |
| El Reno | 1.5 ± 0.8 f | 0.0 ± 0.0 i |
| Guymon | 59.6 ± 4.4 abc | 54.2 ± 8.1 a |
| Cherokee | 49.7 ± 1.2 bcd | 34.9 ± 3.3 bc |
| OK-06-1 | 69.4 ± 5.4 ab | 22.0 ± 2.5 ded |
| OK-06-2 | 47.1 ± 3.9 bcd | 0.0 ± 0.0 i |
| OK-06-3 | 61.4 ± 5.7 abc | 11.7 ± 2.9 fg |
| Atoka | 35.3 ± 4.2d | 23.8 ± 1.4 cd |
| Kiowa | 47.7 ± 3.4 bcd | 22.3 ± 1.2 cde |

[a] Mean ± standard deviation; means following the same letter within a column did not differ significantly by Fisher's least significance difference test ($\alpha = 0.05$).
[b] Data were analyzed after arcsine root transformation.
[c] Two-inch wheat straw of cv. Deliver was infested by mycelial plug and pseudothecia counted on it after 14 days of incubation.

# CHAPTER IV

## VIRULENCE OF *PYRENOPHORA TRITICI-REPENTIS* ISOLATES, YIELD LOSS, AND EVALUATION OF WHEAT RESISTANCE TO TAN SPOT IN OKLAHOMA


### ABSTRACT

In recent years, prevalence of tan spot on wheat has become more noticeable in Oklahoma. Selection of pathogen isolates for use in identifying cultivar resistance is critical to crop improvement. Virulence of sixteen isolates of *Pyrenophora tritici-repentis* collected over three decades was estimated on three wheat cultivars Deliver, TAM 105 and Red Chief. Three of these isolates, OKD1, RBB6 and OK-06-1, collected in 1983, 1996 and 2006, respectively, were tested on cultivar Deliver in the field in 2008 and 2009. Twelve isolates (out of sixteen) were used across eleven cultivars to evaluate resistance to tan spot. All sixteen isolates differed significantly ($P < 0.0001$) in virulence based on percent leaf area infection on the wheat cultivars Deliver, TAM 105 and Red Chief. Isolates collected from the 2000s exhibited more virulence than those collected in the 1980s and 1990s. Four isolates (out of sixteen) in this study were lowly virulent and produced the least percent leaf area infection on the three cultivars. Isolates differed significantly ($P < 0.0001$) in virulence and yield losses, but did not affect thousand kernel weight ($P < 0.2591$). A maximum of 25.2% yield loss was observed for OK-06-1..

In an isolate X cultivar study Kiowa was the most virulent isolate across all eleven cultivars followed by Cherokee, OKD2, OKD5 and OKA1. Significantly ($P < 0.0001$) less disease was observed on cultivar OK-Rising, Pete, Jagger and OK-Bullet across all twelve isolates. A greater amount of disease was observed on cultivars Chisholm, Duster and Triumph-64. Increased virulence in isolates was able to detect an increased variability in susceptibility (percent leaf area infection) in wheat cultivars ($r = 0.53$; $P < 0.05$), and increased susceptibility in cultivars detected increased variance in virulence of the isolates ($r = 0.76$; $P < 0.01$). Thus, developing resistant cultivars should be conducted using virulent isolates to enhance management of tan spot.

## INTRODUCTION

Oklahoma typically ranks second in winter wheat production in the USA, and wheat is grown on nearly six million acres (NASS 2009). To prevent soil erosion, conserve soil water and reduce fuel cost, low- or no-tillage is being practiced on about 30% of the wheat production area in Oklahoma (Dr. Jeff Edwards, OSU Plant and Soil Science, *personal communication*). As this shifting in cultivation practices from clean-till to no-till has occurred in Oklahoma, the incidence of tan spot has increased. The ascomycete fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoemaker) is the causal organism of the wheat foliar disease 'tan spot' or 'yellow spot' (Francl et al. 1992, Weise 1987). Prevalence of tan spot has been reported in Oklahoma since the 1970s (Hunger and Brown 1987), but was not considered a serious problem to wheat production. However, with the increasing shift to no- or low-

till practices, an increase in tan spot has been observed. In a field study near Stillwater, a 21% yield loss due to tan spot was reported (Kader et al. 2009).

*P. tritici-repentis* survives and overwinters on plant residue left in the field (Odvody et al. 1982). Ascospores, ejected from sexual fruiting bodies (pseudothecia) on wheat residue, are the primary inoculum to initiate tan spot in the next cropping season (Shabeer and Bockus 1998). Previous research showed a positive correlation between the amount of residue in the field and tan spot severity (Adee and Pfender 1989). Thus, residue on which the fungus completes its life cycle, contributes to an increase in inoculum of tan spot which increases tan spot incidence and severity if a favorable environment occurs.

Variation in the virulence of a pathogen is a problem in disease management (Stakman, 1957). Previous studies showed that isolates of *P. tritici-repentis* can significantly differ in virulence (Krupinsky 1992, Krupinsky 1992, Sah and Fehrmann 1992, Schider and Bergstrom 1990, Singh et al. 2006). Eight Mexican isolates varied in lesion number produced on seedlings and adult plants on the wheat cultivar Morocco (Gilchrist et al. 1984). Luz and Bergstrom (1980) compared 40 isolates on wheat and, by using a 0-6 scale, found twelve virulence patterns among the isolates. Schilder and Bergstrom (1990) observed a significant isolate X cultivar interaction with isolates from New York. Hunger and Brown (1987) used nine isolates to inoculate the susceptible wheat cultivar TAM 101, and found different lesion lengths. Different races were reported to vary in their virulence (Singh et al. 2006).

The use of host resistance is the best option in disease management, and wheat resistance to tan spot is governed by recessive genes (Cuiffetti and Touri 1999, Strelkov and Lamari 2003). Using partial resistance, Singh et al. (2008) reported a 50-75% reduction of tan spot in wheat. Resistance to tan spot might vary with wheat cultivar, growth stage, leaf position, wetness period, and fungal isolates (Cox 1987, Hosford et al. 1990, Shabeer and Bockus 1988). Wheat lines and cultivars differed significantly in lesion lengths when they were tested against tan spot using a bulk of three isolates (Evans et al. 1999). Wheat cultivars varied significantly in tan spot reaction (0-5 scale) in both the greenhouse and the field when inoculated by a bulk of *P. tritici-repentis* isolates (Raymond et al. 1985). While increasing of no-tillage wheat cultivation is a reality in Oklahoma, it is necessary to evaluate wheat cultivars to tan spot that were developed for this area.

Carson (1987) observed that an increased level of virulence in isolates is able to detect an increased level of variability in cultivar reaction (percent leaf area infection). Conversely, increased susceptibility in cultivars is able to detect maximum variability in virulence in isolates. Thus, in the *P. tritici-repentis*-wheat pathosystem, the relationship between the mean cultivar reaction and variance in virulence among isolates is positive linear. Schilder and Bergstrom (1990) also observed this relationship between isolate virulence in *P. tritici-repentis* and cultivar reaction in wheat. This relationship between isolates virulence and cultivar reaction also allows excluding lowly virulent isolates from an isolate X cultivar study because they cannot detect variability in cultivars' reaction efficiently.

Isolates of *P.tritici-repentis* might also vary in virulence over time and space. It is reported in other fungi that more recently collected isolates are more virulent than older isolates (Cai and Scheider 2008, Peters et al. 1999). Hence, a thorough evaluation of the virulence of isolates of a fungus and resistance in wheat cultivars to those isolates is necessary. The objective of this research was to determine the virulence of isolates of *P. tritici-repentis* collected from wheat in three decades in Oklahoma, and to test wheat cultivars for reaction against them.

## MATERIALS AND METHODS

### Inoculum preparation

All isolates of *P. tritici-repentis* were maintained on potato dextrose agar (PDA) (200 g potato, dextrose 20 g, agar 15 g in l L) at 4$^{o}$C during the period of study. Conidia were produced from each isolate by following the procedure reported in Raymond et al. (1985) with slight modifications. A 5-mm diameter mycelial plug, excised with a sterilized cork borer from the edge of an actively growing isolate, was removed and placed on clarified V8 juice agar (CV8) (150 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 850 ml water) in a 90 cm petri plate. V8 juice was clarified by centrifugation at 7,500 rpm for 5 min. After keeping in an incubator (Percival, Boone, IA) at 23$^{o}$C in the dark for 5 days, about 10 drops of sterile water was added and mycelia were matted down using a sterile bent glass rod. Plates were then kept in an incubator and exposed to cool-white fluorescent lighting (40W, 30 $\mu Es^{-1}m^{-1}$) for 12 hr at 23$^{o}$C to produce conidiophores, followed by 12 hr dark at 16$^{o}$C to induce conidia production. Conidia were collected in a beaker by washing with a

stream of sterile water. Conidia were adjusted to 2000 per ml of suspension before inoculation.

