WATER POTENTIAL INTERACTION WITH HOST AND PATHOGEN AND DEVELOPMENT OF A MULTIPLEX PCR FOR *SCLEROTINIA* SPECIES

By

AHMED ABD-ELMAGID

Bachelor of Science in Agriculture
Assiut University
Assiut, Egypt
1999

Master of Science in Plant Pathology
Assiut University
Assiut, Egypt
2003

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2012

WATER POTENTIAL INTERACTION WITH HOST AND PATHOGEN AND DEVELOPMENT OF A MULTIPLEX PCR FOR SCLEROTINIA SPECIES

Dissertation Approved:

Dr. Hassan Melouk

Dissertation Adviser

Dr. Robert Hunger

Dr. Carla Garzon

Dr. Mark Payton

Outside Committee Member

Dr. Sheryl A. Tucker

Dean of the Graduate College

.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

Chapter II

Chapter III

LIST OF FIGURES

Figure Page

Chapter I

Figure Page

Chapter III

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Water, fungi and plants

Plant growth can only be optimal when there is no environmental stress. Plants are always being exposed to one or more stress factors as conditions in the environment are probably never optimal. Moderate stress usually causes a reversible growth inhibtion, whereas severe or extreme stress usually causes an irreversibl injury to plants (Levitt, 1978). According to Cook and Duniway (1980), water relation in the biology of plant pathogens has two major aspects: 1) the role of water potential in the development of plant diseases, especially diseases caused by soil-borne plant pathogens, and 2) the role of water potential in the growth, reproduction, and survival of pathogens in the soil and in crop debris.

Water potential is a fundamental concept widely used in the biological and soil sciences for quantifying the energy of water in plants, microorganisms, soils, and other related

systems (Papendick and Campbell, 1981). Water potential is an abbreviated expression for the “potential energy of water” (Papendick and Mulla, 1986). By definition, water potential is the potential energy of water relative to pure water (i.e. deionized water) in reference conditions. It explains the tendency of water to move from one area to another due to its components of osmosis, gravity, mechanical pressure, and matric potentials. Water potential is measured in units of pressure and is commonly represented by the Greek letter Ψ (Psi).

Pure water at standard temperature and pressure (or other suitable reference condition) is defined as having a water potential of zero. The addition of solutes to water lowers its potential (makes it more negative). Water moves from an area of higher water potential to an area of lower water potential (Papendick and Mulla, 1986). Fungi as a part of any thermodynamic system tend to achieve water potential equilibrium with the surrounding environment. Water flows spontaneously from high to low potentials (from low negative to more negative) and the availability of water for physiological processes decreases as the potential is lowered (Papendick and Mulla, 1986). High to low potentials (from low negative to more negative) and the availability of water for physiological processes decreases as the potential is lowered (Papendick and Mulla, 1986).

Total water potential in plants is the sum of four main components: $\Psi = \Psi\pi + \Psi m + \Psi p + \Psi g$,where $\Psi\pi$ (also sometimes indicated as Ψs), Ψm, Ψp and Ψg are the osmotic, matric, pressure (turgor) and gravitional potentials, respectively (Cook and Duniway 1980). Inside intact plant cells, the osmotic and matric components are always negative meaning that work is expended in moving water fom the cell to reference state, while the turgor pressure is always positive or zero (Papendick and Mulla, 1986). Inside intact cells, the components of water potential are the osmotic potential due to solutes, a small matric

component arising from liquid-solid interfaces, and a positive (or sometimes zero) turgor potential due to the presence of semipermeable membranes and the cell wall. Gravitational potential is only taken in consideration in the case of water movement in tall trees. It is usually omitted in the case of agricultral plants where the elevation distances across cell walls and membranes are minute for water exchange (Papendick and Mulla, 1986).

In fungi, cells readjust their water potential to approach equilibrium with any change that happens in the surrounding environment through biochemical and biophysical mechanisms (Harris, 1981). Turgor pressure is responsible in various ways for the control of cell growth when external water potential is changed from an optimum to a stressful condition (Luard and Griffin, 1981). Fungi keep their turgor potential positive through osmoregulation via an effect on membrane permeability to solutes and ions (Luard, 1982a), or via the effect on the electrical properities of the cell membrane (Zimmerman, 1978). Luard and Griffin, (1981) stated that when the external water potential to various fungi is lowered by addition of solutes such as sucrose or glucose, or salts such as NaCl and KCl, the internal osmotic potential is continuously lowered.They also mentioned that internal osmotic potential levels are always lower (more negative) than the external osmotic potential by an amount of -1 to -4 MPa to maintain water flow into the cells from the surrounding environment. The principal ions which appear to be involved in osmoregulation include Na^+ and K^+, with charge balance being maintained by the movement of Cl^- , or solutes like proline, sucrose, glycerol and mannitol (Papendick and Mulla, 1986).

The great majority of plant pathogenic fungi have at some stage in their life established an intimate relationship with plant tissue where water has a critical role in the relationship (Eamus and Jennings, 1986). Water affects metabolic processes, and it is

essential for the transport of nutrients within and outside fungal mycelium and plants. Free moisture is required for growth and development of fungal pathogens, so variations in water availability before, during, and after infection affect disease development. Osmotic potential has been identified as an important parameter in the ecology and growth of plant fungi (Cervantes-Garcia *et al*, 2003). Soil-borne fungi show variable metabolic responses, growth patterns and reproductive strategies in response to variable soil water potential conditions.

The term "predisposition" is always used to refer to the interaction between plant diseases and water stress. Predisposition has been defined by Schoeneweiss (1975), as an effect on the "disposition" or "proneness" of the host plant to be attacked by a pathogen. Water availability can alter the reaction of a plant to be attacked by a pathogen, which usually is an increase of susceptibility to the pathogen (Kramer and Boyer, 1995). Leaphart and Stage (1971) concluded that extended drought during the period from 1916 to 1940 in the United States was the main reason for the origin and severity of pole blight of western white pine because the pathogen was favored by drought and reduced the root regeneration capacity of the host.

The influence of soil moisture on development of root rot diseases is often reflected by its effect on the ecology of soil fungi. Long periods of drought or relatively low water available to roots may predispose plants to non-aggressive pathogens, which usually cannot infect plants under normal conditions. Moore *et al*, (1963), showed that water stress predisposed Kentucky bluegrass to *Sclerotinia homoeocarpa*, and highland bentgrass to *Pythium ultimum*. Also, Ma *et al*, (2001) reported that drought stress was the major predisposing factor of pistachio to infection by *Botryosphaeria dothidea*. Some pathogens such as *Fusarium spp*. are tolerant of a wider range of water stress than are their host plants.

The correlation between comparative dry soil and the occurrence of seedling blight caused by *Fusarium spp*., has long been recognized, and low water levels in soil has been shown to increase the severity of Fusarium diseases of mature wheat plants (Schoeneweiss, 1975).

Few studies have investigated the possible effects of water potential on sclerotia development. In nature, sclerotia are produced either on diseased host tissues or on plant debris, where both osmotic potential and matric potential may have an influence. The limited data on the effects of water potential on sclerotia forming species indicate that their requirements differ little from those of fungi at large and that, as might be expected, sclerotia are produced over a narrower range of water potential than will support hyphal extension. However, details of the manner in which water availability may affect the size and number, and hence disease-inciting capacity, of sclerotia remains to be determined (Cook and Al-Hamdani, 1981).

Sclerotinia blight of peanut

Sclerotinia blight of peanut (*Arachis hypogaea*) is caused by the soilborne fungi *Sclerotinia minor* and *S. sclerotiorum*. The first report of this disease in the United States was in Virginia in 1971 (Kokalis-Burelle *et al*, 1997). Sclerotinia blight has become widespread in Virginia and North Carolinia, and in parts of Oklahoma and Texas (Smith *et al*, 2006). In general, peanut cultivars are susceptible to Sclerotinia blight, but some moderately resistant Virginia-type cultivars are available. The partially resistant cultivar Virginia 93B was registered in 1994 (Smith *et al*, 2006), followed by the partially resistant cultivars VA 98R and Perry (Smith *et al*, 2006). Various factors may be responsible for the partial resistance observed in these cultivars. Physiological and enviromental factors play an important role in

the interaction between pathogens and their hosts, and hence, can either strengthen or weaken the resistance (Royle and Butler, 1986).

Sclerotinia minor

S. minor Jagger, is a soil-borne plant pathogenic fungus that is known to infect and cause economic losses in a wide range of plant hosts including the cultivated peanut (Kokalis-Burelle *et al*, 1997). *S. minor* causes disease known by a number of names depending on the host, which include Sclerotinia blight, cottony rot, white mold, stem rot, and crown rot (Agrios, 2005). *S. minor* survives mainly by producing sclerotia. The sclerotia have a black outer rind, are irregularly shaped, and are approximately 0.5-3.0 mm (Agrios, 2005). Apothecia are rarely seen in nature, so infection occurs primarily through eruptive germination of sclerotia that gives rise to white and fluffy mycelia that come in contact with stems and pegs of peanut. *S. minor* generally infects the lower branches of the peanut plant when viable sclerotia are present on the surface of the soil, but infection of upper roots is also possible from buried sclerotia (Agrios, 2005). One infection path of infection involves colonization of pegs at the soil line and subsequently growth to the lateral branches and other parts of the peanut plant (Porter and Beute, 1974). Stem infections are often the most economically important because pegs are directly attached to the stem, which allows quick colonization of the reproductive parts of the plant (Chappell *et al*., 1995). Infected areas are quickly covered with white, fluffy mycelia, eventually producing tan colored, water-soaked lessions with discrete demarcation present between infected and uninfected tissue. The tissue above the lesion often wilts and dies quickly after infection (Agrios, 2005). These lesions progress to a dark brown color. Stem tissue becomes heavily shredded, and collapses. When

plants are heavily infected, pods are geneally rotted, and healthy pods are often left behind in the soil during digging due to weakening of pegs (Porter and Beute, 1974).

Sclerotinia Sclerotiorum

Sclerotinia blight also is caused by *Sclerotinia sclerotiorum* (Lib.) de Bary. *S. sclerotiorum* is a necrotrophic pathogen, which causes high levels of crop loss on more than 400 species of plants from a wide range of taxonomic groups worldwide (Bolton *et al*., 2006). Porter and Beute (1974) were the first to note peanut as a host for *S. sclerotiorum* in Virginia, with Wadsworth (1979) later noting presence of the pathogen on peanut in Oklahoma. *S. sclerotiorum* produces sclerotia as survival structures and spreads from field to field by internally infected seeds and sclerotia mixed with seed (Grau, 1988). Few reports exist that describe *S. sclerotiorum* causing disease in peanut. In other crops, during a growing season, sclerotia in infested fields germinate by producing mycelia arising from the sclerotia or by airborne ascospores produced in apothecia. Ascospores are the primary inoculum for epidemics in many crops (Boland and Hall, 1987). This rarely occurs in peanut (Phipps and Porter, 1982). The infection initiates near the soil line where plant tissues are in contact with the soil, indicating that infections are likely from direct sclerotial germination producing a mycelium (Phipps and Porter, 1982). After a mycelium is produced in the senescent tissue, the *S. sclerotiorum* infection can progress to succulent tissue, where it produces symptoms similar to those produced by *S. minor.*

Impact of water potential on *S. minor* and *S. sclerotiorum*

Many factors affect survival and germination of sclerotia of the two species in the field (Wu *et al*, 2008). Some of these factors are constant soil temperature (35 ºC for 3 weeks or more that reduced survival of sclerotia) (Adams, 1987), sclerotial position and duration in

soil (Abawi and Grogan, 1979), sclerotial shape (Huang and Kozub, 1994), soil gases or chemicals (Imolehin et al, 1980), activities of other microorganisms (Abawi and Grogan, 1979) and nutrition (Burgess and Hepworth, 1996). Temperature and moisture are commonly mentioned as significant factors affecting development of diseases caused by species of *Sclerotinia spp.* (Willets and Wong, 1980).Viability of sclerotia also declines rapidly over time at high soil water potential (Abawi and Grogan, 1979). Moore, (1949) stated that almost 100% of the sclerotia of *S. sclerotiorum* were totally decayed when soil was flooded with water for 24 to 45 days. In general sclerotia of *S. minor* survived better in dry soil than in moist soils, and better in shallow rather than at a deeper depth in soil where higher moisture usually exists (Imolehin *et al*, 1980).

Lower soil water potential in lettuce fields increases survival, and sclerotia only survive short periods in saturated soils at 0 MPa (Hao *et al*, 2003). Sclerotial viability decreased at soil water potential ≥ - 0.02 MPa when the soil temperature increased from 15 to 40 ºC. No sclerotia were viable after 2 weeks at 40 ºC, but the viability of sclerotia of both species remained relatively high in dry soil (Matheron and Porchas, 2005). Sclerotia of *S. minor* can germinate directly at soil moisture levels between -0.03 and -1.5 MPa (Imolehin *et al*, 1980), while sclerotia of *S. sclerotiorum* germinate between 0 and -0.6 MPa (Duniway *et al*, 1977). Most of the research on *S. minor and S. sclerotiorum* was done on lettuce drop disease using *S. minor* and *S. sclerotiorum* lettuce isolates under environmental factors significantly differ from those found in peanut producing areas. Imolehin *et al* (1980) investigated the effects of temperature and moisture tension on growth, sclerotial production, germination and infection of lettuce by *S. minor*.

They found that optimum radial growth occurred on the basal medium with osmotic potential of -1.2 MPa but at -10 MPa they did not get any growth. They got an average of 140 sclerotia production at -0.1 MPa. When they decreased the osmotic potential from -0.1 to -2.4 MPa, sclerotial production increased from 140 to 236. Further decreases in osmotic potential resulted in decreased sclerotial production and none were produced at osmotic potential from -6.4 to -10 MPa. For germination, sclerotia produced over the range of -0.1 to – 4.35 MPa did not differ significantly in ability to germinate eruptively when moistened (29-32%) (Imolehin *et al*, 1980). Matheron and Porchas (2005) studied the influence of soil temperature and moisture on eruptive germination and viability of sclerotia of *S. minor* and *S. sclerotiorum* under conditions found in a lettuce field. They found that soil moisture has a significant effect on germination of the sclerotia of *S. minor* and *S. sclerotiorum*. They observed that sclerotia within wet soil (≥ 0.02 MPa) maintained for 4 weeks at 40 C did not germinate when placed on potato dextrose agar. Sclerotia maintained within dry soil (≤ - 100 MPa) for 4 weeks at 40 ºC germinated when plated on PDA. No work has been done before to investigate the effect of water potential on the sclerotial production and pathogenicity fitness of peanut isolates of *S. minor* and *S. sclerotiorum*.

In order to develop an effective integrated disease management program for Sclerotinia blight of peanut, we first need to understand the factors that affect the biology of the host and the fungus. Research on soil water potential and its effects on *S. minor* and *S. sclerotiorum* needs to be investigated more in depth to determine the true environmental influences on the behavior of both species. Understanding these conditions is crucial to better control the fungus.

Tan spot of wheat

Tan spot or yellow leaf spot is an economically important fungal foliar disease of wheat. It occurs worldwide in most major wheat growing areas. Tan spot was detected in the United States in New York in 1940 and in Kansas in 1947 (Watkins *et al*, 1978). By the end of the 1970's tan spot was detected and became a major disease on wheat in Oklahoma and the southern plains of the United States (Hunger and Brown, 1987). In Canada, the first serious outbreak occurred in 1974 (Ciuffetti and Tuori, 1999).

Symptoms and yield losses

Symptoms of tan spot appear on leaf surfaces during the fall and spring in winter wheat. Two distinct symptoms are usually seen, tan necrosis and chlorosis (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). Toxins produced by the pathogen are responsible for these two symptoms (Engle *et al*., 2006). 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 (Weise 1987). 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 (Hosford, 1982). During grain filling, the fungus can infect wheat seeds and cause a reddish discoloration (Schilder and Bergstorm, 1994). A lower thousand-kernel weight, reduced number of grain per head, shriveling and discoloration of seeds and reduced milling quality have been reported due to tan spot (Bockus and Classen, 1992).

Pyrenophora tritici-repentis

Pyrenophora tritici-repentis (Died.) Drechs. (synonym *P. trichostoma* (Fr.) Fuckel), anamorph *Drechslera tritici-repentis* (Died.) Shoemaker (Synonym *Helminthosporium tritici-repentis* Died.), is a homothallic ascomycete that is the causal agent of tan spot on bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*). This fungus produces a multi-nucleated (haploid) mycelium with cross walls (Zillinsky 1983). It produces sexual and asexual spores. The sexual spores or the perfect stage are called ascospores. They form within asci, which are formed when the female sex cell, called an ascogonium, is fertilized by either an antheridium or a minute male sex spore called spermatium. The fertilized ascogonium produces one to many ascogenous hyphae, the cells of which contain two nuclei, one male and one female. The cell at the tip of each ascogenous hyphae develops into an ascus, in which the two nuclei fuse to produce a zygote, which then undergoes meiosis to produce four haploid nuclei. The cell containing these nuclei elongates, and all four nuclei, like in most Ascomycetes, undergo mitosis and produce eight haploid nuclei. Eventually, each nucleus is surrounded by a portion of the cytoplasm and is enveloped by a wall to become an ascospore. Usually, there are eight ascospores in each ascus. Asci are formed directly in cavities within a stroma or matrix of mycelium, which is called a pseudothecium or an ascostroma is black with double walls. Pseudothecia are 0.2 to 0.35 mm in diameter with dark spines surrounding the short beaks (Zillinsky 1983).

Ascospores are brown with three transverse septa and are oval to globose (Ciuffetti and Tuori, 1999). The asexual spores, (anamorph, or the imperfect stage) are called conidia and are born on septate conidiophores measuring 80 to 400 x 6 to 9 μm with a swollen base. The conidia are subhyaline, cylindrical, four to seven septa and 80 to 250 x 14 to 20 μm in size. On potato dextrose agar (PDA), pathogen growth is dense, fluffy, greenish-grey

mycelium without sporulation (Schilder and Bergstrom, 1993). When grown on V8 juice agar, the mycelium is white to light grey. To produce conidia, cultures on V8 juice agar are exposed to UV light for 12 to 24 hours, followed by 12 to 24 hours of darkness (Schilder and Bergstrom, 1993). The fungus survives through summer, fall and winter primarily as pseudothecia on wheat straw and residue on the soil. Wheat straw and residue are considered to be the main source of primary inoculum in areas of intensive wheat production in North America (Rees and Platz, 1980).

Ascospores are generally thought to be the primary source of incolum. They are discharged from pseudothecia under humid conditions at night early in the spring and infect the lower leaves. Secondary infection on upper leaves is caused by conidia and this infection is directly related to yield losses (Rees and Platz, 1983). McMullen and Hosford (1989) stated that fungal conidial spores germinate and infect leaves over a wide range of temperatures when leaves are wet. Severe spots usually occur on susceptible varieties when leaves are wet for 12 hours, but 18 to 24 hours may be needed on more resistant varieties.

Resistance to tan spot is partly affected by temperature and nitrogen availability (Duveiller and Dubin, 2002). Spores of fungi usually have a low level of respiration and metabolic activity. The presence of substrates such a cereal residues or other nutrients results in a transformation of spores to an active phase characterized by adsorption of water, increase in respiration and biosynthesis of cell components (Magan, 1988). Morphological changes including germ tube formation and elongation occur and ultimately an active vegetative mycelium is formed. This process is influenced by stress imposed by water availability. Spores of fungal species able to overcome such stress would have maximum ecological advantage, resulting in preferential colonization and exploitation of substrata

(Magan, 1988). The effect of water potential on the maturation of pseudothecia on wheat straw has not been investigated before. In soil and in cereal crop residues matric potential is the major component of the total water potential (Griffin, 1981). The matric potential affects growth of soil fungi and maturation of fungal spores on residues more than osmotic potential (Griffin, 1981).

No work has been done before to investigate the effect of osmotic and matric potential on the maturation and viability of the pseudothecia of *P. tritici-repentis*. Also, the effect of water potential on mycelial growth, conidia formation, and germination on artificial media *in vitro*, has seldom been considered. Better understanding of the interaction between abiotic factors and conidia sporulation, germination and pseudothecia maturation is important to developing improved control programs.

Multiplex PCR

Sclerotinia species are destructive and cosmopolitan plant pathogens that cause stem and crown rot on various agronomic and horticultural crops and wild species (Andrew and Kohn, 2009). *Sclerotinia* spp. belongs to the *Sclerotiniaceae*, which is an important family of the class Asco-mycotina (Willetts and Wong, 1980). The distribution of these fungi is cosmopolitan but they are most common in temperate regions (Reichert, 1958). Two hundred forty-six species of *Sclerotinia* have been reported (Andrew and Kohn, 2009). The main species of phythopathological interest in the genus *Sclerotinia* are *S. sclerotiorum* (Lib.) de Bary, *S. minor* Jagger, *S. trifoliorum* Erikss., *Sclerotinia homoeocarpa F.T. Bennett (*Bennett, 1937) and the undescribed species *Sclerotinia* species 1 (Winton *et al*. 2006).

Sclerotinia sclerotiorum is a destructive and cosmopolitan plant pathogen that causes white mold and watery soft-rot diseases in a wide variety of agricultural, ornamental, and

wild plants in the families Solanaceae, Cruciferae, Compositae, Chenopodiaceae, and Leguminosae; but under favorable environmental conditions the fungus will probably infect many more (Boland & Hall, 1994). *S. minor* has a similar but somewhat narrower host range to that of *S. sclerotiorum* (Willetts and Wong, 1980). *S. minor* infects very important crops such as lettuce, sunflower, spinach, tomato, pepper, or peanut (Melzer *et al*. 1997). *S. trifoliorum* was reported to cause crown and stem rot of forage legumes such as alfalfa (*Medicago sativa* L.), red clover (*Trifoliorum pratense* L.), and white clover (*Trifoliorum repens* L.), as well as several other legumes (Njambere *et al*. 2010). Recently *S. trifoliorum* was reported to cause severe losses on chickpea (*Cicer arientinum* L.) (Njambere *et al*., 2010). Some early articles reported *S. trifoliorum* on sunflowers, lettuces, beans and tomatoes (Brooks, 1953), and on cauliflowers (Henderson, 1962).

Diseases caused by the three species *are* generally known as "white molds" (Abawi and Grogan, 1979).White molds are easily identified by the characteristic white cottony mycelia that grow on the surfaces of infected tissues (Abawi and Grogan, 1979). *S. homeocarpa* causes dollar spot of turfgrass (Smiley *et al*. 1993). *S. homeocarpa* is a major pathogen of turfgrass worldwide and causes tremendous annual losses in the U.S.A. (Smiley *et al*., 1993). Currently, the dollar spot pathogen is classified as *Sclerotinia homoeocarpa*. However, this classification is under revision, and once completed, the fungus may be reclassified as *Lanzia*, *Moellerodiscus*, or *Rutstroemia*. Reasons for the suggested reclassification of the fungus are *S. homoeocarpa* does not form sclerotia which is a characteristic of *Sclerotinia* spp.; apothecial morphology of *S. homoeocarpa* differs from that of other *Sclerotinia* spp.; electrophoretic protein patterns and ribosomal DNA of *S. homoeocarpa* are similar to those of *Lanzia*, *Moellerodiscus*, and *Rutstroemia* (Rotter *et al.*

2009). However, *S. homeocarpa* still listed under Sclerotiniaceae in (www.mycobank.org) and (http://www.indexfungorum.org/Index.htm).

Sclerotinia species produce apothecia and sclerotia but lack an obvious conidial stage (Bardin and Huang, 2001).

The main criteria used to distinguish between these species have been size and general characteristics of the sclerotia, host range, and dimensions of ascospores and asci (Bardin and Huang, 2001). Identification studies on fungi are often complicated by the relatively few stable characters available for comparison. Multigenic involvement and responses to environment may lead to variability within each character and result in overlap between species (Cruickshank, 1983). No differences in the structure of hyphae have been reported between *S. sclerotiorum*, *S. trifoliorum*, and *S. minor* (Willets and Wong, 1980). It is not always accurate or rapid to separate *S. sclerotiorum*, *S. trifoliorum*, *S. minor* and *S. homeocarpa* into distinct species based on traditional morphological traits such as cultural characteristics, sclerotial size, ascus and ascospore dimensions, time of apothecial development in the field, host association and disease symptoms. *S. sclerotiorum* produces large smooth sclerotia, *S. trifoliorum* produces large irregular sclerotia, while *S. minor* produces smaller rough sclerotia (Morrall *et al*. 1972). There is an overlap in sclerotia size and shape, and under certain circumstances this character is not reliable for identification (Willets and Wong, 1980). Reports in the literature suggest that mycelial characteristics of theses fungal species do not show distinctive differences and are of only limited use for identification purposes (Willets and Wong, 1980). Analyses based on sequences of the 18S rDNA or the ITS region, common methods for the identification of filamentous fungi and yeasts (Freeman *et al.* 2002), revealed that the Sclerotiniaceae have almost identical

sequences and are not useful for species identification. Other gene loci, e.g. the glyceraldehyde-3-phosphate dehydrogenase, heat-shock protein 60, or DNA-dependent RNA polymerase subunit II have been used for identification instead (Staats *et al*. 2004). Hirschhäuser and Fröhlich (2007) reported novel laccase 2 (lcc2) sequences localized in the genome among different Sclerotiniaceae for a fast and novel detection and identification of *S. sclerotiorum* and *S. minor*. They suggested that this gene could be used for the identification of more members of the *Sclerotiniaceae*.

PCR-based methods have been developed to detect *S. sclerotiorum* (Yin *et al*. 2009; Rogers *et al*. 2009; Freeman *et al*. 2002). Also, Njambere *et al.* (2010) developed microsatellite markers for *S. trifoliorum*. A multiplex PCR was developed by Hirschhauser & Frohlich, (2007) to discriminate some fungal members of the *Sclerotiniaceae* but *S. trifoliorum* or *S. homeocarpa* were not included in their study. A single nucleotide polymorphism (SNPs) was developed by (Andrew and Khon, 2009) to identify *S. sclerotiorum*, *S. minor, S. trifoliorum* and the undescribed species *Sclerotinia* species 1. Their protocol, however, is time consuming if compared to multiplex PCR and it requires costly equipments. Methods for use in diagnostics and detection of plant pathogens need to be quick, simple, reliable, and cost effective. In spite of the importance of the agriculture associated species of *Sclerotinia spp,* there is no rapid and accurate procedure for routine detection of these pathogens. Rather than perform individual PCR amplification reactions for each region or locus, it is often desirable to amplify all sequences of interest simultaneously in a "multiplex" reaction. Multiplex PCR also offers a significant time and cost saving advantage. Another benefit of multiplex PCR is that only a single aliquot of DNA or RNA is required rather than an aliquote for each marker to be analyzed. One of the first multiplex

PCR systems was designed for the detection of mutations in the dystropphin gene (Chamberlain *et al*., 1988). Nine PCR products were amplified simultaneously and analyzed by gel electrophoresis (Chamberlain *et al*., 1988). Developing a sensitive multiplex PCR for the detection of the most common four species of the genus *Sclerotinia* is crucial for accurate and fast diagnostics.

LITERATURE CITED

1- Abwai, G. S. and Grogan, R. G. 1979. Epidemiology of diseases caused by *Sclerotinia* species. *Phytopathology* 69: 899-904.

2- Adams, P. B. 1987. Effect of soil tempertaure moisture, and depth on survival and activity of *Sclerotinia minor*, *Sclerotium cepivorum*, and *Sporidesmium sclerotivorum*. *Plant Disease* 71: 170-174.

3- Agrios, G. N. 2005. Sclerotinia diseases pages 546-550, in *Plant Pathology* 5th Edition. Academic Press, San Diego, CA.

4- Akkasaeng, C., Tantisuwichwong, N., Chairam, I., Prakrongrak, N., Jogloy, S. and Pathanothai, A. 2007. Isolation and identification of peanut leaf proteins regulated by water stress. *Pakistan Journal of Biological Sciences* 10 (10): 1611-1617.

5- Altschul, S. F, Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W.,and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

6- Andrew, M. and Kohn, L. M. 2009. Single Nucleotide Polymorphism-Based Diagnostic System for Crop-Associated *Sclerotinia* Species. *Applied and Environmental* microbiology 5600-606.

7- Bardin, S. D. and Huang, H.C. 2001. Research on biology and control of Sclerotinia diseases in Canada. *Canadian Journal of Plant Pathology* 23, 88-98.

8- Barr, H. D. and Weatherely, P. E. 1962. A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Australian Journal of Biological Science* 15: 413-428.

9- Bennett, F.T. 1937. Dollar spot of turf and its causal organism *Sclerotinia homoeocarpa* N.SP. *Annals of Applied Biology* 24, 236-257.

10- Bockus, W. W. and Classen, M. M. 1992. Effect of crop rotation and residue management practices on severity of tan spot of winter wheat. *Plant Disease* 76: 633-636.

11- Boland, G. J. and Hall, R. 1987. Epidemiology of white mold of white bean in Ontario. *Canadian. Journal of. Plant Pathology* 9: 218-224.

12- Boland, G.J. and Hall, R. 1994. Index of plant hosts of Sclerotinia sclerotiorum. *Canadian Journal of Plant Pathology* 16, 93-108.

13- Brooks, F.T. 1953. Plant Diseases. Geoffrey Cumberlege (ed.). Oxford University Press, London and New York. 457 pp.

14- Burgess, D. R. and Hepworth, G. 1996. Biocontrol of Sclerotinia stem rot (*Sclerotinia minor*) in sunflower by seed treatment with *Gliocladium virens*. *Plant Pathology* 45: 583-592.

15- Campbell, G. S. and Gardner, W. H. 1971. Psychrometric measurement of soil water potential: temperature and bulk density effects. *Soil Science Society of America proceedings* 35: 8-12.

16- Cervantes-Garcia, D., Padilla-Ramirez, S. J., Simpson, J. and Mayek-Perez, N. 2003. Osmotic potential effects on *in vitro* growth, morphology and pathogenicity of *Macrophomina phaseolina*. *Journal of Phytopathology* 151, 456-462.

17- Chamberlain, J. S., Gibbs, R. A., Rainer, j. E., Nguyen, P. N. and Caskey, C. T. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex PCRs. In *The Polymerase Chain Reaction* (K. b. Mullis, F. Ferre, and R. A. Gibbs, Eds.). Birkhäuser, Boston.

18- Chapell, G. F., Shew, B.B., Ferguson, J. M. and Beute, M. K. 1995. Mechanisms of resistance to *Sclerotinia minor* in selected peanut genotypes. *Crop Science* 35: 692-696.

19- Ciuffetti, L. M. and Tuori, R. P. 1999. Advances in the characterization of the *Pyrenophora tritici-repentis* wheat interaction. *Phytopathology* 89: 444-449.

20- Coley-Smith, J. R. and Cooke, R. C. 1971. Survival and germination of fungal sclerotia. *Annual Review of Phytopathology* 9: 65-92.

21- Cook, R. C. and Al-Hamdani, A. M. 1981. Water relations of sclerotia and other infective structures. In: Ayres PG, Boddy L (eds), *Water, Fungi and plants*. Cambridge University Press, Cambridge, pp. 49-63.

22- Cook, R. J. and Duniway, J. M. 1980. Water relations in the life cycles of soilborne plant pathogens. In: Parr JF, Gardner WR, Elliot LF (eds), *Water Potential Relations in Soil Microbiology*. American Society of Agronomy, Wisconsin, pp. 119-139.

23- Cote, M-J, Tardif, M-C. and Meldrum, A. J. 2004. Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa*, and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Disease* 88, 1219–1225.

24- Cruickshank, R.H. 1983. Distinction between *Sclerotinia* species by their pectic zymograms. *Transactions of the British Mycological Society* 80 (1), 117-19.

25- Dallyn, H.and Fox, A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould, G. H., Corry, E. L., eds. *Microbial growth and survival in xtreme environments*. London and New York: Academic Press. P 129-139.

26- Duniway, J. M., Abawi, G. S. and Steadman, J. R. 1977. Influence of soil moisture on the production of apothecia by *Whetzelinia sclerotiorum*. (Abstr.) *Proceedings of American Phytopathological Society* 4:115.

27- Duveiller, E. and Dubin, H. J. Helminthosporium leaf blights: spot blotch and tan spot. 2002. In Bread wheat, improvement and production (B.C. Curtis, S. Rajaram and H. Gómez Macpherson, eds). *FAO Plant Production and Protection Series* 30, 1-6.

28- Eamus, D. and Jennings, D. H. 1986.Water, turgor and osmotic potentials in fungi. In: Ayres PG, Boddy L (eds), *Water, Fungi and plants*. Cambridge University Press, Cambridge, pp. 27-48.

29- Engle, J. S., Madden, L. V. and Lipps, P. E. 2006. Distribution and pathogenic characterization of *Pyrenophora tritici-repentis* and *Stagonospora nodorum* in Ohio. *Phytopathology* 96: 1355-1362.

30- Fan, S. and Blake, T. J. 1997. Comparison of polyethylene glycol 3350 induced osmotic stress and soil drying for drought simulation in three woody species. *Trees* 11: 342-348.

31- Faske, T., Melouk, H. A. and Payton, M. E. 2006. Comparison of *Sclerotinia minor* inocula for differentiating the reaction of peanut genotypes to Sclerotinia blight. *Peanut Science* 33:7-11.

32- Freeman, J. Ward, E. Calderon, C. and McCartney, A. 2002. A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. *European Journal of Plant Pathology* 108, 877–886.

33- Gottlieb, D. (1978). *The germination of fungus spores*. Durham, U.K.: Meadowfield Press, PP. 1-166.

34- Graf. F. and Schumacher T. 1995. *Sclerotinia glacialis* sp. nov., from the alpine zone of Switzerland. *Mycological Research* 99, 113–17.

35- Grau, C. R. 1988. Sclerotinia stem rot of soybean. Pages 55-66 in: *Soybean Diseases of the North Centeral Region*. T. D. Wyllie and D. H. Scott, eds. The American Phytopathological Society, St. Paul, MN.

36- Griffin, D. M. 1981. Water and microbial stress. In *Advances in Microbial Ecology* (ed. M. Alexander). PP. 91-136. Plenum Publishing Co.

37- Hao, J. J., Subbarao, K. V. and Duniway, J. M. 2003. Germination of *Sclerotinia minor* and *S. sclerotiorum* sclerotia under various soil moisture and temperature combinations. *Phytopathology* 93: 443-450.

38- Harris, R. F. 1981. Effect of water potential on microbial growth and activity. In: Parr JF, Gardner WR, Elliot LF (eds), *Water Potential Relations in Soil Microbiology*. American Society of Agronomy, Wisconsin, pp. 23-95.

39- Henderson, R. M. 1962. Some aspects of the life cycle of the plant pathogen *Sclerotinia sclerotiorum* in Western Australia. *Journal Proceedings of the Royal Society* 45, 133-35.

40- Hirschhäuser, S. and Fröhlich, J. 2007. Multiplex PCR for species discrimination of Sclerotiniaceae by novel laccase introns. *International Journal of Food Microbiology* 118, 151–157.

41- Hosford, R. M. 1982 Tan Spot. Pages 1-24 in: *Proc. Tan Spot Wheat disease Workshop*. R. M. Hosford ed. N. D. Agric. Exp. Stn, Fargo.