**Virulence testing in the greenhouse**

To determine virulence of 16 isolates (Table 2), the wheat cultivars Deliver (moderately susceptible), TAM 105 (susceptible) and Red-Chief (resistant) were used. Seeds were sown in 6-in. x 1.5 in. dia plastic cones containing a commercial 'Ready-earth' soil (Sun Gro, Bellevue, WA). Plants were raised in a growth chamber at 21$^{o}$C with a 14 h photoperiod (550 $\mu Em^{-1}s^{-1}$). When three leaves were fully expanded, plants were inoculated with a conidial suspension (2000 conidia per ml) of each isolate until incipient run-off by using an atomizer (DeVilbiss Co., Sommerset, PA). Isolates (main plot) and cultivars (sub plot) were arranged in a spit-plot randomized complete block design with 4 replications. About one hr after inoculation during which time plants dried so conidia would adhere to leaves, plants were placed in a mist chamber that provided near 100% relative humidity for 48 hr. Plants were then placed in a greenhouse at 21-23$^{o}$C. One week after inoculation, leaves were scanned using an EPSON 1650 scanner and the percentage of leaf area infection was calculated using ASSESS software (American Phytopatholigical Society, St. Paul, MN). As leaf position affects tan spot severity (Hosford et al. 1990), only the 2$^{nd}$ and 3$^{rd}$ leaves were rated. Ten plants (20 leaves) were rated for one replication, lesion size was recorded, and the experiment was conducted twice.

**Virulence in field and yield loss**

To evaluate virulence of isolates in the field, experiments were conducted in 2007-08 and 2008-09 at the Oklahoma State University Plant Pathology farm in Stillwater. Seeds of the wheat cultivar 'Deliver' were planted on 10 October 2007 and on 20 October in 2008 in 7 x 10.5 $ft^2$ plots of 9 rows 7.5 in. apart. Three isolates, OKD1, RBB6 and OK-06-1, randomly chosen from the 1980s, 1990s and 2000s, respectively, and inoculum was prepared following the procedure of Raymond et al. (1985). After water-soaking overnight in 130 ml distilled water, oat kernels (150 g) in a conical flask were autoclaved. Conidial plugs (5 mm diameter) from each isolate were added aseptically. Flasks containing inoculated oat kernels were maintained for 14 days at room temperature with shaking every 3 days. Infested kernels were dried by spreading on aluminum foil for one day before placement in the trial. In mid November, infested oat kernels (125 g) were applied in an area of 2.5 X 4 ft (10 $ft^2$) of each plot on the soil surface between the middle rows following the procedure by Sone et al. (1994). Uninoculated oat kernels were applied as a control. The trial was designed as a randomized complete block with 4 replications. Sprinkler irrigation was provided to maintain adequate moisture for disease development. The fungus produced pseudothecia and mature asci during the winter and early spring on the infected kernels, thus, providing primary inoculum (ascospores) in late February to early March. Disease severity was rated starting with the second week of March at two weeks interval from the F-3, F-2, F-1 and flag leaves, and averaged disease severity from these leaves were analyzed. Each time ten leaves from a leaf position were selected randomly from each plot. Leaves were taken to the lab and rated for percent leaf

area infection by using ASSESS software as described earlier. Yield and thousand kernel weight (TKW) were also recorded.

**Isolate virulence X cultivar resistance**

To evaluate wheat reaction to tan spot, eleven cultivars and twelve isolates were used (Table 6). Four lowly virulent isolates were excluded, and thus, twelve isolates were included in this study. Seedlings of each cultivar, inoculum preparation, inoculation and disease rating were done as described earlier. The experiment was conducted in the greenhouse following a split-plot in a randomized complete block design with four replications (five plants per replication). Isolates and cultivars were used as the main plot and sub-plot, respectively. The relationship between variance among cultivar to cultivar resistance was determined by regressing the variance among cultivars to mean isolate virulence. Similarly, the variance among isolates was regressed against the mean percent leaf area infection of cultivars (Carson 1987).

**Statistical analysis**

All analyses were performed using SAS 9.1 statistical software (SAS Institute, Cary, NC). In the isolate X cultivar study, untransformed data were analyzed. Analysis of variance was carried out using the PROC MIXED option, and treatment means were separated using the pairwise option (PDIFF) at 5% level of probability. Field data were analyzed assuming year is a random affect and isolates are fixed affect ($\alpha = 0.05$).

RESULTS

**Virulence of isolates in the greenhouse**

Isolates differed significantly ($P < 0.0001$) in virulence on three wheat cultivars, cultivars differed in response to isolates ($P < 0.0001$), and a significant interaction ($P < 0.0001$) between isolate and cultivar was observed (Table 1). Overall, Cherokee was the most virulent isolate across three cultivars followed by Kiowa, OK-06-1, OK-06-3 and OKA1. El Reno was the least virulent followed by OKD3, OKD5 and OKA2 (Table 2). On an average all 16 isolates produced 29.52%, 43.42% and 7.75% leaf area infection on cultivar Deliver (moderately susceptible), TAM 105 (susceptible) and Red Chief (resistant), respectively. On an average, isolates collected in the 2000s produced 32.96% LAI while isolates of the 1980s and 1990s produced 21.37% and 19.26% LAI, respectively (Table 2). The mean disease severity for the recent (2000s) and older (1980s and 1990s) isolates across the three cultivars was 32.9% and 20.3%, respectively.

**Virulence of isolates in field and yield loss**

In the field study, isolates significantly differed ($P < 0.0001$) in disease severity (percent leaf area infection) and yield. Statistically, thousand kernel weight (TKW) was not affected by the isolates (Table 3). A year affect was found for disease severity, yield and TKW ($P < 0.0077$, $P < 0.0001$ and $P < 0.0001$, respectively) (Table 3).

Disease severity, yield, yield reduction (%) and TKW are presented in Table 4. Highest disease was observed for OK-06-1 while it was least for OKD1. Mean yields from plots infected by OKD1, RBB6 and OK-06-1 were 52.9, 42.6 and 42.9 bu/A, respectively. A

25.22% yield reduction was observed for isolate OK-06-1 compared to the control while it was 19.83% for RBB6 and 7.26% for OKD1. Isolates did not affect TKW (Table 4).

**Isolate virulence X cultivar resistance**

Cultivars differed significantly ($P < 0.0001$) in their percent leaf area infection in response to isolates in the greenhouse (Table 5). Isolates also varied significantly ($P < 0.0001$), and an interaction between cultivar and isolate was detected (Table 5). The highest percent leaf area infection was observed for the susceptible check TAM 105, followed by Chisholm, Duster and Triupmh-64 (Table 6). OK-Rising, Pete, Jagger, OK-Field, OK-Bullet, Karl-92 produced significantly less disease, and thus, were resistant as compared to the resistant check Red Chief. The variance in disease reaction (percent leaf area infection) among wheat cultivars was positively correlated ($r = 0.53$; $P < 0.05$) with mean isolates virulence (Fig. 1). A positive correlation ($r = 0.76$; $P < 0.01$) was also observed between the disease reaction (percent leaf area infection) of wheat cultivars and the variance in virulence among isolates (Fig. 2). The lowly virulent isolates were excluded from this study, and thus, overall mean of isolate virulence from each decade across all eleven cultivars were not presented.

## DISCUSSION

Selection of isolates for gremplasm screening is critical because isolates can vary in virulence. The use of lowly virulent isolates would not be as beneficial as using highly virulent isolates because lowly virulent isolates cannot differentiate disease reaction (percent leaf area infection) in cultivars compared to highly virulent isolates (Krupinsky

1992). Thus, less virulent isolates may be excluded from further isolate X cultivar interaction studies where cultivar resistance is investigated. In this study, lowly virulent isolates OKD3, GYA3, El Reno and OK-06-2 were not included in the isolate X cultivar interaction (Table 6).

Disease severity, yield and TKW differed significantly ($P < 0.05$) between years (Table 3). Isolates also differed significantly to disease severity and grain yield, and a considerable yield loss was observed in this study (Table 3 and 4) although TKW was not affected by isolates (Table 4). Yield and TKW were lowered in 2009 as compared to 2008 due to freezing and hail events. However, these results indicate that tan spot can substantially damage wheat in Oklahoma.

Screening of wheat cultivars is routinely practiced to evaluate reaction to tan spot (Singh et al. 2006), and durable resistance to a wide variety of isolates is always sought by breeders in wheat improvement. Isolates representing a range of virulence should be used in screening wheat cultivars to provide a basis of stringent selection (Schilder and Bergstrom 1990). Carson (1987) suggested the resistance of wheat to tan spot would be stable when highly virulent isolates of *P. tritici-repentis* are used to inoculate diverse genotypes of wheat. In this study, cultivars were inoculated by isolates showing a range of virulence. Thus, we expect the resistance in OK-Rising, Pete, Jagger, OK-Bullet and OK-Field to tan spot should be durable to this pathogen in the field.

We did not study resistance in the field with all the isolates, and field conditions might affect isolate virulence or wheat resistance. Compared to a field study, testing of virulence in the greenhouse is convenient and also indicative of results obtained in a field study. Evans et al. (1999) observed a high similarity ($r$ = 0.75 to 0.93, $P < 0.05$) between greenhouse and field testing in identifying wheat resistant to tan spot. Raymond et al. (1985) reported a correlation of 0.91 between greenhouse and field study of cultivars reaction to tan spot. Thus, use of many isolates and cultivars in the greenhouse is quick, cost-effective and reliable. However, results of resistance screening in wheat improvement programs could change considerably, depending on the isolates used and field conditions (Schilder and Bergstrom 1990).