42- Huang, H. C. and Kozub, G. C. 1994. Longevity of normal and abnormal sclerotia of *Sclerotinia sclerotiorum*. *Plant Disease* 78: 1164-166.

43- Hunger, R. M. and Brown, D. A.1987. Colony color, growth, sporulation, fungicide sensitivity, and pathogenicity of *Pyrenophora tritici-repentis*. *Plant Disease* 71: 907-910.

44- Imolehin, E. D., Grogan, R. G. and Duniway, J. M. 1980. Effect of temperature and moisture on growth, sclerotial production, germination and infection by *Sclerotinia minor*. *Phytopathology* 70: 1153-1157.

45- Kohn, L. M. 1979a. A monographic revision of the genus *Sclerotinia*. *Mycotaxon* 9:365–444.

46- Kohn, L.M. 1979b. Delimitation of the economically important plant pathogenic *Sclerotinia* species. *Phytopathology* 69, 881-86.

47- Kokalis-Burelle, N., Porter, D. M., Rodríguez-Kabana, R., Smith, D. H. and Subrahmanyam, P. 1997. Sclerotinia Blight. Pages 34-36 in: *Compendium of Peanut Diseases*. Second edition. American Phytopathological Society.

48- Kramer, P. J. and Boyer, J. S. 1995. *Water relations of plants and soils*. Academic Press Inc., New York (482 pp).

49- Lamari, L. and Bernier, C. C. 1989. Evaluation of wheat lines and cultivars to tan spot (*Pyrenophora tritici-repentis*) based on type lessions. *Canadian Journal of Plant Pathology* 11, 49-56.

50- Lamari, L. and Bernier, C. C. 1991. Genetics of tan necrosis and extensive chlorosis in tan spot wheat caused by *Pyrenophora tritici-repentis*. *Phytopathology* 81:1092-1095.

51- Leaphart, C. D. and Stage, A. R. 1971. Climate: a factor in the origin of the pole blight disease of *Pinus monticola* Dougl. *Ecology* 52: 229-239.

52- Levitt, J. 1978. An overview of freezing injury and survival and its interrelationships to other stresses. *In* PH Li, A Sakai, eds, *Plant Cold Hardiness and freezing stress*, Academic Press, New York.

53- Liddell, C. M. 1993. Measurement and control of soil temperature and water potential. In: Singleton, L. L., Mihail, J. D., Rush, C. M, eds. *Methods for research on soilborne phytopathogenic fungi*. Eds,. The American Phytopathological society, St. Paul, Minnesota.

54- Luard, E. J. and Griifin, D. M. 1981. Effect of water potential on fungal growth and turgor. *Transactions of the British Mycological Society* 76, 33-40.

55- Luard , E. J. 1982*a.* Accumulation of interacellular solutes by two filamentous fungi in response to growth at low steady-state osmotic potential. *Journal of General Microbiology* 128, 2563-74.

56- Luard , E. J. 1982b. Effect of osmotic shock on some intracellularsolutes in two filamentous fungi. Journal of General Microbiology. 128, 2575-81. In Ayres PG, Boddy L (eds), *Water, Fungi and plants*. Cambridge University Press, Cambridge, pp. 1-25.

57- Ma, Z., Boehm, E.W.A., Luo, Y. and Michailides, T. J. 2001. Population structure of Botryosphaeria dothidea from pistachio and other hosts in California. *Phytopathology* 91, 665–672.

58- Magan, N. 1988. Effects of water potential and temperature on spore germination and germ-tube growth *in vitro* and on straw leaf sheaths. *Transactions of the British Mycological Society* 90 (1), 97-107.

59- Martin, R. R., Delano, J. and Levesque, C.A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology* 38, 207-239.

60- Matheron, M. E. and Porchas, M. 2005. Influence of soil temperature and moisture on eruptive germination and viability of *Sclerotinia minor* and *S. sclerotiorum. Plant Disease* 89:50-54.

61- McMullen, M. P. and Hosford, D. R. 1989. *Tan spot of wheat*. NDSU extension service pp-766. Fargo, ND, USA. 3 pp.

62- Melouk, H. A. 1978. Determination of leaf necrosis caused by *Cercospora arachidicola* Hori in peanut as measured by loss in total chlorophyll. *Peanut Science* 5: 17-18.

63- Melzer, M.S., Smith, E. A. and Boland, G.J. 1997. Index of plant hosts of Sclerotinia minor. *Canadian Journal of Plant Pathology* 19, 272–80.

64- Moore, W. D. 1949. Flooding as a means of destroying the sclerotia of *Sclerotinia sclerotiorum*. *Phytopathology* 39: 920-927.

65- Moore, L. D., Couch, H. B. and Bloom, J. R. 1963. Influence of environment on diseases of turfgrasses III: effect of nutrition, PH, soil temperature, air temperature, and soil moisture on Pythium blight of highland bentgrass. *Phytopathology* 53: 53-57.

66- Moreno, M. V., Stenglein, S. A., Balatii, P. A. and Perell, A. E. 2008. Pathogenic and molecular variability among isolates of *pyrenophora tritici-repentis* agent of tan spot of wheat in Argentina. *European Journal of Plant Pathology* 122 (2), 239-252.

67- Morrall, R. A. A., Duczek, L. J. and Sheard, J. W. 1972. Variations and correlations within and between morphology, pathogenicity, and pectolytic enzyme activity in *Sclerotinia* from Saskatchewan. *Canadian Journal of Botany* 50, 767-86.

68- Nicholas, K. B. and Nicholas, Jr. H. B. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. Available from: www.psc.edu/biomed/genedoc.

69- Njambere, E. N., Chen, W., Frate, C., Temple, S. R., Wu, B. M. and Muehlbauer, F. J. 2007. Cultural and DNA-based identification of *Sclerotinia trifoliorum* infecting chickpea in the U.S. *PISUM Genetics* 39, 30-32.

70- Njambere, E. N., Vandemark, G. and Chen, W. 2010. Development and characterization of microsatellite markers of the fungal plant pathogen *Sclerotinia trifoliorum*. *Genome* 53, 494-500.

71- Papendick, R. I. and Campbell, G. S. 1981. Theory and Measurement of water potential. In *Water Potential Relations in Soil Microbiology*, Soil Science Society of America special Publication No. 9. Madison, Wisconsin.

72- Papendick, R. I. and Mulla, D. J. 1986. Basic principles of cell and tissue water relations. In: Ayres PG, Boddy L (eds), *Water, Fungi and plants*. Cambridge University Press, Cambridge. pp. 1-25.

73- Phipps, P. M. and D. M. Porter. 1982. Sclerotinia blight of soybean caused *Sclerotinia minor* and *Sclerotinia sclerotiorum*. *Plant Disease* 66: 163-165.

74- Porter, D. M. and M. K. Beute. 1974. Sclerotinia blight of peanuts. *Phytopathology* 64: 263-246.

75- Ramires, M. L., Chulze, S. N. and Magan, N. 2004. Impact of osmotic and matric wtaer stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugars alchols in Fusarium graminearum. *Mycologia* 96 (3): 470-478.

76- Rees, R. G. and Platz, G. J. 1980. The epidimiology of yellow spot of wheat in southern Queensland. *Australian. Journal of. Agricultural Research* 31: 259-267.

77- Rees, R. G. and Platz, G. J. 1983. Effects of yellow spot on wheat: comparison of epidemics at different stages of crop development. *Australian Journal of Agricultural Research* 34: 39-46.

78- Reichert, I. 1958. Fungi and plant diseases in relation to biogeography. *Transactions of the New York Academy of Sciences* 20, 333-39.

79- Rogers, S. L., Atkins, S. D. and West, J. S. 2009. Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology* 58, 324-331.

80- Rotter, D., Amundsen, K., Bonos, S. A., Meyer, W.A., Warnke, S. E. and Belanger, F.C. 2009. Colonial bentgrass genetic linkage mapping. In: Yamada T, Spangenberg G, eds. *Molecualr Breeding of Forage and Turf*. Springer Science, 309-322.

81- Royle, D. J. and Butler, D. R. 1986. Epidemiological significance of liquid water in crop canopies and its role in disease forecasting. In: Ayres PG, Boddy L (eds), *Water, Fungi and plants*. Cambridge University Press, Cambridge, pp. 139-156.

82- Schilder, A. M. C. and Bergstrom, G. C. 1993. Tan spot. In S. B. Mathur and B. M. Cunfer, eds. *Seedborne diseases and seed health testing of wheat*, P. 113-122. Copenhagen, Denmark, Jordburgsforlaget

83- Schoeneweiss, D. F. 1975. Predisposition, stress, and plant disease. *Annual Review of phytopathology* 13: 193-211.

84- Smiley, R.W., Dernoeden, P. H. and Clarke, B. B. 1993. *Compendium of Turfgrass Diseases*. APS Press, Minneapolis-St. Paul, MN. 98 p.

85- Smith, D. L., Hollowell, J. E., Isleib, T. G. and Shew, B. B. 2006. Analysis of factors that influence the epidemiology of sclerotinia minor on peanut. *Plant Disease* 90: 1425-1432.

86- Staats, M., van Baarlen, P. and van Kan, J. A. L. 2004. Molecular phylogeny of the plant pathogenic genus Botrytis and the evolution of host specificity. *Molecular Biology and Evolution* 22, 333–346.

87- Strelkov, S. E., Lamari, L., Sayoud, R. and Smith, R. B. 2002. Comparative virulence of chlorosis-inducing races of *Pyrenophora tritci-repentis.Canadian Journal of Plant Pathology* 24: 29-35.

88- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24, 4876–4882.

89- Wadsworth, D. F. 1973. Research on the nature and control of peanut diseases in Oklahoma. *Oklahoma Agricultural Experiment Station Report* P-683. 17 pp.

90- Watkins, J. E., Odvody, G. N., Boosalis, M. G. and Partridge, J. E. 1978. An epidemic of tan spotof wheat in Nebraska. *Plant Disease Reporter* 62:132-134.

91- Weise, M. V. 1987. *Compendium of Wheat Diseases*. 2nd edition. APS Press. The American Phytopathological Society. St. Paul, MN, USA. 112 pp.

92- White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, Hite TJ (Eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press Inc, pp. 315–322.

93- Willets, H. J. and Wong, J. A. L. 1980. The biology of *Sclerotinia sclerotiorum, S. trifoliorum, S. minor* with emphasis on specific nomenclature. *The Botanical Review* 46:101-165.

94- Winton, L. M., Leiner, R. H. and Krohn, A. L. 2006. Genetic diversity of Sclerotinia species from Alaskan vegetable crops. *Canadian Journal of Plant Pathology* 28, 426-34.

95- Wu, B. M., Subbarao, K. V. and Liu, Y.-B. 2008. Comparative survival of sclerotia of *Sclerotinia minor* and *S. sclerotiorum*. *Phytpathology* 98:659-665.

96- Yin, Y., Ding, L., Liu, X., Yang, J. and Ma, Z. 2009. Detection of *Sclerotinia sclerotiorum* in planta by a real-time PCR assay. *Journal of Phytopathology* 157, 465-469.

97- Zillinsky, F. 1983. Common diseases of small grain cereals, a guide to identification. Mexico, DF, CIMMYT. 141 pp.

98- Zimmerman, U. 1978. Physics of turgor- and osmoregulation. *Annual Review of Plant Physiolog* 29,121-48.

CHAPTER II

EFFECT OF OSMOTIC AND MATRIC POTENTIALS ON *SCLEROTINIA MINOR* AND *SCLEROTINIA SCLEROTIORUM* AND THEIR INTERACTION ON PEANUT

ABSTRACT

The effect of osmotic and matric potentials on mycelial growth , sclerotia production, germination and virulence of two isolates of *S. sclerotiorum* and one isolate of *S. minor* were studied on potato dextrose agar (PDA) adjusted with KCl , glycerol or agar.Osmotic potentials created by KCl and glycerol, significantly reduced vegetative growth of the three isolates. On matrically adjusted PDA, vegetative growth of the three isolates was not negatively affected by matric stress up to -3.5 MPa. When KCl was the osmoticum, sclerotia number did not follow a consistent pattern. However, sclerotia number decreased when osmotic stress created by glycerol was increased. Matric stress was not a consistent factor affecting sclerotia production by both species. However, there appear to be a statistical trend to support that the highest levels of matric stress (-3.0 and -3.5 MPa) favorably affected sclerotia production by both species. In general, there was a numerical trend toward lower sclerotial germination with increasing osmotic stress and matric stress Pathogenicity of *S. sclerotiorum* and *S. minor* on peanut (Okrun) cultivar, was numerically reduced by high concentrations of KCl. Mycelia of both species grown at a

high matric potential (-3.5 MPa) did not differ in their pathogenicity on Okrun from the mycelia grown on non-amended PDA. When Okrun was placed under water stress using polyethylene glycol 8000, the Area Under Disease Progress Curve (AUDPC) significantly decreased when the water stress on Okrun increased. The relevance of these results to the behaviour of *S. minor* and *S. sclertiorum* and their pathogenicity on peanut is discussed.

INTRODUCTION

Sclerotinia blight of peanut (*Arachis hypogaea L.*) caused by the soilborne fungi *Sclerotinia minor* Jagger and *S. sclerotiorum* (Lib.) de Bary was first reported in the United States in Virginia in 1971 (Kokalis-Burelle *et al*, 1997). Sclerotinia blight has become widespread in Virginia, North Carolinia, Oklahoma and Texas (Smith *et al*, 2006). *S. minor*, and *S. sclerotiorum* survive mainly by producing sclerotia (Wu *et al*, 2008). Infection occurs primarily through eruptive germination of sclerotia that gives rise to white and fluffy mycelia that come in contact with stems and pegs of peanut.

Many factors affect survival and germination of sclerotia of the two species in the field (Wu *et al*, 2008). Constant soil temperature for 3 weeks or more at 35 ºC reduces survival of sclerotia. Other factors such as sclerotial position and duration in soil, sclerotial shape, soil gases or chemicals, activities of other microorganisms and nutrition affect survival of sclerotia (Adams, 1975; Abwai and Grogan, 1975; Abwai and Grogan, 1979; Huang and Kozub, 1994; Imolehin *et al*, 1980; Burgess and Hepworth, 1996). Temperature and moisture are significant factors affecting development of diseases caused by species of *Sclerotinia spp.* (Willets and Wong, 1980). Viability of sclerotia also declines rapidly over time at high soil water potential (i.e., low water stress) (Abwai

and Grogan, 1979). Almost 100% of the sclerotia of *S. sclerotiorum* were totally decayed when soil was flooded with water for 24 to 45 days. In general, sclerotia of *S. minor* survive better in dry soil than in moist soils, and better in shallow rather than at a deeper depth in soil where higher moisture usually exists (Imolehin *et al*, 1980).

Lower soil water potential (i.e., high water stress) in lettuce fields increases sclerotia survival, and sclerotia only survive short periods in saturated soils at 0 MPa (Hao *et al*, 2003). Sclerotia viability decreased at soil water potentials ≥ - 0.02 MPa when the soil temperature increased from 15 to 40 ºC. Sclerotia of *S. minor* can germinate directly at soil moisture levels between -0.03 and -1.5 MPa (Imolehin *et al*, 1980), while sclerotia of *S. sclerotiorum* germinate between 0 and -0.6 MPa (Duniway *et al*, 1977). Optimum radial growth occurred on basal medium with osmotic potential of -1.2 MPa and at -10 Mpa there was no growth (Imolehin *et al*, 1980). Sclerotia of *S. minor* produced over the range of -0.1 to -4.35 MPa did not differ significantly in its ability to germinate eruptively when moistened (Imolehin *et al*, 1980). Sclerotia of *S. minor* and *S. sclerotiorum* maintained within wet soil ≥ 0.02 MPa for 4 weeks at 40 ºC did not germinate while sclerotia maintained within dry soil ≤ - 100 MPa for 4 weeks at 40 ºC were viable (Matheron and Porchas, 2005).

Most research on the effect of water potential on *S. minor and S. sclerotiorum* was performed on isolates infecting lettuce under environmental factors significantly different from those found in peanut fields. Our research was performed with Sclerotinia isolates pathogenic to peanuts. Development of more effective integrated disease management strategies for Sclerotinia blight of peanut could benefit from new knowledge on the factors that affect the biology of the host, the fungus, and their interaction.

Therefore, the objectives of this research were to: 1) study the effect of water potential on the vegetative growth and sclerotia production of *S. minor* and *S. sclerotiorum*, 2) determine germination of sclerotia produced on nutrient media at various water potential, 3) study the pathogenicity of *S. minor* and *S. sclerotiorum* produced on media at various matric and osmotic potentials and 4) determine the impact of water stress on the infection of peanut with *S. minor and S. sclerotiorum.*

MATERIALS AND METHODS

Plant materials and fungal cultures

The cultivar Okrun, a sclerotinia blight-susceptible runner type peanut, was used in this study. Seeds germinated on wet filter paper at 30 ºC in an incubator for two days, and then planted in pots (10 cm dia) containing a 2:1:1 (sand: soil: shredded peat moss), respectively. Plants were grown in a climate-controlled greenhouse, watered daily, and fertilized with 0.45 % ammonium nitrate solution on a weekly basis starting on the third week after planting to promote the production of highly succulent stems.

Three *Sclerotinia* isolates were used that included one isolate of *S. minor* from peanut, and two *S. sclerotiorum* isolates; one from peanut grown in Nebraska, and the other was isolated from pumpkin fruit that was bought from a supermarket in Stillwater, OK. Isolates were maintained at 25±2 ºC in darkness on potato dextrose agar (Difco Laboratories, Detroit, MI) containing 100 ppm of streptomycin sulfate (SPDA).