A significant ($P < 0.0001$) interaction between isolates and cultivars was observed (Table 1). This interaction was not expected because isolates should produce disease independently of the cultivars tested (Van der Plank 1982). However, significant interactions between isolates of *P. tritici-repentis* and wheat cultivars were also observed by Krupinsky (1992), Sah and Fehrmann (1992) and Schilder and Bergstrom (1990). Schilder and Bergstrom (1990) indicated that every leaf might not receive the same amount of inoculum, thus, difference in inoculum coverage may contribute to the variation. An interaction might also be significant if there is presence of physiological specificity of the isolates (races) (Krupinsky 1992, Van der Plank 1984). However, all the isolates in this study were race 1 (data not shown). Another possible explanation might be that we analyzed untransformed data. Typically this kind of experiment

produces high variance and data transformation usually is not useful (Sah and Fehrmann 1992, Schilder and Bergstrom 1990).

Evaluation of varietal resistance is most likely to be meaningful if diverse isolates of a pathogen are utilized (Raymundu et al. 1999). Inclusion of highly virulent isolates also is desirable because this enhances detection of increased genetic variance in cultivar response to tan spot (Carson 1987). We observed a positive correlation between the isolate variability in virulence and susceptibility of wheat cultivars (Fig. 1 and 2). Thus, the highly virulent isolates differentiated resistance in wheat cultivars better then lowly virulent isolates. Conversely, the most susceptible cultivars were able to differentiate isolate virulence better than resistant cultivars. This phenomenon was also observed by other researchers (Carson 1987, Shah and Fehrmann 1992, Shilder and Bergstrom 1990). Use of cultivars with a range of resistance can also efficiently determine virulence of an isolate. For example, mean virulence for OK-06-1 was high when tested on three cultivars, such as, Deliver, Red Chief and TAM 105 (Table 2), but its overall mean virulence was low when exposed to eleven cultivars (Table 6).

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Table 1. Analysis of variance for percent leaf area infection of sixteen isolates of *Pyrenophora tritici-repentis* on three wheat cultivars Deliver, TAM 105 and Red Chief under greenhouse study

| Source | DF | MS | *F* value | *P* value |
|---|---|---|---|---|
| Isolate | 15 | 2398.34 | 36.53 | <0.0001 |
| Cultivar | 2 | 20679.23 | 389.77 | <0.0001 |
| Isolate*Cultivar | 30 | 409.93 | 7.73 | <0.0001 |

Table 2. Virulence of sixteen isolates of *Pyrenophora tritici-repentis* on three wheat cultivars Deliver, TAM 105 and Red Chief under greenhouse inoculation

| | Percent leaf area infection (Mean ±SE)[a] | | | Decade |
|---|---|---|---|---|
| Isolates | Deliver | TAM 105 | Red Chief | mean |
| OKA1(1983)[c] | 46.8[b] ±5.3 B, a | 54.6±8.5 BCD, a | 4.0±0.3 CDE, b | |
| OKA2(1983) | 16.9 ±2.2 HI, a | 27.1±4.6 F, a | 6.3±0.3 BCDE, b | |
| OKD1(1983) | 27.9 ±2.3 EFG, b | 44.2±3.7 DE, a | 4.0±0.9 CDE, c | |
| OKD2(1983) | 17.9 ±1.5 GHI, b | 47.6±4.4 CDE, a | 12.2±2.3 ABCD,b | |
| OKD3(1983) | 11.3 ±1.6 I, ab | 14.8±1.9 G, a | 1.6±0.2 E, b | |
| OKD5(1983) | 19.8 ±1.5 GHI, a | 24.9± 4.9 FG, a | 2.9±0.4 DE, b | 21.4[d] |
| RBB6 (1996) | 23.8 ±3.7 EFGH, b | 53.5±2.4 BCD, a | 6.2±0.8 BCDE, c | |
| GYA3(1996) | 11.8 ±1.2 I, ab | 18.6±1.6 FG, a | 1.6±0.2 E, b | 19.3[e] |
| El Reno(2005) | 0.4 ±0.1 J, a | 0.7±0.1 H, a | 0.1±0.0 E, a | |
| Guymon(2006) | 34.1 ±3.5 DE, b | 58.5±12.2 B, a | 5.2±1.2 BCDE, c | |
| Cherokee(2006) | 70.0 ±2.0 A, a | 74.7±9.5 A, a | 21.4±1.9 A, b | |
| OK-06-1(2006) | 51.3 ±3.8 B, a | 57.6± 2.1 BC, a | 6.0±0.9 BCDE, b | |
| OK-06-2(2006) | 23.0 ±.1 FGH, b | 57.3± 3.4 BC, a | 1.8±0.3 DE, c | |
| OK-06-3(2006) | 39.0 ±4.2 CD, b | 53.4±6.5 BCD, a | 14.4±2.1 ABC, a | |
| Atoka (2007) | 30.5 ±2.3 DEF, a | 37.8±4.2 E, a | 15.0±1.4 AB, b | |
| Kiowa (2007) | 47.7 ±1.6 BC, b | 69.5±5.8 A, a | 21.4±1.5 A, c | 33.0[f] |
| Mean | 29.5 | 43.4 | 7.8 | |

[a] Means following the same letter in a column did not differ significantly by pairwise difference (PDIFF) in PROC MIXED option ($\alpha = 0.05$); SE = Standard error.
[b] Mean leaf area infection from the bottom 2nd and 3rd leaves after seven days of inoculation; each mean is the average of four replications.
[c] Year of collection in parenthesis.
[d,e,f] Mean isolate virulence from 1980s, 1990s and 2000s, respectively.

Table 3. Analysis of variance for percent leaf area infection (LAI), yield and thousand kernel weight (TKW) for isolates of *Pyrenophora tritici-repentis* on wheat cultivar Deliver in inoculated field plots in Stillwater, Oklahoma (2008 and 2009 combined)

| | | % LAI | | Yield | | TKW | |
|---|---|---|---|---|---|---|---|
| Source | DF | *F* | *P* > *F* | *F* | *P* > *F* | *F* | *P* > *F* |
| Year | 1 | 9.01 | 0.0077 | 172.55 | <0.0001 | 25.61 | <0.0001 |
| Rep*Year | 3 | 0.09 | 0.9656 | 3.44 | 0.0391 | 0.43 | 0.7345 |
| Isolate | 3 | 83.70 | <0.0001 | 12.38 | <0.0001 | 1.46 | 0.2591 |
| Year*Isolate | 3 | 6.51 | 0.0036 | 0.16 | 0.9203 | 1.38 | 0.2804 |

Table 4. Mean[a] (± standard error) disease severity, yield, yield reduction and thousand kernel weight (TKW) of wheat cultivar Deliver inoculated with three isolates of *Pyrenophora tritici-repentis* near Stillwater, Oklahoma (2008 and 2009 combined)

| Isolate | Disease severity[b] | Yield (bu/A) | Yield reduction (%) | TKW[d] |
|---|---|---|---|---|
| OKD1 (1983)[c] | 15.7 ± 1.6 c | 52.9 ± 5.0 a | 7.3 | 34.9 ± 1.0 a |
| RBB6 (1996) | 24.9 ± 1.1 b | 46.2 ± 4.8 b | 19.8 | 33.3 ± 0.7a |
| OK-06-1 (2006) | 31.1 ± 2.7a | 42.9 ± 4.9 b | 25.2 | 34.1 ± 0.9 a |
| Control | 4.6 ± 0.4 d | 56.7 ± 4.6 a | - | 34.1 ± 0.6 a |

[a] Means having same letter in a column did not differ significantly by Fisher's least significant difference test ($\alpha = 0.05$). Mean is the average of four replications.
[b] Disease severity measured as leaf area infection (%) from the average of third, second and first leaf below the flag leaf and the flag leaf.
[c] Year of collection in the parenthesis.
[d] Thousand kernel weight (gm).

Table 5. Analysis of variance for percent leaf area infection on eleven winter wheat cultivars inoculated by each of twelve isolates of *Pyrenophora tritici-repentis* under greenhouse study

| Source | DF | MS | *F* | *P* > *F* |
|---|---|---|---|---|
| Isolate | 11 | 472.31 | 13.31 | <0.0001 |
| Cultivar | 10 | 8785.16 | 247.61 | <0.0001 |
| Isolate*Cultivar | 110 | 269.72 | 7.60 | <0.0001 |