Preparation of media at various water potentials

Potato-dextrose agar medium (Difco Laboratories, Detroit, MI) containing 100 ppm of streptomycin sulfate (SPDA) was used as a basal medium. SPDA medium was osmotically modified over the range of -0.5 to -4 MPa with potassium chloride (Ritchie *et*

al, 2006) or glycerol (Dallyn and Fox 1980) and sterilized for 20 minutes. Total water potential was the sum of the water potential of the SPDA -0.34 MPa and the osmotic potential of the added osmotica (potassium chloride or glycerol) (Campbell and Gardner, 1971; Dallyn and Fox, 1980); Osmotic potential was calculated according to (Liddell, 1993). The actual osmotic potential of all media were also checked by Vapor Pressure Osmometer (VAPRO 5520, Wescor, Utah, USA).

Various matric potentials of SPDA were adjusted by granulated agar (fisher scientific, Fair Lawn, New Jersey). Matric potentials of media equivalent to -1, -1.5, -2.0, -2.5, -3.0 and -3.5 MPa at 25 ºC were determined using Vapor Pressure Osmometer (VAPRO 5520, Wescor, Utah, USA). The total matric potential was the sum of the water potential of SPDA and the matric potential of the added agar.

Mycelia growth and sclerotia production on nutrient medium

Petri dishes containing 15 ml of nutrient medium were each inoculated in the center with a 3-mm dia mycelial disc taken from the periphery of 2-day old cultures of *S. minor* and *S. sclerotiorum* grown on SPDA. Inoculated plates were incubated at 25±2 ºC in darkness. Radial growth (mm) of colony was measured up to 4 days after inoculation. Sclerotia harvested from 21 days old cultures with the aid of camel hair brush. Harvested sclerotia were dried at 22 ºC for two weeks in a desiccator containing anhydrous $CaSO_4$. Sclerotia from each 9.0 cm plate were counted. This experiment was conducted twice with five plates as replications in each treatment. Data were analyzed with SAS (SAS Institute, Cary NC) using a 0.05 level of significance.

Viability of sclerotia

Sclerotia produced under different osmotic and matric potentials were tested for viability by plating on SPDA medium. Before plating, sclerotia were surface sanitized with a sodium hypochlorite solution (Melouk *et al*., 1999). For each treatment, five sclerotia were plated on each of five plates, and incubated at room temperature 25±2 ºC in darkness. Percentage of sclerotial germination was determined after 7 days of incubation.

Pathogenicity of mycelial inoculum of *S. minor* and *S. sclerotiorum* produced on media at various water potentials

Plant inoculations were performed on peanut plants (6-8 weeks-old) according to Faske *et al*., (2006). A total of eight pots (4replicates) were used for each one of the osmotic and matric potentials used in this study. Plants were then placed in humidity chambers (150 x 60 x 60 cm) built from PVC pipe and clear plastic. Temperature was maintained at 19±2 ˚C at night and 26±2 C during the day and relative humidity was maintained at 95 to 100%. Light in the incubation chamber was adequate (13.5 µmol/s/m^2) to sustain healthy plants throughout experiments. Inoculated plants were watered when necessary for the duration of the experiments. Starting three days after inoculation, lesion length measurements were recorded for the infected stems and continued on a 24 hour to day 7 post inoculation. The plants were then left to dry for one week in the chambers to facilitate production of sclerotia on infected tissue. To facilitate further drying, the infected stems were clipped at soil level and placed in brown paper bags for one week more. Sclerotia were collected from both the stem surface and from within the pith cavity of the stem, and quantified based on number and weight.

Experimental design was a random complete block design (RCBD) with 4 replicates (chambers).

The effect of water stress on the infection of peanut by *S. minor* and *S. sclerotiorum*

Plants were divided into 9 groups (8 different water potentials + water control). Each group of plants was placed in fabricated humidity chambers (58.7 cm x 42.9 cm x 40 cm). Six to eight weeks old Okrun plants that received (PEG 8000) solutions were prepared for inoculation as described by (Faske *et al*, 2006). Water stress was applied to plants with polyethylene glycol 8000 (Union Carbide Chemicals and Plastics, Danbury, Conn.). PEG solutions of various water potentials were prepared according to (Michel and Kaufmann, 1973) and are presented in (Table 1). PEG 8000 was applied by pouring each solution into the bottom of its assigned plastic chamber on the fourth week after planting. In the water control group water was used to keep seedling well irrigated. Six-to -eight week old plants were prepared for inoculation as described by Faske *et al* (2006). Total number of plants in the experiment was 72 (9 treatments x 8 replicates). The experiment was performed using the same methods with each of the three isolates. Lesion length was taken at the fourth day post inoculation. This experiment was repeated once. Statistical analyses were done using SAS 9.1 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Mycelial growth of *Sclerotinia* isolates on SPDA with various water potentials

In osmotic potential (ψ_s) experiments, mycelial growth response of *Sclerotinia* isolates to ψ_s was similar for the two osmotica (Table 2). On both KCl and glycerol amended SPDA, the vegetative growth of *S. sclerotiorum* (peanut isolate) was

consistently reduced at osmotic stress values below -1.5 MPa (Table 2). On both KCl and glycerol amended SPDA, the vegetative growth of *S. sclerotiorum* (pumpkin isolate) was consistently reduced at osmotic stress values below -2.5 MPa (Table 2). On both KCl and glycerol amended SPDA, the vegetative growth of *S. minor* has been significantly (P=0.05) reduced at osmotic stress values below -1.5 MPa (Table 2). This suppression of vegetative growth suggests that *S. sclerotiorum* (pumpkin isolate) may tolerate higher levels of osmotic stress for survival than *S. sclerotiorum* (peanut isolate) and *S. minor*.

In matric potential (ψ_m) studies, vegetative growth of *S. minor*, *S. sclerotiorum* (peanut isolate), and *S. sclerotiorum* (pumpkin) was not negatively affected by matric stress of up to -3.5 MPa (Table 3). Trend of mycelial growth (as indicated by AUMGPC) of the three isolates on metrically amended SPDA is shown in (Figure 1).

This pattern of mycelial growth was similar to that of the observed by Ferrin and Stanghellini (2006) with the fungus *Monosprascus cannonballus*, which indicates that the observed responses were indeed caused by changes in osmotic stress rather than by toxicity of the osmotica. Also, the mycelial growth responses of *S. minor* and *S. sclerotiorum* to different osmotic stress in this study are similar to those previously observed for other soil borne pathogens (Ritchie *et al*, 2006). For instance, mycelial growth of *Rhizoctonia solani* (Kumar *et al*, 1999), *Gaeumannomyces graminis* (Grose *et al*, 1984), *Typhula idanoensis* and *Typhula incarnata* (Bruehl and Cunfer 1971), *Macrophomina phaseolina* (Cervantes-Garcia *et al*, 2003), and *Aspergillus niger* and *Fusarium moniliforme* (Subbarao *et al*, 1993) were reduced when the osmotic stress increased.

Solutes present in agar medium trap water molecules, therefore water will not be available to *S. minor* and *S. sclerotiorum*. The energy spent by the fungi in order to obtain water molecules from the medium is increased as the solute concentrations in the agar medium increase and therefore reduction of fungal growth occurs. Ionic solutes such as KCl and NaCl and nonionic solutes such glycerol and sucrose have been used in several water potential studies involving various plant pathogenic fungi like *Phytophthora cryptogea* and *Fusarium moniliforme* (Woods and Duniway, 1986) and *Verticillium dahlia* (Ioannou *et al*, 1977). *S. minor* and *S. sclerotiorum* isolates grew on KCl and glycerol adjusted PDA over all levels of the test osmotica (Table 2). The ability of a fungus to grow under osmotic stress and the exact optimal water potential depends on the fungus species and in some cases on the osmoticum, temperature, or other factors in the environment (Cook, 1981). Mycelial growth under KCl osmotic stress may result from uptake of potassium ions and its accumulation by microbial cells, which lower the water potential of the protoplasm to a value more ideal for cellular processes or may increase turgor and hence acceleration of growth (Olaya *et al*, 1996).

On matrically modified SPDA, *S. sclerotiorum* (peanut isolate) had the highest mycelial growth at -3.5 MPa, however, there was no significant differences over the range -2.0 to -3.5 MPa. *S. sclerotiorum* (pumpkin isolate) and *S. minor* grew greatest at -1.0 MPa. However, AUMGC values produced by the three isolates at the lowest matric potential were greater than those recorded at the lowest osmotic potential used in this study. Moreover, the mycelial growth of the three isolates have not been inhibited at the lowest matric potential used in this study -3.5 MPa which is lower than the permanent wilting point of mesophytic higher plants -1.5 MPa (Slayter, 1967).

Sclerotia number produced on nutrient media

Different levels of osmotic potentials (ψ_s) created by KCl and glycerol significantly (P=0.05) affected sclerotia number produced by the three *Sclerotinia* isolates (Table 4). In general, when KCl was the osmoticum sclerotia number did not follow a consistent pattern. However, when glycerol was the osmoticum, sclerotia number decreased when osmotic stress increased (Table 4).

In matric potential (ψ_m) studies, different levels of ψ_m significantly affected the mean sclerotia number produced by the three isolates (Table 5). In general, matric stress has not shown to be a consistent factor affecting the number of sclerotia produced by the three Sclerotinia isolates. However, there appears to be a statistical trend to support that highest level of matric stress (-3.5 MPa) favorably affected the number of sclerotia produced by *S. sclerotiorum* and *S. minor* (Table 5).

Total sclerotia production by any test isolate was bigger on glycerol amended PDA than on KCl amended PDA (Table 4). This may be due to the utilization of the glycerol as a carbon source by *S. sclerotiorum* and *S. minor* (Sommers *et al*, 1970). On matrically amended SPDA, the three isolates of *S. sclerotiorum* and *S. minor* produced the biggest numbers of sclerotia on -3 and -3.5 Mpa (Table 5). This indicates these isolates of *S. sclerotiorum* and *S. minor* are well adapted to wider ranges of soil water potentials well beyond the limits of their peanut host, provided that other environmental factors are conducive. Also, osmotic stress forces the isolates of *S. sclerotiorum* and *S. minor* to produce sclerotia as survival structure. This could be one of the factors involved in its fitness as a soil-borne plant pathogen (Ritchie *et al*, 2006).

Sclerotial germination

Germination of sclerotia of *S. sclerotiorum* (pumpkin isolate) and *S. minor* produced at different levels of ψ_s created by KCl was significantly (P=0.05) affected (Table 6). However, germination of the sclerotia of *S. sclerotiorum* (peanut isolate) produced at various levels of ψ_s was not affected (Table 6). The difference in sclerotial germination between the two isolates of *S. sclerotiorum* in response to osmotic stress suggests that within each species there may exist ecotypes with variability in their response to environmental factors. This needs future research. In case of matric potential (ψ_m), significant differences observed between treatments for *S. sclerotiorum* isolates but not for *S. minor*. At the lowest ψ_m -3.5 Mpa, the percentage of seclerotia germination was 80% of *S. sclerotiorum* (peanut isolates), 55% of *S. sclerotiorum* (pumpkin isolate) and 98% of *S. minor*. Ability of sclerotia to germinate at low osmotic potential is perhaps due to solute uptake by the sclerotiorum causing a reduction in its internal osmotic potential and so allowing maintenance of germination processes (Cook and Al-Hamdani, 1986). In this study, the sclerotial formation and germination of *S. minor* and *S. sclerotiorum* occurred at osmotic and matric potentials lower than those at which most crops seeds germination and roots development are curtailed -1.4 to -2.0 MPa (Tommerup, 1984). This could be of importance to understand the ecological factors that could affect the pathogenicity.

Virulence of mycelia produced on media at various water potentials

In osmotic potential (ψ_s) studies, mycelia of *S. sclerotiorum* (peanut isolate), and *S. minor* produced on different ψ_s were inconsistent in its virulence against the runner peanut cv. Okrun (Table 7). Only mycelia of *S. sclerotiorum* (pumpkin isolate) produced at osmotic stress at -2.0 MPa and lower were statistically less virulent (Table 7). In matric

potential studies, mycelia of *S. minor* and *S. sclerotiorum* produced at different matric levels were inconsistent in its virulence against peanut cv. Okrun (Table 8). Different matric potentials significantly (P=0.05) did not affect AUDPC produced by the three isolates (Table 8).

No research has been done before to investigate the effect of osmotic and matric potentials on the virulence of *S. minor* and *S. sclerotiorum*. Few studies in the literature investigated the effect of water potential on the virulence of plant pathogenic fungi. Cervantes-Garcia *et al* (2003) observed a reduction in the pathogenicity of *Macrophomina phaseolina* on seeds of common beans, as NaCl concentrations increased in potato-glucose-agar medium. The results reported herein shows that *S. minor* and *S. sclerotiorum* can grow vegetatively under relatively low water potentials. The ability of *S. minor* and *S. sclerotiorum* to grow in a wide range of water potentials indicates the presence of adaptive mechanisms for life under variable environmental conditions. Adapting to a wide range of water potentials may be a strategy to exist as saprophyte.

Determine the effect of water stress on the infection of peanut by *S. minor and S. sclerotiorum*

Water stressed seedlings of the cultivar Okrun differed significantly (P=0.05) in their reaction to the infection by the three isolates of *S. minor, S. sclerotiorum* (Table 9). Stressed plants exhibited less disease when inoculated with *S. minor* or *S. sclerotiorum* (peanut isolate), AUDPC produced by both isolates decreased when the water stress level increased (Table 9). In case of *S. sclerotiorum* (pumpkin isolate), AUDPC decreased significantly (P=0.05) as the water stress applied on plants increased but there was an

eruption in the amount of the disease observed on plants when water stressed to -1.76 MPa (Table 9).

Plant stress was measured by determining the relative water content (RWC) in the leaves (Teulat *et al*, 1997) 1 cm^2 discs. Also, photosynthesis efficiency was measured by a Chlorophyll Fluorometer (OS1-FL, Opti-Sciences, Inc) was used to further confirm the status of water deficiency of plants. Our search of the literature has found no previous research that examined the effect of the status of water hydration on peanut and its infection by *S. minor* and *S. sclerotiorum*. Short term droughts of days or weeks during the growing season may predispose plants to diseases (Schoeneweiss, 1975). For example, larger cankers were induced by *Lasiodiplodia theobromae* on water stressed dogwood (*Cornus florida* L.) (Mullen *et al*, 1991), by *Hypoxylon prunatum* on water stressed *Populus tremuloides* (Bagga and Smalley, 1969), and drought stress increased the severity of Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea* (Ma *et al*, 2001). Our results contradict these observations. Our data indicate that water stressed plants had smaller lesions than non-water stressed plants or plants were under less water stress. This information can be used to disease management by applying less irrigation in infected peanut plants. Reduction in mycelial growth of *S. minor* and *S. sclerotiorum* under increased osmotic stress suggests that the reduced growth of both species may partly explain the reduction in AUDPC on plants under high level of water stress. *S. sclerotiorum* (pumpkin isolate), caused larger lesions when the stress level increased. There was no published data, to our knowledge, concerning effects of water potential on mycelial growth, sclerotial number and germination of *Sclerotinia sclerotiorum* and *Sclerotinia minor* collected from peanut fields. Therefore, this study is

the first to show the negative effects of osmotic and matric stress on mycelial growth and sclerotial formation of the two *Sclerotinia* species. Furthermore, this study stated for the first time the effect of water stress on the infection of peanut by *S. sclerotiorum* and *S. minor*.

LITERATURE CITED

1- Abwai, G. S. and Grogan, R. G. 1975. Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. *Phytopathology* 65: 300-309.

2- Abwai, G. S. and Grogan, R. G. 1979. Epidemiology of diseases caused by *Sclerotinia* species. *Phytopathology* 69: 899-904.

3- Adam, P.B. 1975.Factors affecting survival of *Sclerotinia sclerotiorum* in soil. *Plant Disease Reporter* 59: 599-603.

4- Bagga, D. K. and Smalley, E. B. 1969. Factors affecting canker development on Populus tremuloides artificially inoculated with Hypoxylon pruinatum. *Canadian Journal of. Botany* 47: 907-914.

5- Brownell, K. H. and Schneider, R. W. 1985. Roles of matric and osmotic components of water potential and their interaction with temperature in the growth of *Fusarium oxysporum* in synthetic media and soil. *Phytopathology* 75: 53-57.

6- Bruehl, G. W. and Cunfer, B. 1971. Physiologic and environmental factors that affect the severity of snow mold of wheat. *Phytopathology* 61:792-799.

7- Burgess, D.R. and Hepworth, G. 1996. Biocontrol of Sclerotinia stem rot (*Sclerotinia minor*) in sunflower by seed treatment with *Gliocladium virens*. *Plant Pathology* 45: 583-592.

8- Campbell, G. S., and Gardner, W. H. 1971. Psychrometric measurement of soil water potential: temperature and bulk density effects. *Soil Science Society of America, Proceedings* 35: 8-12.

9- Cervantes-Garcia, D., Padilla-Ramirez, J. S., Simpson, J.and Mayek-Perez, N. 2003. Osmotic potential effects on in vitro growth, morphology and pathogenicity of *Macrophomina phaseolina*. *Journal of Phytopathology* 151: 456-462.

10- Cook, R. J. 1981. Water relations in the biology of *Fusarium*. Pages 236-244 in: *Fusarium: Diseases, Biology, and Taxonomy*. P. E. Nelson, T. A. Toussoun, and R. J. Cook, eds. The Pennsylvania Satate University Press, University Park.

11- Cook, R. C., and Al-Hamadani, A. M. 1986. Water relations of sclerotia and other infective structures. Pages 49-63 in: *Water, Fungi and Plants*. P. G. Ayres and L. Boddy, eds. Cambridge University Press, Cambridge.

12- Dallyn, H. and Fox, A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould, G. H., Corry, E. L., eds. *Microbial growth and survival in extreme environments*. London and New York: Academic Press. P 129-139.