Table 6. Mean[a] percent leaf area infection on eleven winter wheat cultivars by twelve isolates of *Pyrenophora tritici-repentis* collected from heat field in Oklahoma

| | Wheat cultivars | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolate | Chisholm | Duster | Jagger | Karl-92 | OK-Bullet | OK-Field | OK-Rising | Pete | Red Chief | TAM-105 | Triumph-64 | Mean |
| OKA1 | 13.5[b] FG, bcd | 20.4 DE,b | 5.3 B,de | 13.8 ABC,bc | 5.5 CD,de | 5.3 A,de | 10.5 A,cde | 9.3 ABC,cde | 4.0 C,e | 54.6 BC,a | 13.5CD,bcd | 14.1 |
| OKA2 | 9.3 G,c | 36.8 B,a | 4.3 B,d | 17.3A,c | 5.8 CD,d | 5.3 A,d | 9.0 A,c | 9.8 AB,cd | 5.4 C,d | 27.2 F,b | 8.3 DE,d | 12.6 |
| OKD1 | 46.8 A,a | 7.0 FG,b | 9.3 B,b | 4.4 DE,b | 5.0 CD,b | 5.3 A,b | 1.8 A,b | 2.5 BC,b | 2.5 C,b | 52.1 BCD,a | 6.0 DE,b | 13.0 |
| OKD2 | 54.3 A,a | 10.8 FG,c | 21.5 A,b | 5.8 CDE,c | 8.0 CD,c | 7.0 A,c | 2.5 A,c | 2.8 BC,c | 7.8 C,c | 45.0 DE,a | 24.8 AB,b | 16.8 |
| OKD5 | 33.2 BC,b | 45.8 A,a | 3.8 B,de | 11.3 A-D,cd | 11.0 BC,cd | 15.5 A,c | 1.0 A,e | 4.8 BC,de | 2.3 C,e | 25.5 F,b | 7.0 DE,de | 14.6 |
| RBB6 | 19.8 DEF,b | 22.0 CDE,b | 2.3 B,c | 1.5 E,c | 4.8 CD,c | 7.5 A,c | 1.5 A,c | 1.4 BC,c | 4.8 C,c | 49.9 CDE,a | 24.9 AB,b | 12.8 |
| Cherokee | 15.3 EFG,cd | 27.8 CD,b | 1.6 B,f | 14.3 AB,cde | 16.4 B,cd | 9.1 A,df | 6.3 A,ef | 2.1 BC,f | 18.0 AB,c | 67.0 A,a | 2.9 E,f | 16.4 |
| Guymon | 21.7 DEF,b | 10.3 FG,c | 1.8 B,d | 2.9 E,cd | 2.8 CD,cd | 2.7 A,cd | 3.9 A,cd | 1.7 BC,d | 5.5 C,cd | 50.8 B-E,a | 6.1 DE,cd | 10.0 |
| OK-06-1 | 25.5 CD,b | 14.3 EF,c | 3.0 B,d | 2.0 E,d | 4.3 CD,d | 1.8 A,d | 1.7 A,d | 2.0 BC,d | 6.5 C,cd | 58.0 BC,a | 4.0 E,d | 11.2 |
| OK-06-3 | 15.6 EFG,bc | 6.2 FG,de | 3.5 B,de | 6.2 B-E,de | 7.3 CD,cde | 1.0 A,e | 10.0 A,cd | 1.8 BC,de | 8.8 C,cde | 53.4 BC,a | 20.0 BC,b | 12.1 |
| Atoka | 23.3 DE,b | 3.8 G,cd | 2.5B,cd | 2.5 E,cd | 1.0 D,d | 2.0 A,cd | 7.0 A,cd | 1.0 C,d | 10.3 BC,c | 43.3 E,a | 2.6 E,cd | 9.0 |
| Kiowa | 37.6 B,b | 29.1 BC,c | 1.6 B,f | 4.5 DE,f | 28.3 A,c | 5.7 A,ef | 1.1 A,f | 13.4 A,de | 19.3 A,d | 58.8 AB,a | 31.1 A,bc | 20.9 |
| Mean | 25.9 | 19.5 | 5.0 | 7.2 | 8.3 | 5.7 | 4.7 | 4.4 | 7.9 | 48.8 | 12.6 | - |

[a] Means having same letter within a column (upper case) and row (lower case) did not differ significantly by pairwise difference (PDIFF) in PROC MIXED option ($\alpha = 0.05$).
[b] Mean is the average of four replications.

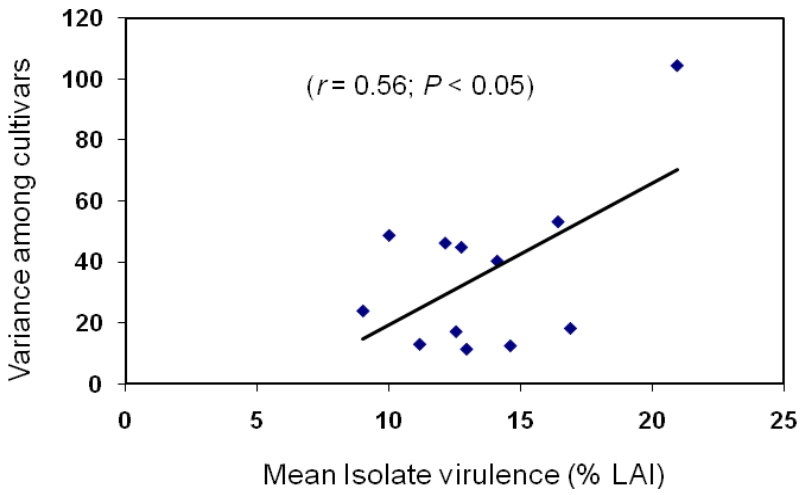


Fig. 1. Relationship between the mean virulence of twelve isolates of *Pyrenophora tritici-repentis* and variance in disease reaction among eleven wheat cultivars.

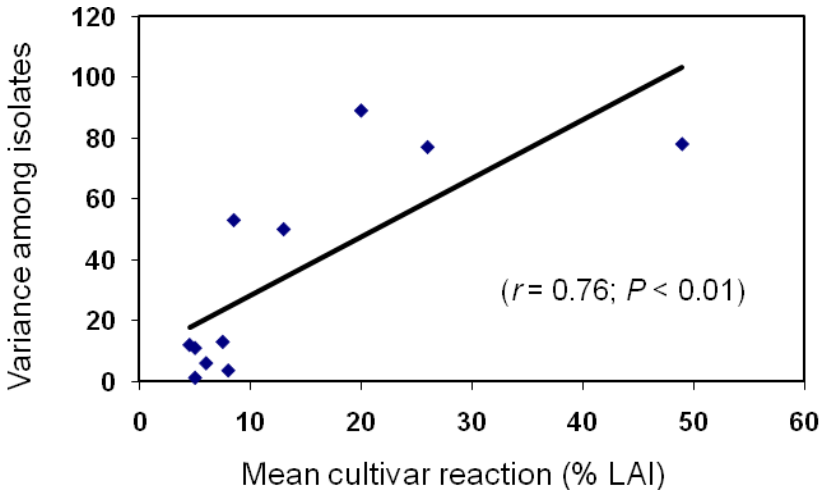


Fig. 2. Relatioship between the mean disease reaction of eleven wheat cultivars and the variance in virulence among twelve isolates of *Pyrenophora tritici-repentis*.

CHAPTER V

# RACE STRUCTURE, SYMPTOMATOLOGY, AND GENETIC RELATIOSHIP OF *PYRENOPHORA TRITICI-REPENTIS* ISOLATES COLLECTED OVER THREE DECADES FROM WINTER WHEAT IN OKLAHOMA


## ABSTRACT

In recent years, tan spot of wheat caused by the fungus *Pyrenophora tritici-repentis* has become more prevalent in Oklahoma and variability in this pathogen has made sustained management of tan spot difficult to attain. Experiments were conducted to investigate the race structure, disease symptoms and genetic relationship in *P. tritici-repentis* isolates from seventeen isolates collected from winter wheat over three decades. Races were determined based on the symptoms of necrosis and/or chlorosis produced on wheat differentials by using 16 isolates. Variability in symptom development for 12 isolates was tested on 13 wheat cultivars. Genetic relationship among 17 isolates was determined by amplified fragment length polymorphism- polymerase chain reaction (AFLP-PCR).

All isolates except one (El Reno), were classified as race 1. Isolates varied widely in producing necrosis and/or chlorosis symptoms on wheat cultivars, but necrosis with a chlorotic halo was predominate (56.4%). AFLP-PCR analysis using 13 primer pairs produced a total of 494 alleles of which 285 were polymorphic. The overall genetic diversity among the isolates was 25.2%. Genetic relationships based on cluster analysis and principal component analysis showed only minor differences between isolates, and isolates did not form tight clusters or groups. Based on the date of collection and location, no association could be detected among the isolates. The lack of distinct grouping indicates that the isolates of *P. tritici-repentis* used in this study are likely from a single lineage and did not vary genetically to a great extent over the past three decades. The results will facilitate selection of isolates for use in wheat resistance screening and other management practice.

## INTRODUCTION

Tan spot of wheat (*Triticum aestivum* L.) is caused by the necrotrophic ascomycetes fungus *Pyrenophora tritici-repentis* (anamorph: *Dreschlera tritici-repentis* (Died.) Shoemaker) and is a major disease in many wheat growing areas in the world (Francl et al. 1992, Weise 1987). A range of 3 to 50% yield loss in wheat has been observed for tan spot depending on the isolate virulence, cultivar susceptibility and the environmental conditions (Rees and Platz 1983, Shabeer and Bockus 1988). In Oklahoma in the southern Great Plains, wheat is grown annually on nearly six million acres, which typically ranks second across the nation (NASS 2009). For many years, tan spot was not a major problem in wheat in Oklahoma although its occurrence has been reported since

the 1970s (Hunger and Brown 1987). In recent years the presence of tan spot in Oklahoma has become more prevalent. In a field study in Stillwater, a 21% yield loss of wheat has been reported due to tan spot (Kader et al. 2009). Shifting cultivation practices from clean tillage to a no-till system, which leaves residue on the field surface, is favorable for the survival and seasonal carry over of this fungus (Baily 1996, Bockus and Classen 1992). Adee and Pfender (1989) found a positive correlation between the amount of infected residue in the field and tan spot severity. Moreover, genetic variability in *P.tritici-repentis* might increase as the fungus completes its life cycle every year by going through a sexual stage.