13- Duniway, J. M., Abawi, G. S. and Steadman, J. R.1977. Influence of soil moisture on the production of apothecia by Whetzelinia sclerotiorum. (Abstr.). *Proceedings of the American Phytopathological Society*. 4:115.

14- Faske, T., Melouk, H. A. and Payton, M. E. 2006. Comparison of *Sclerotinia minor* inocula for differentiating the reaction of peanut genotypes to Sclerotinia blight. *Peanut Science* 33: 7-11.

15- Ferrin, D. M. and Stanghellini, M. E. 2006. Effect of water potential on mycelial growth and perithecial production of *Monosprascus cannonballus* in vitro. *Plant Pathology* 55: 421-426.

16- Griffin, D. M. 1981. Water and microbial stress. In: Alexander A, ed. *Advances in microbial ecology* 5. New York. Plenum Publishing Co. P 91-136.

17- Grose, M. J., Parker, C.A. and Sivasithamparam, K. 1984. Growth of *Gaeumannomyces graminis* var. *Tritici* in soil: effects of temperature and water potential. *Soil Biology and Biochemistry* 16:211-216.

18- Hao, J. J., Subbarao, K. V. and Duniway, J. M. 2003. Germination of *Sclerotinia minor* and *S. sclerotiorum* sclerotia under various soil moisture and temperature combinations. *Phytopathology* 93: 443-450.

19- Huang, H. C., and Kozub, G. C. 1994. Longevity of normal and abnormal sclerotia of *Sclerotinia sclerotiorum*. *Plant Disease* 78: 1164-166.

20- Imolehin, E. D., Grogan, R. G. and Duniway, J. M. 1980. Effect of temperature and moisture tension on growth, sclerotial production, germination, and infection by *Sclerotinia minor*. *Phytopathology* 70: 1153-1157.

21- Ioannou, N., Scheider, R. W., Grogan, R. G. and Duniway, J. M. 1977. Effect of water potential and temperature on growth, sporulation, and production of microsclerotia by *Verticillium dahlia*. *Phytopathology* 67:637-644.

22- Jennings, D. H. 1995. *The physiology of fungal nutrition*. Cambridge, UK: Cambridge University Press.

23- Jennings, D. H. and Burke, R. M. 1990. Compatible solutes-the mycological dimension and their role as physiological buffering agents. *New Phytologist* 116: 277-283.

24- Kokalis-Burelle, N., Porter, D. M., Rodríguez-Kabana, R., Smith, D. H. and Subrahmanyam, P. 1997. Sclerotinia Blight. Pages 34-36 in: Compendium of Peanut Diseases. Second edition. *American Phytopathological Society*.

25- Kumar, S., Sivasithamparam, K., Gill, J. S. and Sweetingham, M. W. 1999. Temperature and water potential effects on growth and pathogenocoty of *Rhizoctonia solani* Ag-11 to lupin. *Canadian Journal of Microbiology* 45: 389-395.

26- Lang, A. R. G. 1967. Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40 C. *Australian Journal of Chemistry* 20: 2017-2023.

27- Liddell, C. M. 1993. Measurement and control of soil temperature and water potential. In: Singleton, L. L., Mihail, J. D., Rush, C. M, eds. Methods for research on soilborne phytopathogenic fungi. Eds,. *The American Phytopathological society*, St. Paul, Minnesota.

28- Ma, Z., Morgan, D. P. and Michailides, T. J.2001. Effect of water stress on Botryosphaeria blight of pistachio caused by *Botryosphaeria dothidea*. *Plant Disease* 85: 745-749.

29- Magan, N. and Lynch, J. M. 1986. Water potential, growth and cellulolysis of fungi involved in decomposition of cereal residues. *Journal of General Microbiology* 132: 1181-1187.

30- Matheron, M. E. and Porchas, M. 2005. Influence of soil temperature and moisture on eruptive germination and viability of *Sclerotinia minor* and *S. sclerotiorum. Plant Disease* 89:50-54.

31- Melouk, H. A., Bown, C. and Aboshosha, S. S. 1999. A procedure for isolation and determination of incidence of *Sclerotinia minor* in peanut seed. *Alexanderia Journal of Agricultural Research* 44: 261-270.

32- Michel, B. E. and Kaufmann, M. R. 1973. The osmotic potential of polyethylen glycol 6000. *Plant Physiology* 51, 914-916.

33- Mullen, J. M., Gilliam, C. H., Hagan, A. K. and Morgan-Jones, G. 1991. Canker of dogwood caused by *Lasiodiplodia theobromae,* a disease influenced by drought stress or cultivar selection. *Plant Disease* 75: 886-889.

34- Olaya, G. and Abawi, G. S. 1996. Effect of water potential on mycelial growth and on production and germination of sclerotia of *Macrophomina phaseolina*. *Plant Disease* 80: 1347-1350.

35- Ramirez, M. L., Chulze, S. N. and Magan, N. 2004. Impact of osmotic and matric water stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugar alcholos in *Fusarium graminearum*. *Mycologia* 96 (3), 470-478.

36- Ritchie, F., McQuilken, M. P. and Bain, R. A. 2006. Effect of water potential on mycelial growth, sclerotial production, and germination of *Rhizoctonia solani* from potato. *Mycological Research*. 110: 725-733.

37- Schoeneweiss, D. F. 1975. Predisposition, stress, and plant disease. *Annual Review of Phytopathology* 13: 193-211.

38- Slayter, R. D. 1967. *Plant-water relationships*. Academic Press, New York.

39- Smith, D. L., Hollowell, J. E., Isleib, T. G. and Shew, B. B. 2006. Analysis of factors that influence the epidemiology of sclerotinia minor on peanut. *Plant Disease* 90: 1425-1432.

40- Sommers, L. E., Harris, R. F., Dalton, F. N. and Gardner, W. R. 1970. Water potential relations of three root-infecting *Phytophthora* species. *Phytopathology* 60: 932-934.

41- Subbarao, K. V., Michailides, T. J. and Morgan, D. P. 1993. Effects of osmotic potential and temperature on growth of two pathogens of figs and a biocontrol agent. *Phytopathology* 83: 1454-1459.

42- Teulat, B., Monneveux, P., Wery, J., Souyris, B. I., Charrier, A. and This, D. 1997. Relatioships between relative water content and growth parameters under stress in barely: a QTL study. *New Phytologist* 137: 99-107.

43- Tommerup, I. C. 1984. Effect of soil water potential on spore germination by vesicular-arbuscular mycorrhizal fungi. *Transactions of the British Mycological Society* 83: 193-202.

44- Willets, H. J.and Wong, J. A. L. 1980. The biology of *Sclerotinia sclerotiorum, S. trifoliorum, S. minor* with emphasis on specific nomenclature. *The Botanical Review* 46:101-165.

45- Woods, D. M. and Duniway, J. M. 1986. Some of effects of water potential on growth, turgor, and repiration of *Phytophthora cryptogea* and *Fusarium moniliforme*. *Phytopathology* 76:1248-1253.

46- Wu, B. M., Subbarao, K. V. and Liu, Y.-B. 2008.Comparative survival of sclerotia of *Sclerotinia minor* and *S. sclerotiorum*. *Phytpathology* 98:659-665.

Table 1.1. Required concentrations of polyethylene glycol (PEG 8000) solutions to attain corresponding water stress on peanut plants at 25 ºC.

% PEG 8000	Osmotic stress in MPa
0	<-0.05
5	-0.05
10	-0.15
15	-0.30
20	-0.49
25	-0.73
30	-1.03
35	-1.37
40	-1.76

Table 2.1. Mean area under mycelial growth progress curve (AUMGC) for *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* grown on SPDA with different osmotic potentials (ψ_s) using KCl and Glycerol.

Isolates	Osmotic potentials in MPa	AUMGC[1] KCl	AUMGC[2] Glycerol
SS[3]	-0.34	19.190 b[4]	21.580 b[5]
SS	-0.50	18.480 b	25.010 a
SS	-1.00	20.440 a	23.310 ab
SS	-1.50	19.120 b	18.630 c
SS	-2.00	19.090 b	15.495 d
SS	-2.50	14.925 c	14.826 d
SS	-3.00	13.980 c	10.560 ef
SS	-3.50	9.830 d	12.092 e
SS	-4.00	10.390 d	10.160 f
SSP	-0.34	16.910 c	28.560 a
SSP	-0.50	19.040 b	26.810 a
SSP	-1.50	21.325 a	17.886 b
SSP	-2.00	22.255 a	13.490 c
SSP	-2.50	18.750 b	13.270 c
SSP	-3.00	16.980 c	8.380 d
SSP	-3.50	13.500 d	7.745 d
SSP	-4.00	12.905 d	5.220 d
SM	-0.34	16.155 a	29.710 a
SM	-0.50	16.514 a	29.050 a
SM	-1.00	16.670 a	26.240 b
SM	-1.50	13.015 b	22.328 c
SM	-2.00	9.945 c	20.102 d
SM	-2.50	10.545 c	20.090 d
SM	-3.00	7.795 d	14.121 e
SM	-3.50	7.895 d	11.972 f
SM	-4.00	5.425 e	9.630 g

[1] Means of area under mycelial growth progress curve values on KCl amended SPDA.
[2] Means of area under growth progress curve values on glycerol amended SPDA.
[3] SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*
[4, 5] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 3.1. Mean area under mycelial growth progress curve (AUMGC) for *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* grown on SPDA with different matric potentials (ψ_m).

Isolates	Matric potentials in MPa	AUMGC[1]
SS[2]	-0.34	21.95 b[3]
SS	-1.00	22.81 a
SS	-1.50	21.91 b
SS	-2.00	22.24 ab
SS	-2.50	22.88 a
SS	-3.00	22.46 ab
SS	-3.50	22.89 a
SSP	-0.34	21.62 bc
SSP	-1.00	24.49 a
SSP	-1.50	22.03 bc
SSP	-2.00	22.75 ab
SSP	-2.50	21.58 bc
SSP	-3.00	21.14 bc
SSP	-3.50	20.34 c
SM	-0.34	26.93 bc
SM	-1.00	28.62 a
SM	-1.50	26.85 bc
SM	-2.00	26.25 cd
SM	-2.50	25.49 d
SM	-3.00	25.39 d
SM	-3.50	27.52 b

[1] Means of area under mycelial growth progress curve values on matrically amended SPDA.
[2] SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 4.1. Mean of sclerotia number of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* produced on SPDA amended to various osmotic potentials using KCl and glycerol.

Isolates	Osmotic potentials in MPa	Sclerotia number[1] KCl	Sclerotia number[2] Glycerol
SS[3]	-0.34	9.8 bcd[4]	20.40 a[5]
SS	-0.50	8.6 cd	19.20 ab
SS	-1.00	9.6 bcd	17.80 abc
SS	-1.50	15.2 a	15.40 bc
SS	-2.00	11.2 bcd	15.20 bc
SS	-2.50	10.8 bcd	14.00 cd
SS	-3.00	8.2 d	13.80 cd
SS	-3.50	11.6 bc	10.00 ed
SS	-4.00	11.8 b	9.60 e
SSP	-0.34	13.8 de	70.80 a
SSP	-0.50	17.0 cde	48.20 b
SSP	-1.00	20.2 bcd	39.60 bc
SSP	-1.50	22.0 abc	36.80 c
SSP	-2.00	28.4 a	35.20 c
SSP	-2.50	22.8 abc	33.00 cd
SSP	-3.00	25.2 ab	30.60 cd
SSP	-3.50	20.4 bcd	25.00 d
SSP	-4.00	13.0 e	24.60 d
SM	-0.34	460.2 d	1018.80 a
SM	-0.50	546.0 d	992.20 a
SM	-1.00	492.4 d	948.00 a
SM	-1.50	735.2 bc	924.40 a
SM	-2.00	776.8 b	731.20 b
SM	-2.50	760.8 b	682.80 b
SM	-3.00	788.0 b	644.00 b
SM	-3.50	1069.6 a	535.20 c
SM	-4.00	593.6 cd	520.00 c

[1] Means of sclerotia number produced on KCl amended SPDA.
[2] Means of sclerotia number produced on glycerol amended SPDA.
[3] SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*
[4, 5] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 5.1. Mean of sclerotia number of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* produced on SPDA amended to various matric potentials of 0.0 to -3.5 MPa.

Isolates	Matric Potentials in MPa	Sclerotia number[1]
SS[2]	-0.34	15.6 cd[3]
SS	-1.00	11.6 d
SS	-1.50	15.8 cd
SS	-2.00	18.8 bc
SS	-2.50	19.0 bc
SS	-3.00	20.6 ab
SS	-3.50	23.8 a
SSP	- 0.34	28.2 c
SSP	-1.00	36.0ab
SSP	-1.50	33.0 bc
SSP	-2.00	34.4 bc
SSP	-2.50	32.8 bc
SSP	-3.00	42.2 a
SSP	-3.50	34.6 bc
SM	-0.34	509.2 c
SM	-1.00	478.2 c
SM	-1.50	726.8 a
SM	-2.00	659.2 ab
SM	-2.50	608.8 abc
SM	-3.00	543.2 bc
SM	-3.50	740.0 a

[1] Means of sclerotia number produced on SPDA amended to various matric potentials.
[2] SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor.*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 6.1. Percentage of sclerotia germination of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor*, produced on SPDA with various osmotic potentials of 0.0 to -4.0 MPa.

Isolates	Osmotic Potentials in Mpa	% sclerotia[1] germination[1]
SS[2]	-0.34	100.0 a[3]
SS	-0.50	100.0 a
SS	-1.00	100.0 a
SS	-1.50	100.0 a
SS	-2.00	100.0 a
SS	-2.50	95.0 a
SS	-3.00	95.0 a
SS	-3.50	90.0 a
SS	-4.00	90.0 a
SSP	-0.34	100.0 a
SSP	-0.50	100.0 a
SSP	-1.00	100.0 a
SSP	-1.50	100.0 a
SSP	-2.00	100.0 a
SSP	-2.50	100.0 a
SSP	-3.00	95.0 ab
SSP	-3.50	85.0 bc
SSP	-4.00	80.0 c
SM	-0.34	100.0 a
SM	-0.50	100.0 a
SM	-1.00	100.0 a
SM	-1.50	95.0 ab
SM	-2.00	95.0 ab
SM	-2.50	95.0 ab
SM	-3.00	90.0 ab
SM	-3.50	90.0 ab
SM	-4.00	80.0 b

[1] Percentage of sclerotia germination on SPDA amended to different osmotic potentials.
[2] SS, *S. sclerotiorum*, SSP, *S. sclerotiorum* (pumpkin), and SM, *S. minor*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 7.1. Percentage of sclerotia germination of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor*, produced on SPDA with various matric potentials of 0.0 to -3.5 MPa.

Isolates	Matric potentials in MPa	% sclerotia germination[1]
SS[2]	-0.34	100.0 a[3]
SS	-1.00	100.0 a
SS	-1.50	100.0 a
SS	-2.00	100.0 a
SS	-2.50	100.0 a
SS	-3.00	85.0 b
SS	-3.50	80.0 b
SSP	-0.34	100.0 a
SSP	-1.00	100.0 a
SSP	-1.50	100.0 a
SSP	-2.00	100.0 a
SSP	-2.50	95.0 a
SSP	-3.00	90.0 a
SSP	-3.50	55.0 b
SM	-0.34	100.0 a
SM	-1.00	99.0 a
SM	-1.50	99.0 a
SM	-2.00	99.0 a
SM	-2.50	99.0 a
SM	-3.00	98.0 a
SM	-3.50	98.0 a

[1] Percentage of sclerotia germination on SPDA amended to different matric potentials.
[2] SS, *S. sclerotiorum*, SSP, *S. sclerotiorum* (pumpkin), and SM, *S. minor*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 8.1. Mean area under disease progress curve (AUDPC) on "okrun" inoculated with mycelia of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* produced on osmotic amended SPDA.

Isolates	Osmotic Potentials in (-MPa)	AUDPC[1]
SS[2]	-0.34	16.108 ab[3]
SS	-0.50	15.731 abc
SS	-1.00	17.781 a
SS	-1.50	12.956 cbd
SS	-2.00	9.844 d
SS	-2.50	12.200 cbd
SS	-3.00	11.000 d
SS	-3.50	10.419 d
SSP	-0.34	40.375 ab
SSP	-0.50	47.032 a
SSP	-1.00	18.000 c
SSP	-1.50	30.875 b
SSP	-2.00	18.813 c
SSP	-2.50	19.281 c
SSP	-3.00	14.656 c
SSP	-3.50	15.094 c
SM	-0.34	33.500 a
SM	-0.50	33.815 a
SM	-1.00	30.313 ab
SM	-1.50	30.719 ab
SM	-2.00	31.219 ab
SM	-2.50	24.813 b
SM	-3.00	26.406 b
SM	-3.50	29.438 ab

[1]Means of area under disease progress curve values caused by mycelia produced on KCl amended SPDA.
[2]SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 9.1. Mean area under disease progress curve (AUDPC) on "okrun" inoculated with mycelia of *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor* produced on metrically amended SPDA.

Isolates	Matric Potentials in (Mpa)	AUDPC[1]
SS[2]	-0.34	19.925 a[3]
SS	-1.00	21.743 a
SS	-1.50	23.893 a
SS	-2.00	28.050 a
SS	-2.50	20.225 a
SS	-3.00	28.431 a
SS	-3.50	25.225 a
SSP	-0.34	15.937 a
SSP	-1.00	15.543 ab
SSP	-1.50	15.718 ab
SSP	-2.00	15.293 ab
SSP	-2.50	13.718 b
SSP	-3.00	15.469 ab
SSP	-3.50	15.398 ab
SM	-0.34	20.093 a
SM	-1.00	16.587 a
SM	-1.50	15.550 a
SM	-2.00	17.293 a
SM	-2.50	16.931 a
SM	-3.00	16.906 a
SM	-3.50	13.468 a

[1]Means of area under disease progress curve values caused by mycelia produced on metrically amended SPDA using agar.