Variability of plant pathogens is a major problem in achieving disease control, and the knowledge of genetic variability is essential for developing sustainable management practices including development of resistant cultivars (Stakman 1957). Genetic variability is significant and common among isolates of *P.tritici-repentis* (De Wolf 1998). This fungus is able to induce two distinct types of symptoms (necrosis and/or chlorosis) on wheat leaves (Cuiffetti and Touri 1999, Lamari and Bernier 1989). Isolates of this fungus can also induce both necrosis and chlorosis symptoms on a single cultivar (Lamari and Bernier 1991) or induce different symptoms on different wheat cultivars (Moreno et al. 2008). Based on the necrosis and/or chlorosis symptoms on wheat differentials, Lamari and Bernier (1989) classified this fungus into eight races (Andrie et al. 2007) (Appendix 2). Race 1 is the most prevalent in the USA, although other races (race 2 and 3) are reported occasionally (Ali and Francl, 2003). Race 5 is reported in the northern part of USA and Canada (Ali and Francl 2003, Lamari et al. 1995).

Considerable genetic diversity in wheat is found in the 'center of origin and diversity' (the Fertile Crescent and Caucasus regions, Syria, Azerbaijan, Kazakhstan, Uzbekistan), and all the eight races have been reported in those areas (Lamari et al. 2003, Lamari et al. 2005, Stelkov and Lamari 2003).

Measuring the genetic relationship between pathogen isolates based on isolate morphology and virulence exclusively is often difficult to accomplish because complex pathogen-host-environment interactions can affect phenotypic (disease) development. Employment of molecular tools is useful in determining genetic variability in the population of a species at the genomic level (Williams et al. 1990). Researchers have used different molecular markers to study variability in *P. tritici-repentis* including rapid amplified polymorphic DNA (RAPD) (Mironenko et al. 2007, Peltonen et al. 1996, Santos et al. 2002, Singh and Hughes 2006), inter simple sequence repeats (ISSR) (Moreno et al. 2008), internal transcribed spacer (ITS) sequence analysis (Friesen et al. 2005, Stevens et al. 1998), and restriction fragment length polymorphism (RFLP) (Faris et al. 1997). Amplified fragment length polymorphism (AFLP) is a polymerase chain reaction (PCR)-based marker (Vos et al. 1996), which is highly reproducible and has been used to estimate genetic relationship in different species. AFLP has been employed to study the genetic relationship in other fungi and Oomycetes (Garzon et al. 2005, Hielmann et al. 2006, Majer et al. 1996, O'Neill et al. 1997, Serenius et al. 2007, Zhan et al. 2006). Friesen et al. (2005) used AFLP to study population genetic structure of a global collection of *P. tritici-repentis*, but did not find a grouping of the isolates based on geographic origin or races. To date, elucidation of the genetic relationships in *P. tritici-*

*repentis* isolates from Oklahoma is lacking. The objectives of this study were to investigate race(s), symptomatology and to reveal genetic relationship in *P. tritici-repentis* isolates collected over three decades from winter wheat in Oklahoma.

## MATERIALS AND METHODS

### Fungal isolates

A total of seventeen isolates of *P. tritici-repentis*, derived from single ascospores or single conidia, were collected in the 1980s, 1990s and 2000s from winter wheat in Oklahoma (Appendix 1). Isolates were stored in liquid nitrogen for long-term storage. When needed, isolates were removed from liquid nitrogen and were grown on potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 15 g agar in 1L water). After 3-4 days on PDA, a mycelial plug (5 mm diameter) was aseptically excised from the actively growing colony margin using a cork borer and transfer onto clarified V8 juice agar (CV8) (150 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 850 ml water). After growing on CV8 for 5 days in dark at 23$^{o}$C, mycelia were matted down, and plates were incubated in a 12/12 h light/dark cycle at 23$^{o}$C to induce conidial production. Agar plugs of 5 mm diameter removed from the conidial ring formed on CV8 were transferred into vials and were stored in liquid nitrogen for future use. Isolates were grown on PDA, and were maintained at 4$^{o}$C in the dark during the experiment.

### Inoculum preparation

Conidia were produced by each isolate following the procedure of Raymond et al. (1985). A 5-mm diameter mycelial plug, excised with a sterilized cork borer from the edge of an

actively growing isolate on fresh PDA, was removed and placed on clarified V8 (CV8) juice agar in 90 mm petri plates were maintained in an incubator (Percival model I-36LL, Boone, IA) at 23$^{o}$C in the dark for 5 days. About 10 drops of sterile water were added and mycelia were matted down using a sterile bent glass rod. Plates were then incubated for 12 hr at 23$^{o}$C with cool-white fluorescent tubes (40W, 30 $\mu Es^{-1}m^{-1}$) to produce conidiophores. This was followed by 12 hr dark at 16$^{o}$C to induce conidia production. Conidia were washed from the plate into a beaker using a stream of sterile water. Conidia were adjusted to 2000 per ml before inoculation.

**Race identification**

To determine race(s), the necrosis and/or chlorosis model on wheat differential was followed (Andrie et al. 2007) (Appendix 2). Differential wheat lines, namely 6B662, Glenlea, 6B365, and Salamouni were used (Ali and Francl, 2003). Four seeds of each wheat differential were planted in a plastic container (6 in. X 1.5 in.) filled with Ready-Earth soil (Sun Gro., Bellevue, WA). Seedlings with two leaves fully expanded were inoculated with the conidial suspension (2000 conidia/ml) of each isolate until incipient run-off using an atomizer (DeVilbiss Co. model 5610D, Somerset, PA) following the procedure of Rodriguez and Bockus (1996). Inoculated plants were allowed to dry for 30 min so conidia would adhere to leaves and then were placed in a mist chamber for 48 hr. Inoculated plants then were placed on a lab bench at 21-23$^{o}$C under metal halide lighting following a cycle of 14 hr light (510 $\mu Es^{-1}m^{-1}$) and 10 hr dark.

Seven days after inoculation, symptoms produced by each isolate on the wheat differentials were rated on a scale of 1-5 based on lesion type (Lamari et al. 1989), where 1 = small, dark brown to black spots without any surrounding chlorosis or tan necrosis (resistant), 2 = small, dark brown to black spots with very little chlorosis or tan necrosis (resistant), 3 = small dark brown to black spots completely surrounded by a distinct chlorotic or necrotic ring, lesions not coalescing (susceptible), 4 = spots completely surrounded by chlorotic or necrotic zones, lesion coalescing (susceptible), 5 = spots may not be distinguishable, lesions coalescing and spread over leaf (susceptible). One seedling was considered as one replication.

**Symptom variability**

To study variability in symptoms, 13 wheat cultivars and 12 isolates were used (Table 1). Seedlings of wheat cultivars were raised, and inoculum of each isolates was prepared as described earlier. At the three-leaf stage, four seedlings of each cultivar were inoculated with 2000 conidia/ml until incipient run-off and then maintained as described earlier. Disease symptoms (necrosis and/or chlorosis) were recorded 5 days after inoculation.

**DNA extraction**

Four day-old fungal cultures growing on PDA plates were kept at -20$^{o}$C for 2 hrs, and mycelia of each isolate were scraped from the PDA surface using a sterilized scalpel and placed into a mortar. Mycelia were ground into a fine powder using a mortar and pestle and liquid nitrogen. DNA was extracted from ~0.25 g mycelia powder of each isolate following the salt-extraction method of Aljanabi and Martinez (1997). DNA

concentration (ng/µl) was measured at 260/280 absorbance using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the purity of genomic DNA was examined after agarose gel (1%) electrophoresis of 3 µg DNA from each of the isolate and staining with 0.5 µg/ml ethidium bromide.

**ITS sequence**

Nuclear internal transcribed spacer (ITS) regions (ITS1, 5.8S rDNA and ITS2) were amplified by polymerase chain reaction (PCR) using ITS4 and ITS5 primers (White et al. 1990). A reaction included 25 µl 2X Gotaq Green core mix (Promega, San Luis, CA), 1 µl each of 10 mM 1X- ITS4 and ITS5 primer (IDT technologies, IA), 23 µl DNase free water and 0.5 µl purified DNA. Polymerase chain reaction (PCR) was performed in a PT200 thermocycler (MJ Research, Watertown, MA) using the following program: 95$^{o}$C for 3 min followed by 34 cycles of 94$^{o}$C for 30 sec, 55$^{o}$C for 45 sec, and 72$^{o}$C for 1 min, and a final extension at 72$^{o}$C for 7 min. Electrophoresis was performed in a 1% agarose gel with 1X sodium borate running buffer (pH 8.0), and DNA stained with ethidium bromide and visualized under UV light. Amplified products were purified using Purelink$^{TM}$ PCR Purification Kit (Invitrogen, Carlsbad, CA). ITS region from each isolate was sequenced at OSU Core Facility, and the sequences were compared with *P. tritici-repentis* GenBank accessions in the NCBI database (ncbi.nih.gov) using the BLAST search.