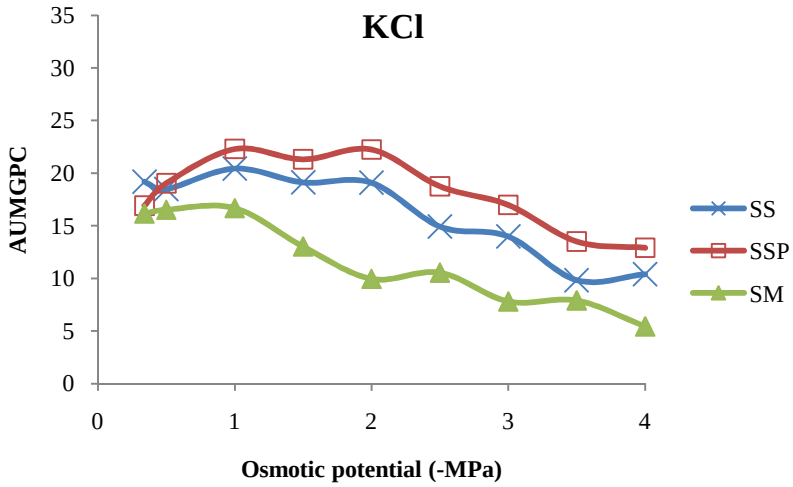
[2]SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*.

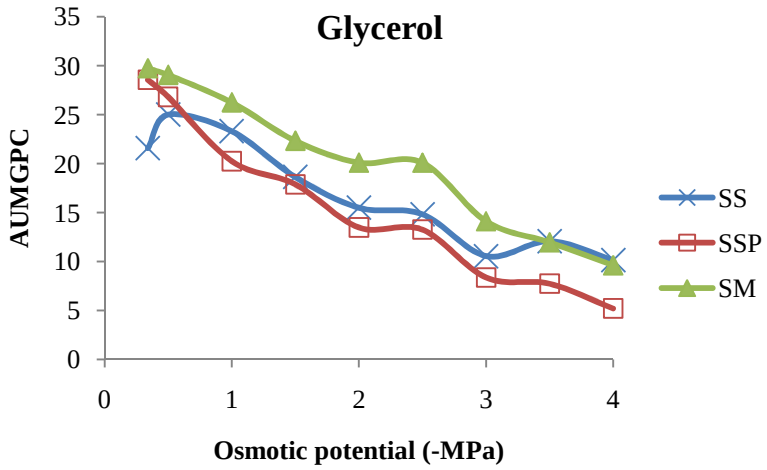
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance..

Table 10.1. Mean area under disease progress curve (AUDPC) on okrun cultivar under water stress and infected by *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor*.

Isolates	Water stress in (MPa)	MN AUDPC[1]
SS[2]	0.00	35.406 a[3]
SS	-0.05	35.953 a
SS	-0.15	32.891 ab
SS	-0.30	26.563 abc
SS	-0.49	26.734 abc
SS	-0.73	24.844 bc
SS	-1.03	22.078 c
SS	-1.37	21.031 c
SS	-1.76	21.016 c
SSP	0.00	34.109 a
SSP	-0.05	32.344 a
SSP	-0.15	33.047 a
SSP	-0.30	30.588 ab
SSP	-0.49	30.438 ab
SSP	-0.73	27.766 bc
SSP	-1.03	25.844 cd
SSP	-1.37	23.828 d
SSP	-1.76	38.625 e
SM	0.00	33.463 a
SM	-0.05	30.225 ab
SM	-0.15	29.038 ab
SM	-0.30	24.797 bc
SM	-0.49	19.984 cd
SM	-0.73	19.234 cd
SM	-1.03	18.784 cd
SM	-1.37	13.531 d
SM	-1.76	14.703 d

[1]Means of area under disease progress curve values on Okrun under water stress created by PEG8000.
[2]SS, *S. sclerotiorum* (peanut isolate), SSP, *S. sclerotiorum* (pumpkin isolate), and SM, *S. minor*
[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.





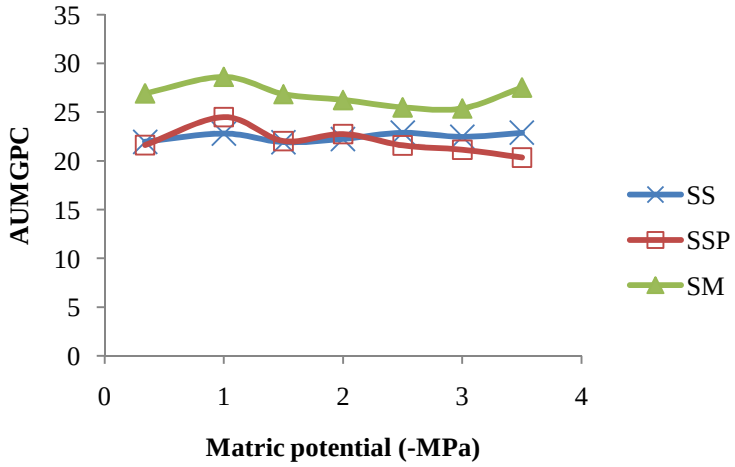


Fig 1. Trend of mycelial growth (as indicated by AUMGPC) of *S Sclerotiorum*, peanut isolate (SS), *S. sclerotiorum*, pumpkin isolate (SSP), and *S. minor* (SM) on potato dextrose agar adjusted to different osmotic and matric water potentials with KCl, glycerol, and agar.

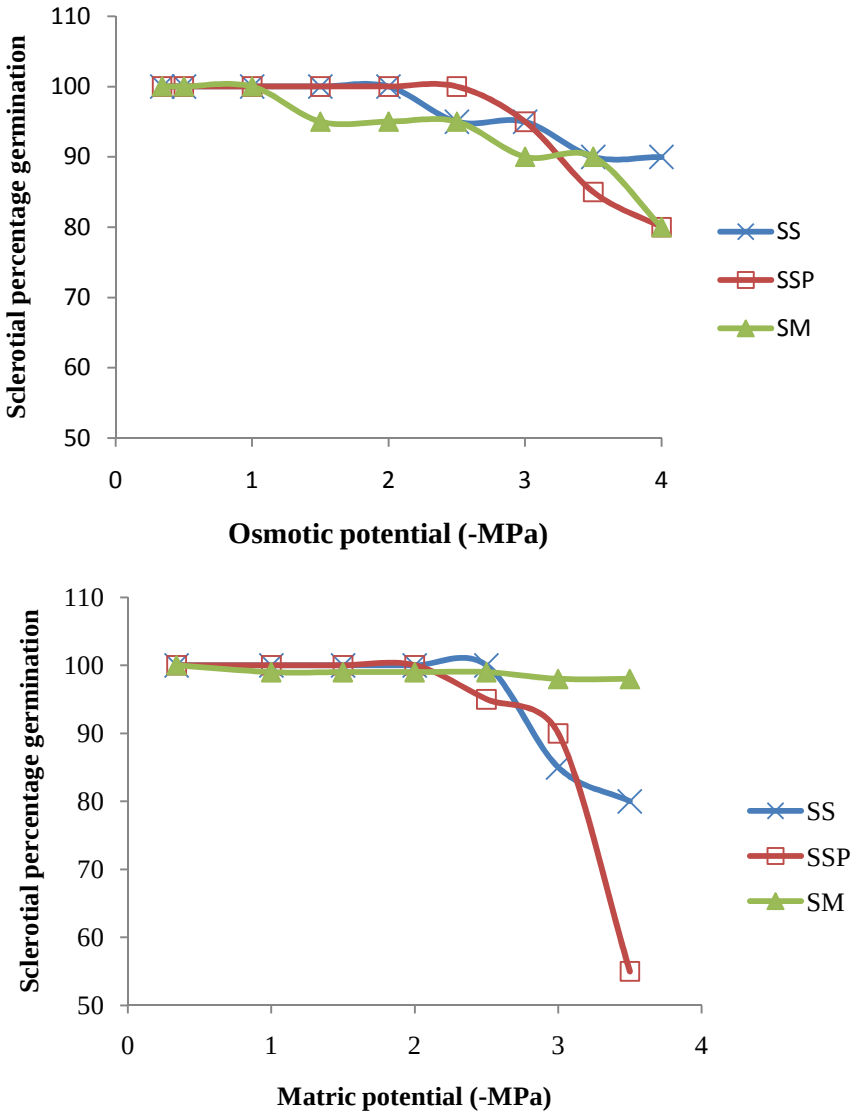


Fig 2. Trend of sclerotial percentage germination of *S Sclerotiorum*, peanut isolate (SS), *S. sclerotiorum*, pumpkin isolate (SSP), and *S. minor* (SM) on potato dextrose agar adjusted to different osmotic and matric water potentials with KCl and agar.

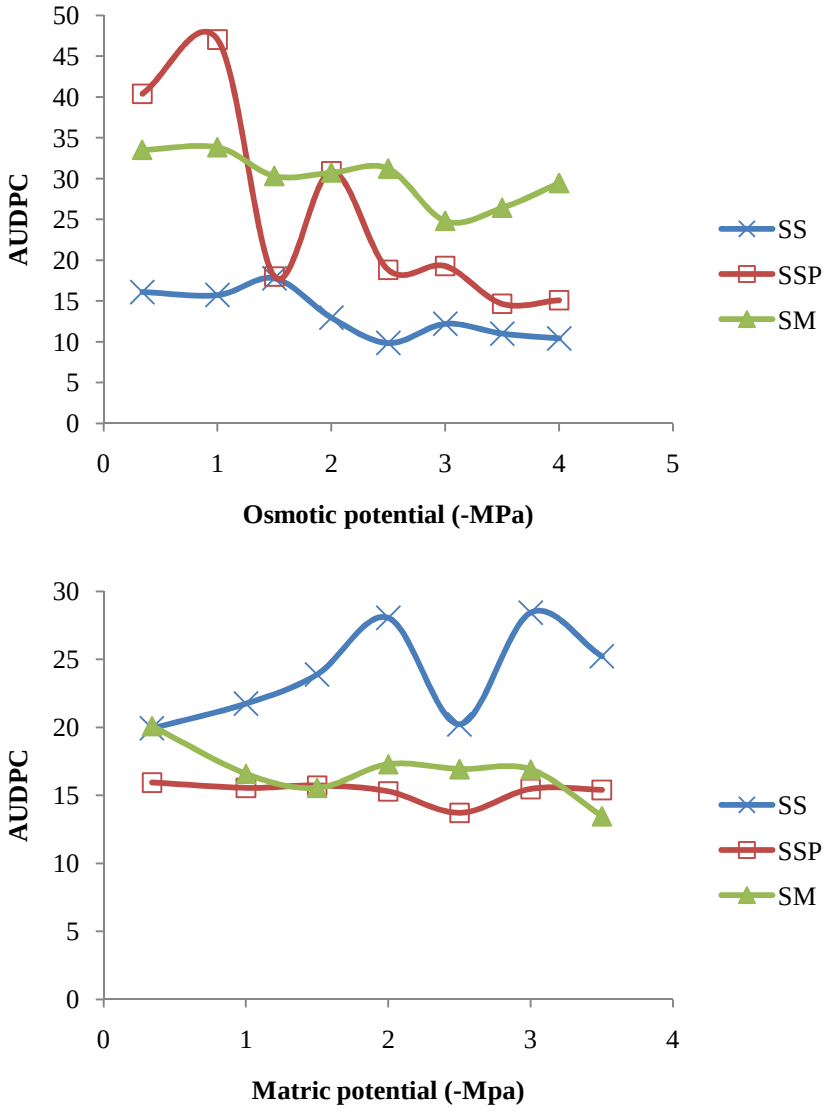


Fig 3. Area Under Disease Progress Curve (AUDPC) caused by S *Sclerotiorum*, peanut isolate (SS), *S. sclerotiorum*, pumpkin isolate (SSP), and *S. minor* (SM) grown on potato dextrose agar adjusted to different osmotic and matric water potentials with KCl and agar, respectively.

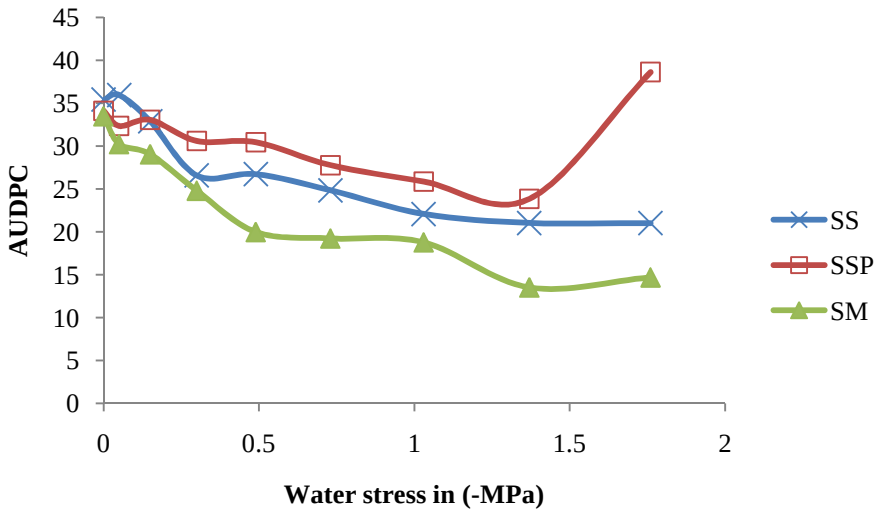


Fig 4. Mean area under disease progress curve (AUDPC) on Okrun cultivar water stressed by PEG 8000 and infected by *S. sclerotiorum* (peanut isolate), *S. sclerotiorum* (pumpkin isolate) and *S. minor*.

CHAPTER III

EFFECTS OF OSMOTIC AND MATRIC POTENTIALS ON *PYRENOPHORA TRITICI-REPENTIS.*

ABSTRACT

The effects of osmotic and matric water potential on mycelial growth, conidia production germination, and pseudothecia production and maturation of *Pyrenophora tritici-repentis* (PTR), the cause of tan spot of wheat, were examined on clarified V8 juice agar amended with KCl or polyethylene glycol 8000 (PEG8000).Patterns of the growth responses of three isolates to decreasing osmotic and matric potentials were similar for KCl and (PEG8000), respectively. Compared with growth on non-amended CV8 agar (-0.24 MPa), growth of all isolates significantly decreased as osmotic and matric potentials reduced to -4.0 MPa and -2.0 MPa, respectively. Conidia production and germination decreased in response to the reduction in osmotic and matric potentials. All isolates produced pseudothecia on wheat straw at all water potentials created by PEG8000 over the range of -0.29 to -2.0 MPa. Mycelial growth, conidia production, germination and pseudothecia production have not been inhibited at any of the osmotic and matric potentials used in this study. Treatment with various concentrations of PEG8000 was used to simulate water stress on the wheat cultivar (TAM105) in the second week after

planting, followed by inoculation with PTR when three leaves were fully expanded. Increasing water stress on TAM105 was associated with a greater susceptibility to tan spot.

INTRODUCTION

Tan spot or yellow leaf spot is an economically important foliar disease of wheat. It occurs worldwide in most major wheat growing areas. Tan spot was detected in the United States in New York in 1940 and in Kansas in 1947 (Watkins *et al*, 1978). By the end of the 1970's tan spot was detected and became a major disease on wheat in Oklahoma and the southern plains of the United States (Hunger and Brown, 1987). In Canada, the first serious outbreak occurred in 1974 (Ciuffetti and Tuori, 1999).The first foliar symptoms of tan spot appear as small, light brown blotches that develop into oval–shaped, necrotic lesions bordered with a chlorotic yellow halo (Schilder and Bergstrom, 1993). Necrosis typically begins near the tip and progresses towards the base of the leaf. As lesions age, they merge and cause senescence of the entire leaf.

Pyrenophora tritici-repentis (Died.) Drechs. (synonym *P. trichostoma* (Fr.) Fuckel), anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker (Synonym *Helminthosporium tritici-repentis* Died.), is a homothallic ascomycete that is the causal agent of tan spot on bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*) (Pfender *et al*., 1991). This fungus produces a multi-nucleated (haploid) mycelium with cross walls. It produces sexual and asexual spores. The sexual spores or the perfect stage are called ascospores.

They form within asci, which are formed when the female sex cell, called an ascogonium, is fertilized by either an antheridium or a minute male sex spore called spermatium. The fertilized ascogonium produces one to many ascogenous hyphae, the cells of which contain two nuclei, one male and one female. The cell at the tip of each ascogenous hyphae develops into an ascus, in which the two nuclei fuse to produce a zygote, which then undergoes meiosis to produce four haploid nuclei. The cell containing these nuclei elongates, and all four nuclei, like in most Ascomycetes, undergo mitosis and produce eight haploid nuclei. Eventually, each nucleus is surrounded by a portion of the cytoplasm and is enveloped by a wall to become an ascospore.

Usually, there are eight ascospores in each ascus. Asci are formed directly in cavities within a stroma or matrix of mycelium which is called a pseudothecium or an ascostroma which is black with double walls. Pseudothecia are 0.2 to 0.35 mm in diameter with dark spines surrounding the short beaks (Zillinsky 1983). Ascospores are brown with three transverse septa and are oval to globose (Ciuffetti and Tuori, 1999). The asexual spores, (anamorph, or the imperfect stage) are called conidia and are born on septate conidiophores measuring 80 to 400 x 6 to 9 μm with a swollen base. The conidia are subhyaline, cylindrical, with four to seven septa and 80 to 250 x 14 to 20 μm in size.

On potato dextrose agar (PDA), pathogen growth is a dense, fluffy, greenish-grey mycelium without sporulation (Schilder and Bergstrom, 1993). When grown on V8 juice agar (CV8), the mycelium is white to light grey. To produce conidia, cultures on CV8 are exposed to UV light for 12 to 24 hours, followed by 12 to 24 hours of darkness (Schilder and Bergstrom, 1993).

The fungus survives through summer, fall and winter primarily as pseudothecia on wheat straw residue on the soil. The disease has become prevalent in several regions of the world, including the Central Plains area of the United States. This is in part due to the widespread employment of conservation-tillage farming, in which crop residue is retained on the soil surface between seasons to reduce erosion losses. Ascospores released from pseudothecia are the primary source of inoculum. They discharge from pseudothecia on wheat straw under humid conditions at night (Rees and Platz, 1980) in late winter and early in the spring and infect the lower leaves. Secondary infection on upper leaves is caused by conidia and this infection is directly related to yield losses (Rees and Platz, 1983).

McMullen and Hosford (1989) stated that fungal conidial spores germinate and infect leaves over a wide range of temperatures when leaves are wet. Severe spots usually occur on susceptible varieties when leaves are wet for 12 hours, but 18 to 24 hours may be needed for more resistant varieties. Resistance to tan spot is partly affected by temperature and nitrogen availability (Duveiller and Dubin, 2002). Spores of fungi usually have a low level of respiration and metabolic activity. The presence of substrates such as cereal residues or other nutrients results in a transformation of spores to an active phase characterized by adsorption of water, increase in respiration and biosynthesis of cell components (Magan, 1988).

Morphological changes including germ tube formation and elongation occur and ultimately an active vegetative mycelium is formed. This process is influenced by stress imposed by water availability. Spores of fungal species able to overcome such stress

would have maximum ecological advantage, resulting in preferential colonization and exploitation of substrata (Magan, 1988).

The effect of water potential on the maturation of pseudothecia on wheat straw has not been investigated before. In soil and in cereal crop residues matric potential is the major component of the total water potential (Griffin, 1981). The matric potential affects growth of soil fungi and maturation of fungal spores on residues more than osmotic potential (Griffin, 1981) No work has been done before to compare the effect of osmotic and matric potential on the maturation and viability of the pseudothecia of PTR. Also, the effect of water potential on mycelial growth, conidia formation, and germination on artificial media *in vitro*, has seldom been considered.

Better understanding of the interaction between abiotic factors and pseudothecia maturation and conidia sporulation and germination is important to developing improved control programs. Hence, the objectives of this research are to (1) Determine the role of water potential on mycelial growth, conidia formation and germination of PTR; (2) Determine the effect of water potential on initiation and maturation of pseudothecia of PTR on wheat straw; and (3) Investigate the impact of water stress on the infection of wheat by PTR.

MATERIALS AND METHODS

Role of water potential on the mycelial growth, conidia formation and germination of *Pyrenophora tritici-repentis*.

Three isolates, RBB6, OKD2, and OK-06-3 were used in this study. The three isolates were collected from Oklahoma in 1996, 1983 and 2006, respectively. The isolates were maintained on PDA (200 g potato, dextrose 20 g, agar 15 g in 1 L water)

acidified to pH 5 with 250 mg chloramphenicol to suppress bacterial growth. Mycelial plugs (5-mm diameter) were excised from the advancing margin of each *P. tritici-repentis* isolate grown for 6 days on PDA. The plugs were placed in the center of 9 cm diam Petri dishes filled with 15 ml of clarified V8 juice agar (200 ml of V8 juice®, 3 g $CaCO_3$, 20 g agar and 800 ml distilled water). CV8 used was osmotically modified over the range of -0.5 to -4 MPa with KCl (Ritchie *et al*, 2006).

Total water potential was the sum of the water potential of the CV8 (-0.24) MPa and the osmotic potential of the added osmoticum. Osmotic potential was calculated according to (Liddell, 1993). CV8 also was adjusted matrically over the range -0.29 to -2.0 MPa using polyethylene glycol 8000 (PEG 8000) (Union Carbide Chemicals and Plastics, Danbury, CT) (Michel and Kaufmann 1973, Magan 1988). It previously has been shown that the water potential generated by PEG 8000 is predominantly (99%) due to matric forces (Steuter *et al*., 1981). The actual osmotic and matric potential of all media were checked using a Vapor Pressure Osmometer (VAPRO 5520, Wescor, Utah, USA). All media were sterilized for 20 minutes. The plates were sealed with parafilm, placed inside plastic bags and incubated for 5 days at 21±2 ºC.

Radial growth was measured by averaging the length of two opposite diameters and substracting 5 mm from each reading. Five replicates (plates) were used for each treatment. The experiement was repeated once. These same CV8 plates were used to determine conidial production. Ten drops of sterile water was added to each plate and mycelia were mated down using a sterile bent glass rod. Plates were then kept in the incubator for 12 hr at 23 ºC with cool-white fluroscent tubes (40 W, 30 μEs^{-1} m^{-1}) to produce conidiophores. This was followed by 12 hr dark at 16 ºC to induce conidia

production (Raymond and Bockus, 1982). Conidia were harvested by flooding each plate with 15 ml of distilled water and dislodging the conidia with a bent glass rod. The resulting suspension was filtered through cheesecloth (Moreno *et al*, 2008). One ml of conidial suspension was pipetted into a segmented petri plate (40 mm) and examined using a stereomicroscope to determine the number of conidia produced.

To determine germination of conidia, 1 ml of conidial suspension was added to 9 ml sterile water amended to the corresponding osmotic or matric potential using KCl and PEG 8000, respectively. Solutions were left at room temperature. After 4-6 hours, 1 ml of each solution was pipetted into a segmented petri plate (40 mm), and a compound microscope was used to determine germination. Spores were considered germinated when the germ-tube length was equal to or longer than the diameter of the spore (Ramirez *et al*., 2004).

Determine the effect of water stress on initiation and maturation of pseudothecia of *Pyrenophora tritici-repentis* on wheat straw

To determine the effect of water stress on pseudothecia production by each isolate on wheat straw, the procedure of James *et al* (1991) as modified by Kazi Kader (Oklahoma State University, personal communication) was followed. Wheat straw collected from the field was cut into pieces (80 mm long) and autoclaved. Three pieces of (9cm) sterilized Whatman filter paper were placed in petri dishes (9cm) dia and 20 ml of each PEG 8000 solution was added to the sterilized filter papers in concentrations as listed in (Table 1) to create different water potentials. Five pieces of wheat straw were placed parallel to each other on the filter papers. Then, three (5 mm) dia mycelia plugs of each isolate of PTR were placed between the wheat straws. Petri plates were sealed with

parafilm to prevent water evaporation. Plates were placed in dark at 21 ºC for two weeks and then transferred to an incubator with 12 h light (30 $\mu Es^{-1} m^{-1}$) and 12 h dark periods at 15 ºC for 24 days.

The total number of pseudothecia and mature pseudothecia per wheat straw were counted. A pseudothecium was considered mature only if at least one mature ascospore was found as indicated by the presence of pigmentation and clear septation using a compound microscope (Friesen *et al*., 2003). The experiment was conducted once in a randomized complete block design with four replicates.

The effect of water stress on the infection of wheat by *Pyrenophora tritici-repentis*

The cultivar TAM 105, which is a tan spot susceptible cultivar, was used in this study. Seedlings were raised in commercially prepared 'Ready-Earth' soil (Sun Gro Co., Bellevue, WA) in 6-inch x 1.5 in dia plastic cylinders. To apply the water stress, PEG 8000 solutions of various water potentials were prepared according to (Michel and Kaufmann, 1973) (Table 1). Four pots (replicates) per water potential (treatment) were used. Plants were divided into nine groups (eight different water potentials plus the water control). Each group of plants was placed in a plastic tray (38.1 cm x 29.2 cm x 15.2 cm) (Sterilite, Townsend, MA, USA). Water stress was applied to plants in the second week by pouring each PEG 8000 solution into the bottom of its assigned plastic tray. In the water control group, water was used to keep seedlings well irrigated.

Conidia were produced on CV8 as described above. A conidial suspension was adjusted to 2 x 10^3 conidia ml^{-1} and 0.05% Tween ® 20 was added as a surfactant. Seedlings with three leaves fully expanded were inoculated with the conidial suspension of each isolate using an atomizer (DeVilbiss Co., Somerest, PA) following the procedure

of Rodriguez and Bockus (1996). Inoculated plants were allowed to dry for 30 min so conidia adhered to leaves and then were placed in a mist chamber at 21 ºC and a light:dark cycle of 14 (510 $\mu Es^{-1}m^{-1}$):10 hr. After 48 hr, plants were placed in a greenhouse at 21ºC. The disease reaction was recorded six days post-inoculation using the rating system of Lamari and Bernier (1989).

The rating system is described as follows: 1 = small dark brown to black spots without any surrounding chlorosis or tan necrosis, 2 = small dark brown to black spots with very little chlorosis or tan necrosis (moderately resistant), 3 = small dark brown to black spots completely surrounded by a distinct chlorotic or tan necrotic ring (lesions generally not coalescing), 4 = small dark brown or black spots completely surrounded with chlorotic or tan necrotic zones (some of the lesions coalescing), 5 = the dark brown or black centers may or may not be distinguishable (most lesions consist of coalescing chlorotic tissues or tan necrotic zones).

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) and treatment means were compared using the least significant difference (LSD) at a probability of 5% (P=0.05). Statistical analysis was performed using SAS 9.1 software (SAS Institute, Inc., Cary, NC).

RESULTS

Mycelial growth, conidia formation and germination of *Pyrenophora tritici-repentis* on CV8 agar amended to various water potentials.

Osmotic (ψ_s) and matric (ψ_m) potentials significantly (P=0.05) affected the vegetative growth of PTR (Tables 2 and 3). Mycelial growth responses of the three PTR

isolates to decreasing ψ_s and ψ_m potentials were similar. Area under mycelial growth curve (AUMGC) decreased when ψ_s and ψ_m potentials decreased. In ψ_s studies, there was no significant (P=0.05) differences among AUMGC values of RBB6 isolate at the control, -0.5 and -1.0 MPA. For OK-06-3 and OKD2, there was no significant (P=0.05) differences among AUMGC values at the control and -0.5 MPa. In general, there is a trend toward lower AUMGC values with increasing osmotic stress (Table 2). OK-06-3 had greater growth than RBB6 and OKD2 over the different osmotic potentials (Table 2).

In ψ_m studies, greatest AUMGC values were observed for the control (-0.24 MPa) for isolates OK-06-3 and OKD2, and for the control and at -0.29 MPa for isolate RBB6. Smallest AUMGC values were recorded at -2.00 Mpa for RBB6 and OK-06-3 and at -1.61 and -2.00 MPa for isolate OKD2.

Osmotic potential significantly (P=0.05) affected conidia production of the three PTR isolates on CV8 (Table 4). RBB6, OK-06-3, and OKD2 produced the greatest number of conidia at the control and -0.5 MPa. The fewest conidia were produced by the three isolates at -4.0 MPa. However, there was no significant difference between -3.5 and -4.0 MPa (Table 4). Osmotic potential did not significantly (P<0.05) affect conidial germination of RBB6 and OK-06-3, but significantly (P=0.05) affected conidia germination of OKD2 (Table 4). Obviously there was a trend toward lower conidia number and germination with increasing osmotic stress (Table 4).

The three isolates behaved similarly in response to decreasing matric potential (Table 5). The greatest number of conidia was produced by the three isolates at control and -0.29 MPa, while the fewest were produced on -2.0 MPa (Table 5). Matric potential significantly (P=0.05) affected conidia germination of RBB6 and OKD2, but did not

affect conidia germination of OK-06-3 (Table 5). Similar trend of lowering conidia number and germination observed in response to matric stress increase.

The effect of water stress on formation and maturation of pseudothecia by *Pyrenophora tritici- repentis* on wheat straw.

A downward trend of pseudothecia production by RBB6, OK-06-3, OKD2 was observed when the matric stress increased (Table 6). However, matric potential significantly (P=0.05) affected pseudothecia production on wheat straw by OK-06-3 and OKD2 but not by RBB6 (Table 6). Different matric potentials significantly (P=0.05) affected the pseudothecia maturation for RBB6 and OK-06-3 isolates, while no significant differences (P<0.05) occurred with OKD2. A similar downward trend of pseudothecia maturation by the three isolates of PTR was observed in response to matric stress increase (Table 6).

The effect of water stress on the infection of wheat by *Pyrenophora tritici-repentis*.

Increasing water stress imposed on TAM 105 was associated with a greater susceptibility to tan spot as indicated by greater disease reactions associated with the greatest three water stresses ranging from -1.03 to -1.76 MPa (Table 7). This was consistently observed with all three isolates.

DISCUSSION

Most research investigating the effect of water potential on the biology of plant pathogenic fungi has focused on soil-borne pathogenic fungi. Few studies have been done in the past investigating the effect of water potential on the biology of air-borne fungi such as *P. tritici repentis*. The likely rationale for this is that air-borne fungi are not in direct contact with soil, and it is in the soil where water potential effects are most

commonly considered. However, a foliar pathogen such as PTR survives on plant residue between agricultural seasons, and the involvement of water potential and its components on such a system may significantly effect survival and infection by the pathogen. In this regard, some research has been conducted studying the presence of microbial antagonists on PTR and the interaction between PTR and potential antagonists on wheat straw under different environmental factors (Pfender *et al*., 1991; Pfender *et al*., 1988; Pfender 1988; and Summerell and Burgess 1989). However, there is no available data in the literature about the effect of osmotic and matric potentials directly on mycelial growth, conidia production and conidia germination of PTR.

In our study, area under mycelia growth curve (AUMGC) values of the three isolates decreased when osmotic (ψ_s) and matric (ψ_m) potentials decreased (i.e, the stress increased). However, mycelia growth of the three isolates was not totally inhibited at any of the ψ_s and ψ_m potentials used in this study. The response of mycelia growth of the three isolates was similar to decreasing osmotic and matric potentials. We do believe that the observed responses were caused by changes in osmotic and matric stresses rather than by toxicity of KCl or PEG 8000 (Ferrin and Stanghellini, 2006). Toxic effects of KCl and PEG 8000 could cause inconsistency in the mycelial growth of the three isolates among different treatments which has not been observed. Ionic solutes such as KCl and NaCl have been used in several water potential studies involving various plant pathogenic fungi such as *Fusarium moniliforme* (Woods and Duniway, 1986) and *Verticillium dahlia* (Ioannou *et al*, 1977).). KCl also has been used in many studies investigating the effect of matric potential on several soil plant pathogenic fungi such as *Rhizoctonia solani* (Ritchie *et al*., 2006) and *Fusarium graminearum* (Ramirez *et al*., 2004).

The water potential generated by PEG 8000 is predominantly (99%) due to matric forces (Steuter *et al*., 1981). The ability of a fungus to grow under osmotic stress and the exact optimal water potential depends on the fungus species and in some cases on the osmoticum, temperature, or other factors in the environment (Cook and Al-Hamadani, 1986). Mycelial growth under KCl osmotic stress may result from uptake of potassium ions and its accumulation by microbial cells, which lower the water potential of the protoplasm to a value more ideal for cellular processes or may increase turgor and hence acceleration of growth (Olaya *et al*., 1996). In this study, the mycelial growth of the three isolates of *P. tritici repentis* were not inhibited at ψ_s and ψ_m potentials below -1.5 MPa, which is lower than the permanent wilting point of mesophytic higher plants, which is approximately -1.5 MPa (Slayter, 1967).

The conidia produced and germination of conidia by the three isolates were reduced significantly in response to increase ψ_s and ψ_m stresses. The reduction of conidia produced may be correlated to reduction of mycelial growth.

Tan spot is a disease favored in wheat produced under conservation tillage because PTR completes its life cycle on wheat residue. Conservation tillage, in which crop residue is left on the soil surface between cropping seasons to reduce soil and water loss, is becoming increasingly common (Pfender, 1988). The production of pseudothecia, and number of mature ascospores per ascus are important to tan spot epidemics. Survival of PTR on and in infested straw differs with its position (i.e. buried or on soil surface), or microenvironment, in the field (Pfender *et al*, 1991). In a study of fungal communities associated with conservation-tillage wheat straw in Kansas, Pfender and Wootke (1988) found that the fungus persisted in straw retained on a mulch layer above the soil surface.

It was rarely recovered from buried straw or straw retained for several months directly on the soil surface beneath the mulch layer. In this study, pseudothecia number and maturation of pseudothecia produced by PTR in artificially infested wheat straw stored without soil contact decreased significantly (P=0.05) when the matric potential decreased.

In soil and cereal crop residue, matric potential is the major component of the total water potential (Magan and Lynch 1986). Griffin (1981) suggested that matric potential would affect growth of soil fungi more than osmotic potential. High water potential is not in itself detrimental to growth or pseudothecia production by PTR. Summer and Burgess (1988) reported that the fungus requires water potentials above approximately -1.5 MPa for pseudothecia production on osmotically adjusted agar or on adjusted wheat residue. Although maximal growth of this pathogen occurs at high water potential (i.e., less water stress); it can grow in wheat residue at water potentials as low as -8.5 MPa (Pfender *et al*., 1988).

Growth at such low water potentials could enable PTR to avoid competition from micro-organisms more limited in their moisture stress tolerance. PTR on wheat straw buried in soil have been displaced by actinomycetes and soil borne fungi than on straws on soil surface (Pfender, 1988). Nevertheless, because of the relatively high water potential requirement for pseudothecia production, PTR must at least occasionally interact with micro-organisms under wet conditions if it is to produce its primary inoculum (Pfender *et al*., 1991).