**AFLP analysis**

AFLP analysis was performed by using an AFLP$^{TM}$ Microbial Fingerprinting kit (Applied Biosystems, Foster city, CA). Genomic DNA (120 ng) was digested by *EcoR*1 and *Mse*1 enzymes (Invitrogen, Carlsbad, CA) and adaptors were ligated by incubating at 37$^{o}$C for 2 hr. The digested and adaptor-ligated templates were diluted 1:19 in 1X TE buffer (pH 8.0) and 4 µl of the diluted DNA sample mixed with 16 µl of pre-selective amplification master mixture (0.5 µl of each *EcoR*1 and *Mse*1 pre-selective primer plus 15 µl core mix). The samples were pre-amplified in a PCR thermocycler (Nyx Technik Inc., San Diego, CA) using 24 cycles of 94$^{o}$C for 20 s, 56$^{o}$C for 30 s, and 72$^{o}$C for 2 min. Amplified pre-selective templates were again diluted 1:19 by 1X TE buffer (pH 8.0), and 3 µl of this diluted pre-selective PCR product was mixed with 17 µl of selective amplification master mixture [1 µl of each fluorescent-labeled *EcoR*1-NN/NNN and unlabelled *Mse*1-N/NNN primer (Table 2) plus 15 µl of core mix]. PCR conditions were of 33 cycles of which 20 cycles of 94$^{o}$C for 20 s, 56$^{o}$C for 30 s and 72$^{o}$C for 2 min; the first 10 cycles started with an annealing temperature of 66$^{o}$C with 1$^{o}$C decreased in each subsequent cycle; and finally a 60$^{o}$C for 30 m hold. The amplified products were separated by capillary electrophoresis by an ABI 3730 DNA Analyzer (Applied Biosystems, Foster city, CA). Rox 500 (Applied Biosystems) was used as the internal standard size. The chromatograms were converted into a binary data matrix as presence (1) or absence (0) of an allele using GeneMapper 4.0 (Applied Biosystems) calibrated against 75-500 bp DNA size standards.

**AFLP data analysis**

Genetic diversity (DI) for each primer combination was determined using the software POPGENE (version 1.32, University of Alberta, Edmonton, Canada) as DI= 1- $\sum P^2 i$, where $P^2 i$ is the frequency of *i*th allele at a locus in a population (Nei 1978). NTSYSpc (version 2.0, Exeter Biological software, Setauket, NY) (Rolf 1993) was used to determine pairwise Jaccard similarity coefficient between isolates (Sneath and Sokal 1973), S*ij*= a/(a+b+c), where a is the number of alleles present in both isolates (*ij*), b is the number of allele present in *i* but not in *j*, c is the number of allele present in *j* but not in *i*. A cluster analysis was performed based on the Jaccard similarity coefficient and an unweighted pair group method with arithmetic average (UPGMA) by using the sequential, agglomerative, hierarchical, nested clustering (SAHN) module of the NTSYSpc. The data were bootstrapped 1000 times to determine robustness of the dendogram by WINBOOT program (Yap and Nelson 1996). In order to highlight the resolution power of the coordination, a principal component analysis (PCA) was carried out using the PROC PRINCOMP option of SAS 9.2 (SAS Institute, Cary, NC) to generate a two-dimensional presentation.

RESULTS

All isolates except one (El Reno) were identified as race 1 based on the symptoms produced on the wheat differential set (1-5 scale; 1-2: resistant, 3-5 susceptible) (Table 1). No disease symptoms other than dark brown to black spots were induced by El Reno, which therefore was considered as race 4.

Isolates varied widely in symptoms induced on wheat cultivars (Table 2). All twelve isolates were pathogenic and produced necrosis with a yellow halo (N), necrosis with extended chlorosis (NC) or only chlorosis extended over the leaf (C) on all thirteen wheat cultivars. Over the combination of 12 cultivars and 13 isolates, necrosis was the dominant symptom (56.41%) followed by necrosis with extended chlorosis (32.05%) and chlorosis (11.54%).

Results from the AFLP analysis are presented in Table 3. Using 13 primer combinations all 17 isolates of *P. tritici-repentis* yielded a total of 494 alleles of which 285 were polymorphic. On an average each primer combination produced 38 alleles of which ~22 were polymorphic. The *EcoR*1-AA and *Mse*1-C combination gave the highest number of alleles of 70 while the *EcoR*1-AT and *Mse*1-CAG combination produced the fewest number of alleles at 16. The highest number of polymorphic alleles (44 out of 70) was detected by the *EcoR*1-AA and *Mse*1-C combination. The percentage of polymorphism ranged from 26.42% to 100%, with an average of 57.69%. An overall genetic diversity among the isolates was 0.252, with a range of variation from 0.104 to 0.394 for the primer combinations.

The dendrogram generated by cluster analyses revealed genetic divergence among the isolates, but did not show any distinct obvious grouping based on the date of collection or race (Fig. 1). Similarity coefficients among isolates ranged over 70-82%. A maximum similarity (82%) was found between OKD3 and El Reno. Bootstrap analysis gave low percentage values (11.7 to 66.7%) of the grouping, indicating that these grouping are not

highly reliable, and all isolates are basically belong to a single group (Fig. 1). All the isolates formed one large group by the principal component analysis (PCA) (Fig. 2). In PCA, the first, second and third PC explained 37.3%, 8.02% and 6.45% of the total variability, respectively.

## DISCUSSION

Resistance to tan spot in breeding programs and management strategies to control this disease should be developed against the most prevalent races. Predominant races that occur in an area are a major challenge to limiting losses due to this disease. So, knowing the races present in the field would help to determine control strategies such as selection of fungicide, crop rotation or the use of cultivar mixtures. In this study, 15 out of 16 isolates were race 1, and one was race 4 (Table 1). Race 1 was also found to be the predominant race in the southern Great Plains by Ali and Francl (2003). In a study of a global collection of *P. tritici-repentis* populations, five isolates were included from Oklahoma, and all of them were race 1 (Friesen et al. 2005). Race 1 is considered more virulent than other races due to the presence of two toxin genes (necrosis and chlorosis). Another reason explaining the predominance of race 1 might be the lack of selection pressure on this pathogen due to the monoculture of wheat with a narrow genetic base with most cultivars being susceptible to tan spot (Ali and Francl 2003). One isolate (El Reno) was race 4. Ali and Francl (2003) tested 22 isolates collected from wheat in southern Great Plains, of which 21 isolates were race 1 except one isolate of race 4. Race 4 has been reported to be most typically isolated from alternative non-cereal grasses (Ali and Francl 2003). It is suspected that the race El Reno might have infected wheat from a

non-cereal host, and likely is non-persistent because it would not compete well with more virulent races.

Presence of races of a pathogen in a specific area can have a practical impact on disease control. A highly host-selective pathogen might survive on other related plant species, but probably would not cause severe disease. In contrast, a non-specialized pathogen has a broad host range and can be equally pathogenic to many hosts. Based on the knowledge of host specialization and cultivation of wheat, the presence of a particular race of *P. tritici-repentis* can also be predicted. Thus, a non-host crop rotation effectively controls a host-specialized pathogen. For example, a crop rotation with canola or sorghum between wheat seasons would more effectively eliminate *P. tritici-repentis* than would rotation with an alternative crop such as barley. Due to the lack of a susceptible host (wheat) the fungus would not survive and complete its life cycle (Bockus and Classen 1992).

Isolates showed different symptoms on the same cultivar, indicating that there is considerable variation in symptom production by the isolates (Table 2). Conversely, one isolate did not induce the same symptoms on every cultivar. This indicates that a cultivar has its own genetic and physiological affect. This agrees with Moreno et al. (2008), who reported that *P. tritici-repentis* isolates induced different symptoms on a particular wheat cultivar and an isolate induced more than one symptom on different cultivars.

Diversity value by the analysis of DNA polymorphism is useful to measure genetic diversity within a species. In a study with *Xanthomonas oryzae* pv. *oryzicola*, the bacterial leaf streak pathogen to rice, Raymundo et al. (1999) found a diversity value of 0.93 reflecting a high genetic diversity in the organism. Our AFLP data demonstrated only slight genetic diversity among the isolates with a diversity value of 0.252. This finding is consistent with other marker studies of *P. tritici-repentis* fungal populations where many primer combinations revealed a genetic diversity value of 0.20 or less (Friesen et al. 2005). Using AFLP, Leisova et al. (2008) found little genetic diversity (0.096) among 100 isolates of *P. tritici-repentis* in the Czech Republic collected during 1998-2005. The reasons for genetic diversity in *P. tritici-repentis* could be regular sexual recombination or vegetative compatibility among isolates (Moreno et al. 2008, Singh and Hughes 2006); however, as *P. tritici-repentis* is homothallic, sexual recombination should contribute only minimally to genetic variation.

In cluster analysis, high similarity coefficient (90% or greater) and a higher percentage of consistency value of the bootstrap analysis determine the stability of grouping among the isolates within a population (Chen et al. 1995, Felsenstein 1985, Yap and Nelson 1996). Felsenstein (1985) suggested that groups only with bootstrap consistency values of 95% or greater would be considered stable in a cluster analysis. Lower bootstrap consistency values (11.7 to 66.7%) in our study suggest that the apparent grouping is not stable, and basically all isolates belong to a single group (Figure 1). However, our data suggest the presence of some genetic diversity in this pathogen, and thus, deploying of resistance to

virulent isolates in wheat should be helpful because diverse isolates would breakdown resistance at a faster rate (Raymodo et al. 1999).

We used AFLP as a molecular marker. AFLP has been shown to produce the maximum number of alleles (Jurgenson et al. 2002). When compared to other markers used in population genetics studies, AFLP performed the best for estimating genetic relationships (Garcia et al. 2004, Powel et, al. 1996). In principle component analysis (PCA), the original variable (in this case alleles) was transformed to new variables or principal components (PCs), which represent the combination of the original data (Hielmann et al. 2006). One of the useful properties of PCs is the lack of correlation, because they are sorted according to the variability they explain. The first PC is the linear combination of the original variable that accounts for the greatest amount of variation; thus, the second PC accounts for the next greatest amount of variation, and so on (Hielmann et al. 2006).

Despite little genetic diversity, cluster analysis and PCA revealed that the isolates of *P. tritici-repentis* used in this study are homogeneous, and therefore, are derived from a common lineage. According to PCA, a high PC value distinguishes the isolates into distinct groups (Gril et al. 2008, Mello, et al. 2008, Priyatmojo et al. 2002), whereas a low PC value fails to sort isolates into groups and the isolates fit around the center of co-ordination (Fernando et al. 2006). In our study, PC1 explained the highest variability (37.3%) of the total variation and put all the isolates in one group (Fig 2, A and B), which confirms that the isolates in this study are genetically similar. In a genetic diversity study from 100 isolates of *P. tritici-repentis* using AFLP analysis, Leisova et al. (2008) found that a high PC value fit isolates into one group while a low PC value failed to group

isolates. In our study PC2 and PC3 accounted for a proportion of the total variance of only 8.02% and 6.45%, respectively. Further, isolates clustered around the center of coordination, suggesting that isolates in this collection could not be characterized into further grouping (Fig. 2C). Fernando et al. (2006) studied genetic relationship among 60 isolates of *Gibberella zeae*, which causes Fusarium head blight in cereals. Although *G. zeae* isolates were collected within 200 miles and the isolates exhibited a high genetic diversity, low values in PCs put all the isolates around the center of ordination suggesting the population is derived from a single lineage. Using AFLP markers, a global collection of *P. tritici-repentis* isolates also did not show any genetic grouping based on geographic origin or races (Friesen et al. 2005).

Host specialization might play some role in determining genetic relationships. In a study with *Monilinia laxa*, the causal agent of apple brown rot, isolates collected from apple trees did not group in different clusters, while isolates collected from peach, nectarine, plum and apricot did group into separate clusters (Gril et al. 2008). In our study, all isolates were collected from Oklahoma and from the same host (wheat). Another reason only a single cluster may have been observed with our isolates is that conidia of *P. tritici-repentis* are windborne and can readily mix together in the field. Hence, it is not possible for an isolate to maintain an isolated pocket of high similarity in Oklahoma.

A complete understanding of variability in the population of *P. tritici-repentis* is critical to resistance screening, and to developing management practices such as the selection of fungicides. For example, representative isolates from different groups or lineages, if any,

should be included in the screening of cultivars to deploy a wider resistance to tan spot. Regular monitoring and virulence testing of this pathogen would benefit tan spot management of wheat in Oklahoma.

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Table 1. Reaction of isolates on wheat differential and identification of races in *Pyrenophora tritici-repentis* collected from 1980s, 1990s and 2000s in Oklahoma

| | Wheat differential | | | | |
|---|---|---|---|---|---|
| Isolates | 6B662 | Glenlea | 6B365 | Salamouni | Race[b] |
| **1980s** | | | | | |
| OKA1 | 1R[a] | 4N | 5C | 1R | Race 1 |
| OKA2 | 1R | 3N | 5C | 1R | Race 1 |
| OKD1 | 2R | 5N | 5C | 1R | Race 1 |
| OKD2 | 2R | 4N | 5C | 1R | Race 1 |
| OKD3 | 1R | 4N | 5C | 1R | Race 1 |
| OKD5 | 1R | 3N | 3C | 1R | Race 1 |
| **1990s** | | | | | |
| RBB6 | 1R | 5N | 5C | 1R | Race 1 |
| GYA3 | 1R | 3N | 4C | 1R | Race 1 |
| **2000s** | | | | | |
| El Reno | 1R | 1R | 1R | 1R | Race 4 |
| Guymon | 1R | 5N | 5C | 1R | Race 1 |
| Cherokee | 2R | 4N | 5C | 1R | Race 1 |
| OK-06-1 | 1R | 4N | 5C | 2R | Race 1 |
| OK-06-2 | 1R | 5N | 5C | 2R | Race 1 |
| OK-06-3 | 1R | 5N | 5C | 1R | Race 1 |
| Atoka | 2R | 5N | 5C | 2R | Race 1 |
| Kiowa | 1R | 4N | 5C | 1R | Race 1 |

[a] Lesion type on a 1-5 scale, where 1= small, dark brown to black spots without any surrounding chlorosis or tan necrosis (resistant), 2= small, dark brown to black spots with very little chlorosis or tan necrosis (resistant), 3= small dark brown to black spots completely surrounded by a distinct chlorotic or necrotic ring, lesions not coalescing (susceptible), 4= spots completely surrounded by chlorotic or necrotic zones, lesion coalescing (susceptible), 5= spots may not be distinguishable, lesions coalescing and spread over leaf (susceptible) (Lamari et al. 1989); R= resistant, N= necrosis, C= chlorosis.

[b] Races identified based on symptoms on leaf as necrosis and/or chlorosis (Andrie et al. 2007).

Table 2. Different symptoms produced by thirteen wheat cultivar in response to twelve isolates of *Pyrenophora tritici-repentis* collected from Oklahoma winter wheat fields

| Sl. No. | Isolates | Wheat cultivars | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Billings | Chisholm | Deliver | Duster | Endurance | Jagger | Karl-92 | OK-Bullet | OK-Field | OK-Rising | Pete | TAM105 | Triumph-64 |
| 1 | OKA1 | NC | N | N | N | C | N | N | N | N | N | N | NC | N |
| 2 | OKA2 | NC | N | N | N | NC | N | N | N | N | N | N | N | N |
| 3 | OKD1 | NC | N | NC | N | C | N | N | N | N | N | N | NC | N |
| 4 | OKD2 | NC | NC | NC | N | C | N | N | N | N | N | N | N | N |
| 5 | OKD5 | C | N | NC | N | C | N | NC | N | N | N | N | NC | N |
| 6 | RBB6 | C | N | NC | NC | C | NC | NC | NC | NC | N | N | NC | NC |
| 7 | Cherokee | C | N | NC | NC | C | N | NC | N | NC | NC | N | N | NC |
| 8 | Guymon | C | NC | NC | NC | C | N | NC | N | N | NC | N | NC | N |
| 9 | OK-06-1 | C | NC | NC | N | C | N | NC | N | N | N | N | NC | NC |
| 10 | OK-06-3 | C | N | NC | NC | C | NC | N | N | N | N | N | NC | NC |
| 11 | Atoka | NC | N | NC | N | C | N | N | N | N | NC | N | NC | N |
| 12 | Kiowa | C | N | NC | N | C | N | N | N | N | N | N | NC | NC |

[N] Necrosis with yellow hallow, [C] Chlorosis extended over leaf, [NC] Both necrosis and chlorosis, usually necrosis with little extended chlorosis. Cultivars Billing and Endurance produced mainly extended chlorosis with little necrosis.

Table 3. Genetic variability in seventeen isolates of *Pyrenophora tritici-repentis* following amplified fragment length polymorphism (AFLP) analysis using thirteen primer combinations

| *EcoR*1- | *Mse*1- | Total allele | Polymorphic allele | Percent polymorphism | Genetic diversity |
|---|---|---|---|---|---|
| AA | C | 70 | 44 | 62.86 | 0.204 |
| AA | CA | 53 | 14 | 26.42 | 0.104 |
| AA | CG | 57 | 32 | 56.14 | 0.196 |
| AA | GC | 24 | 23 | 95.83 | 0.394 |
| AC | CA | 30 | 15 | 50.00 | 0.158 |
| AG | C | 57 | 31 | 54.39 | 0.233 |
| AG | GA | 21 | 19 | 90.48 | 0.374 |
| AT | C | 51 | 25 | 49.02 | 0.193 |
| AT | CA | 20 | 12 | 60.00 | 0.256 |
| AT | CAC | 19 | 19 | 100.00 | 0.397 |
| AT | CAG | 16 | 16 | 100.00 | 0.397 |
| TA | C | 27 | 12 | 44.44 | 0.188 |
| TG | C | 49 | 23 | 46.94 | 0.183 |
| Total | | 494 | 285 | - | - |
| Average | | 38 | 21.92 | 57.69 | 0.252 |

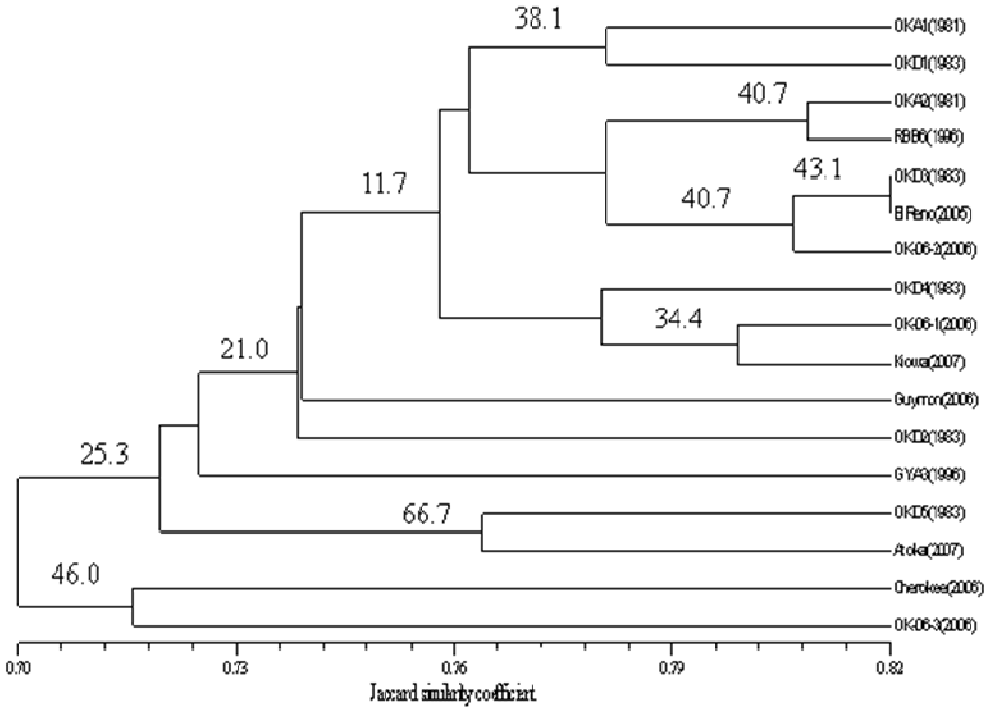
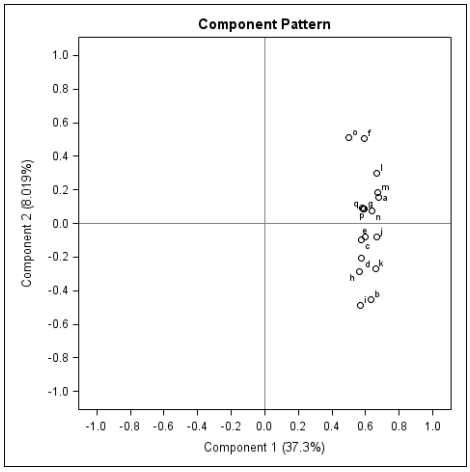
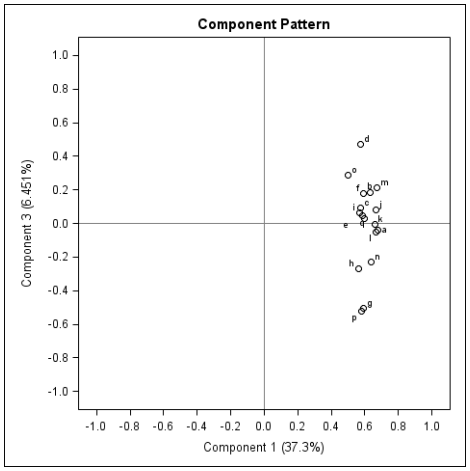


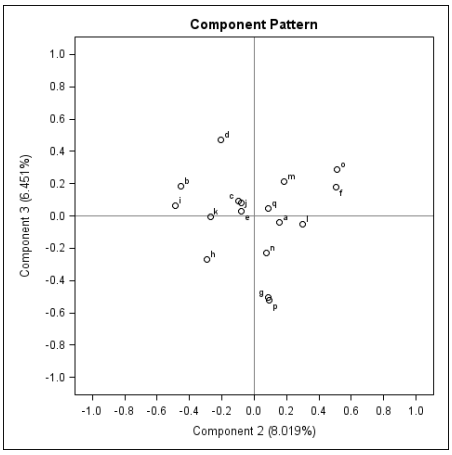

Fig. 1. Cluster analysis based on 494 alleles obtained from amplified fragment length polymorphism (AFLP) showing the relationship among seventeen isolates of *Pyrenophora tritici-repentis* collected from winter wheat in Oklahoma over three decades. Cluster analysis was performed based on unweighted pair group method with arithmetic average (UPGMA). Values in the fork show the percentage of times the grouping was consistent from a 1000 replicated bootstrap analysis (Yap and Nelson 1996).




A




B




C

Fig. 2. Principal component analysis based on amplified fragment length polymorphism (AFLP) for seventeen isolates of *Pyrenophora tritici-repentis* collected from winter wheat in Oklahoma. A higher PC value grouped the isolates in one group (A and B), a lower PC value did not differentiate isolates into group (C).

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# APPENDICES

Appendix 1. *Pyrenophora tritici-repentis* isolates collected from winter wheat fields in Oklahoma that were used in this study

| Isolate | Collection year | County | Propagule of initial isolation |
|---|---|---|---|
| OKA1 | 1983 | Garfield | Ascospore |
| OKA2 | 1983 | Garfield | Ascospore |
| OKD1 | 1983 | Blaine | Ascospore |
| OKD2 | 1983 | Blaine | Ascospore |
| OKD3 | 1983 | Blaine | Ascospore |
| OKD4 | 1983 | Blaine | Ascospore |
| OKD5 | 1983 | Blaine | Ascospore |
| RBB6 | 1996 | Kay | Ascospore |
| GYA3 | 1996 | Texas | Ascospore |
| El Reno | 2005 | Canadian | Conidia |
| Guymon | 2006 | Texas | Conidia |
| Cherokee | 2006 | Cherokee | Conidia |
| OK-06-1 | 2006 | Payne | Ascospore |
| OK-06-2 | 2006 | Payne | Ascospore |
| OK-06-3 | 2006 | Payne | Ascospore |
| Atoka | 2007 | Atoka | Conidia |
| Kiowa | 2007 | Pittsburg | Conidia |

Appendix 2. Races of the fungus *Pyrenophora tritici-repentis*, the causal organism of tan spot in wheat (Andrie et al. 2007)

| Races | Wheat differentials | | | |
|---|---|---|---|---|
| | 6B662 | Glenlea | 6B365 | Salamouni |
| 1 | Resistant | Necrosis | Chlorosis | Resistant |
| 2 | Resistant | Necrosis | Resistant | Resistant |
| 3 | Resistant | Resistant | Chlorosis | Resistant |
| 4 | Resistant | Resistant | Resistant | Resistant |
| 5 | Chlorosis | Resistant | Resistant | Resistant |
| 6 | Chlorosis | Resistant | Chlorosis | Resistant |
| 7 | Chlorosis | Necrosis | Resistant | Resistant |
| 8 | Chlorosis | Necrosis | Chlorosis | Resistant |

VITA

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Dissertation: FITNESS, VIRULENCE AND GENETIC VARIABILITY IN *PYRENOPHORA TRITICI-REPENTIS* ISOLATES CAUSING TAN SPOT OF WHEAT IN OKLAHOMA

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Scope and Method of Study: Isolates of *Pyrenophora tritici-repentis* (causal fungus of tan spot of wheat) collected from the 1980s, 1990s and 2000s were studied for their fitness, virulence and genetic relationship. Mycelial growth was quantified on agar media, and sporulation was quantified on agar and on the three wheat cultivars Deliver, TAM 105 and Red Chief. Wheat straw of cv Deliver was infested by each isolate, and total pseudothecia were determined after 14 days of incubation. Maturity of pseudothecia was determined after 23 days of incubation at 15$^{o}$C. Virulence of isolates was determined by inoculating wheat cultivars with conidia. Three isolates, OKD1, RBB6 and OK-06-1, collected in 1983, 1996 and 2006, respectively, were used in the field study to determine disease severity, yield loss and thousand kernel weight on wheat cv Deliver in 2007-08 and 2008-09. In the greenhouse, the reaction of 11 wheat cultivars was tested to 12 isolates. Race structure and symptom variability was determined by the reaction of wheat cultivars to *P. tritici-repentis* isolates. Amplified fragment length polymorphism (AFLP) was performed to determined genetic relationship among the isolates.

Findings and Conclusions: Recent isolates collected in the 2000s were more fit for growth, sporulation, pseudothecia formation and maturity than isolates collected in the 1980s and 1990s. The recent isolates also were more virulent than the older isolates. Isolate OK-06-1 (2006) produced significantly more tan spot on wheat in both the years in the field compared to the older isolates OKD1 (1983) and RBB6 (1996). A 25% yield loss was observed for the isolate OK-06-1. Among cultivars, OK-Rising, OK-Field, OK-Bullet and Pete were as resistant as the resistant check Red Chief. All isolates were race 1 except El Reno, which was race 4. Isolates also exhibited variability in symptoms induced of which 56, 32, and 12% were necrosis, necrosis and chlorosis, and chlorosis only, respectively. AFLP studies revealed that the *P. tritici-repentis* population in Oklahoma did not change significantly over the last three decades and the isolates were likely from a single lineage.

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