Whether a disease develops depends upon the influence of environmental factors on the genetically controlled response of the host plant to the presence of the pathogen or its metabolites. The tendency of non-genetic factors, acting prior to infection, to affect the

susceptibility of plants to disease is called predisposition (Schoeneweiss 1975). In the course of their development plants may frequently be exposed to temporary water deficiency which is intrinsic to most abiotic forms of stress, not only during drought, but also at low temperature and when the soil contains high concetrations of ions. This can occur not only in arid and semi-arid regions, but also under continental climatic conditions (Hoffmann and Burucs, 2005).

Various plant varieties differ in their ability to survive long periods of water deficiency and in the strategies they employ to counteract the adverse effects of water stress. This depends primarily on the water use efficiency of the variety and on its genetically determined drought tolerance (Janda *et al*., 2008). In the present study, water stress predisposed TAM 105 seedlings to infection by PTR. Disease severity on the wheat variety TAM 105 increased when water stress increased. Major changes in climate over a period of years have been implicated as stress factors affecting the incidence and severity of many diseases (Schoeneweiss 1975). In USA, Ash dieback, maple decline, sweetgum blight, birch dieback, oak decline, dry face of slash pine, and pitch streak of slash pines have been associated with an extended period of below normal precipitation in the 1930s (Schoeneweiss 1975). Short term droughts of days or weeks during the growing season may also predispose plants to diseases (Cook 1973).

In a similar study, Janda *et al* (2008) studied the effect of water stress on winter wheat seedlings using PEG under greenhouse conditions. They reported a significant increase in the susceptibility of a resistant wheat variety (M-3) and a susceptible variety (Bezostaya) to tan spot in response to water stress created by 20% PEG. They concluded that a high level of drought stress may cause a reduction in the level of resistance. In

another study, Beddis and Burgess (1992) found that *Fusarium graminearum* was able to colonize water stressed wheat seedlings to a greater height than seedlings grown under non-stress conditions. Water stress like other abiotic stresses may increase the concentration of reactive oxygen species (ROS), which may cause damage to macromolecules, leading to the death of the cells (Janda *et al*., 2008). The stress response can also vary depending on the developmental stage during which wheat is subject to stress (Pereyra and Torroba, 2003).

In conclusion, low water potential (high stress) decreased vegetative growth, conidia production, conidia germination, pseudothecia production and pseudothecia maturation on wheat straw without soil contact but increased water stress predisposed wheat seedlings (TAM 105) to the infection by PTR. In no till systems in dry years, the pathogenicity parameters that allow PTR to survive on wheat straw may be negatively affected. Future work should be extended to the field and include more wheat varieties to to develop residue management and biological control procedures for reducing primary inoculum (i.e., pseudothecia).of PTR in conservation-tillage wheat residue.

LITERATURE CITED

1- Ciuffetti, L. M. and Tuori, R. P. 1999. Advances in the characterization of the *Pyrenophora tritici-repentis* wheat interaction. *Phytopathology* 89: 444-449.

2- Cook, R. J. 1973. Influence of low plant and soil water potentials on diseases caused by soilborne fungi. *Phytopathology* 63:451-458.

3- Cook, R. C. and Al-Hamadani, A. M. 1986. Water relations of sclerotia and other infective structures. Pages 49-63 in: *Water, Fungi and Plants*. P. G. Ayres and L. Boddy, eds. Cambridge University Press, Cambridge.

4- Dallyn, H. and Fox, A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. In: Gould, G. H., Corry, E. L., eds. *Microbial growth and survival in extreme environments*. London and New York: Academic Press. P 129-139.

5- Duveiller, E. and Dubin, H. J. Helminthosporium leaf blights: spot blotch and tan spot. 2002. In *Bread wheat, improvement and production* (B.C. Curtis, S. Rajaram and H. Gómez Macpherson, eds). FAO Plant Production and Protection Series.30, 1-6.

6- Ferrin, D. M. and M. E. Stanghellini. 2006. Effect of water potential on mycelial growth and perithecial production of *Monosprascus cannonballus* in vitro. *Plant Pathology* 55: 421-426.

7- Friesen, T. L., Ali, S., kianian, S., Francl, L. J. and Rasmussen, J. B. 2003. Role of host sensitivity to Ptr ToxA in development of tan spot of wheat. *Phytopathology* 93, 397-401.

8- Griffin, D. M. 1981. Water and microbial stress. In *Advances in Microbial Ecology* (ed. M. Alexander). PP. 91-136. Plenum Publishing Co.

9- Hoffmann, B. and Burucs, Z. 2005. Adaptaion of wheat (Triticum aestivum L.) genotypes and related species to water deficiency. *Cereal Research communications* 33:681-687.

10- Hunger, R. M. and Brown, D. A.1987. Colony color, growth, sporulation, fungicide sensitivity, and pathogenicity of *Pyrenophora tritici-repentis*. *Plant Disease* 71: 907-910.

11- Ioannou, N., Scheider, R. W., Grogan, R. G. and Duniway, J. M.1977. Effect of water potential and temperature on growth, sporulation, and production of microsclerotia by *Verticillium dahlia*. *Phytopathology* 67:637-644.

12- James, T. D. W., Summerell, B. A. and Burgess, L. W. 1991. Production of pseudothecia and conidia by Pyrenophora tritic-repentis in relation to nutrients and substrate. *Australian Plant Pathology* 20, 92-96.

13- Lamari, L. and Bernier, C. C. 1989. Evaluation of wheat lines and cultivars to tan spot (*Pyrenophora tritici-repentis*) based on type lessions. *Canadian Journal of Plant Pathology* 11, 49-56.

14- Liddell, C. M. 1993. Measurement and control of soil temperature and water potential. In: Singleton, L. L., Mihail, J. D., Rush, C. M, eds. Methods for research on soilborne phytopathogenic fungi. Eds,. *The American Phytopathological society,* St. Paul, Minnesota.

15- Loughman, R. and Deverall, B. J. 1986. Infection of resistant and susceptible cultivars of wheat by *Pyrenophora tritici-repentis*. *Plant Pathology* 35: 443-450

16- Magan, N. 1988. Effects of water potential and temperature on spore germination and germ-tube growth *in vitro* and on straw leaf sheaths. *Transactions of the British Mycological Society* 90 (1): 97-107.

17- Magan, N. and Lynch J. M. 1986 Water potential, growthand cellulolysis of fungi involved in decomposition of cereal residues. *Journal of general applied microbiology* 132: 1181-1187.

18- McMullen, M. P. and Hosford, D. R. 1989. Tan spot of wheat. NDSU extension service pp-766. Fargo, ND, USA. 3 pp.

19- Michel, B. E. and Kaufmann, M. R. 1973. The osmotic potential of polyethylene glycol 6000. *Plant Physiology* 51: 914-916.

20- Moreno, M. V., Stenglein, S. A., Balatii, P. A. and Perell, A. E. 2008. Pathogenic and molecular variability among isolates of *pyrenophora tritici-repentis* agent of tan spot of wheat in Argentina. *European Journal of Plant Pathology* 122 (2): 239-252.

21- Olaya, G. and Abawi, G. S. 1996. Effect of water potential on mycelial growth and on production and germination of sclerotia of *Macrophomina phaseolina*. *Plant Disease* 80: 1347-1350.

22- Pereyra, M. and Torroba, M. del C. 2003. Molecular and physiological responses to water deficit in wheat (*Triticum aestivum* L.). Rev. Fac. Agronomia. 14:13-22.

23- Pfender, W. F. 1988. Suppression of ascocarp formation in *Pyrenophora tritici-repentis* by Limonomyces roseipellis, a Basidiomycete from reduced-tillage wheat straw. *Phytopathology* 78: 1254-1258.

24- Pfender, W., Sharma, U. and Zhang, W. 1991. Effect of water potential on microbial antagonism to *Pyrenophora tritici-repetis* in wheat residue. *Mycological research* 95: 308-314.

25- Pfender, W. F. and Wootke, S. L. 1988. Microbial communities of Pyrenophora-infested wheat straw as examined by multivariate analysis. *Microbial Ecology* 15: 95-113.

26- Ramirez, M. L., Chulze, S. N. and Magan, N. 2004. Impact of osmotic and matric water stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugar alcohols in *Fusarium graminearum*. *Mycologia* 96: 470-478.

27- Raymond, P. J. and Bockus, W. W. 1982. An in-vitro technique for profuse sporulation of *Drechslera tritici-repentis*. (Abstr). *Phyotopathology* 722, 934.

28- Rees, R. G. and Platz, G. J. 1983. Effects of yellow spot on wheat: comparison of epidemics at different stages of crop development. *Australian Journal of Agricultural Research* 34: 39-46.

29- Ritchie, F., McQuilken, M. P. and Bain, R. A. 2006. Effect of water potential on mycelial growth, sclerotial production, and germination of *Rhizoctonia solani* from potato. *Mycological Research* 110: 725-733.

30- Rodriguez, R. and Bockus, W. 1996. Differences among isolates of Pyrenophora tritici-repentis in production of conidia on wheat leaves. *Plant Disease* 80: 686-694.

31- Schilder, A. M. C. and Bergstrom, G. C. 1993. Tan spot. In S. B. Mathur and B. M. Cunfer, eds. *Seedborne diseases and seed health testing of wheat,* P. 113-122. Copenhagen, Denmark, Jordburgsforlaget.

32- Schoeneweiss, D. F. 1975. Predisposition, stress, and plant disease. Annual Review of *Phytopathology* 13: 193-211.

33- Slayter, R. D. 1967. Plant-water relationships. Academic Press, New York.

34- Steuter, A. A., Mozafar, A. and Goodin JR. 1981. Water potential of aqueous polyethylene glycol. *Plant Physiology* 67:64-67.

35- Strelkov, S. E., Lamari, L., Sayoud, R. and Smith, R. B. 2002. Comparative virulence of chlorosis-inducing races of *Pyrenophora tritci-repentis.Canadian Journal of Plant Pathology* 24: 29-35.

36- Summerell, B. A. and Burgess, L. W. 1989. Factors survival of pyrenophora tritici repentis: water potential potential and temperature. *Mycological research* 93: 41-45.

37- Watkins, J. E., Odvody, G. N., Boosalis, M. G. and Partridge, J. E. 1978. An epidemic of tan spot of wheat in Nebraska. *Plant Disease Reporter* 62:132-134.

38- Woods, D. M. and J. M. Duniway. 1986. Some of effects of water potential on growth, turgor, and repiration of *Phytophthora cryptogea* and *Fusarium moniliforme*. *Phytopathology* 76:1248-1253.

39- Zillinsky, F. 1983. Common diseases of small grain cereals, a guide to identification. Mexico, DF, CIMMYT. 141 pp.

Table 1.2. Required concentrations of polyethylene glycol (PEG 8000) solutions to attain corresponding water stress on peanut plants at 25ºC.

% PEG 8000	Osmotic stress in MPa
0	<-0.05
5	-0.05
10	-0.15
15	-0.30
20	-0.49
25	-0.73
30	-1.03
35	-1.37
40	-1.76

Table 2.2. Area under Mycelial Growth Curve (AUMGC) values for *Pyrenophora tritici- repentis* (PTR) grown on clarified V-8 juice (CV8) agar amended to different osmotic potentials (ψ_s) using KCl.

Isolates	Osmotic potentials in Mpa	AUMGC[1]
RBB6	-0.24	33.26 a[2]
RBB6	-0.50	32.99 a
RBB6	-1.00	32.01 a
RBB6	-1.50	30.11 b
RBB6	-2.00	24.59 c
RBB6	-2.50	24.23 c
RBB6	-3.00	19.74 d
RBB6	-3.50	18.25 de
RBB6	-4.00	16.79 e
OK-06-3	-0.24	57.14 a
OK-06-3	-0.50	59.57 a
OK-06-3	-1.00	52.83 b
OK-06-3	-1.50	43.56 c
OK-06-3	-2.00	36.94 d
OK-06-3	-2.50	34.87 d
OK-06-3	-3.00	34.11 d
OK-06-3	-3.50	30.28 e
OK-06-3	-4.00	27.09 e
OKD2	-0.24	35.21 a
OKD2	-0.50	34.43 ab
OKD2	-1.00	32.29 b
OKD2	-1.50	29.80 c
OKD2	-2.00	29.57 c
OKD2	-2.50	25.97 d
OKD2	-3.00	25.85 d
OKD2	-3.50	25.83 d
OKD2	-4.00	23.24 e

[1]AUMGC values for PTR isolates RBB6, OK-06-3, and OKD2 on CV8 agar amended to different osmotic potentials using KCl.

[2] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 3.2. Area under mycelial growth curve (AUMGC) values for *Pyrenophora tritici- repentis* (PTR) grown on clarified V-8 juice (CV8) agar amended to different matric potentials (ψ_m) using polyethylene glycol 8000.

Isolates	Matric potentials in Mpa	AUMGC[1]
RBB6	-0.24	31.28 a[2]
RBB6	-0.29	30.67 ab
RBB6	-0.39	29.30 b
RBB6	-0.54	26.76 c
RBB6	-0.73	24.56 d
RBB6	-0.97	23.16 d
RBB6	-1.27	20.51 e
RBB6	-1.61	19.33 e
RBB6	-2.00	16.46 f
OK-06-3	-0.24	54.49 a
OK-06-3	-0.29	52.15 b
OK-06-3	-0.39	49.85 c
OK-06-3	-0.54	41.49 d
OK-06-3	-0.73	37.67 e
OK-06-3	-0.97	36.74 e
OK-06-3	-1.27	33.52 f
OK-06-3	-1.61	25.19 g
OK-06-3	-2.00	16.12 h
OKD2	-0.24	38.50 a
OKD2	-0.29	32.96 b
OKD2	-0.39	31.78 bc
OKD2	-0.54	30.76 c
OKD2	-0.73	30.25 c
OKD2	-0.97	25.75 d
OKD2	-1.27	24.69 d
OKD2	-1.61	21.62 e
OKD2	-2.00	20.45 e

[1]AUMGC values for RBB6, OK-06-3, and OKD2 isolates of PTR on CV8 agar amended to different matric potentials (ψ_m) using PEG 8000.

[2] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 4.2. Mean conidia number and germination percentage produced by RBBS, OK-06-3, and OKD2 of for *Pyrenophora tritici-repentis* (PTR) grown on CV8 medium amended to different osmotic potentials (ψ_s) using KCl.

Isolate	Osmotic potentials in Mpa	Mean conidia[1]	Conidia germination (%)[2]
RBB6	-0.24	133.6 a[3]	22.47 a[3]
RBB6	-0.50	116.6 a	23.26 a
RBB6	-1.00	83.4 b	22.33 a
RBB6	-1.50	76.8 bc	17.95 a
RBB6	-2.00	63.4 bcd	21.84 a
RBB6	-2.50	56.0 cd	17.05 a
RBB6	-3.00	49.8 de	17.46 a
RBB6	-3.50	44.2 de	19.71 a
RBB6	-4.00	30.4 e	17.41 a
OK-06-3	-0.24	125.0 a	24.26 a
OK-06-3	-0.50	117.0 a	26.49 a
OK-06-3	-1.00	95.0 b	21.80 a
OK-06-3	-1.50	86.2 c	24.62 a
OK-06-3	-2.00	72.8 d	19.70 a
OK-06-3	-2.50	66.8 d	21.23 a
OK-06-3	-3.00	62.6 d	23.08 a
OK-06-3	-3.50	45.8 e	25.74 a
OK-06-3	-4.00	32.2 e	18.19 a
OKD2	-0.24	115.0 a	22.06 a
OKD2	-0.50	112.0 ab	20.06 ab
OKD2	-1.00	98.4 b	20.04 ab
OKD2	-1.50	89.0 c	19.57 abc
OKD2	-2.00	72.8 c	18.32 abcd
OKD2	-2.50	64.6 d	16.16 bcd
OKD2	-3.00	61.8 d	15.01 bcd
OKD2	-3.50	41.6 d	14.52 cd
OKD2	-4.00	24.6 e	13.76 d

[1]Mean conidia per 1 ml produced by RBB6, OK-06-3, OKD2 of PTR on CV8 amended to different osmotic potentials (ψ_s) using KCl.

[2]Percentage of conidia germination.

[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 5.2. Mean conidia number and germination percentage produced by RBBS, OK-06-3, and OKD2 of *Pyrenophora tritici repentis* (PTR) grown on CV8 amended to different matric potentials (ψ_m) using polyethylene glycol 8000.

Isolate	Matric potentials in MPa	Mean conidia number[1]	Conidia germination (%)[2]
RBB6	-0.24	126.2 a[3]	17.94 a[3]
RBB6	-0.29	113.2 a	17.16 a
RBB6	-0.39	90.0 b	17.13 a
RBB6	-0.54	78.6 bc	15.53 ab
RBB6	-0.73	64.6 cd	14.84 ab
RBB6	-0.97	53.4 d	14.50 ab
RBB6	-1.27	48.6 de	13.85 ab
RBB6	-1.61	35.8 e	12.80 ab
RBB6	-2.00	14.8 f	9.22 b
OK-06-3	-0.24	124.8 a	19.39 a
OK-06-3	-0.29	122.8 a	15.32 a
OK-06-3	-0.39	85.6 b	16.90 a
OK-06-3	-0.54	86.2 b	15.96 a
OK-06-3	-0.73	70.0 bc	18.56 a
OK-06-3	-0.97	67.6 c	18.38 a
OK-06-3	-1.27	40.6 d	20.61 a
OK-06-3	-1.61	32.4 de	16.31 a
OK-06-3	-2.00	22.2 e	17.42 a
OKD2	-0.24	116.4 a	16.22 a
OKD2	-0.29	106.0 a	13.18 ab
OKD2	-0.39	84.8 b	13.09 ab
OKD2	-0.54	79.2 bc	12.27 ab
OKD2	-0.73	67.0 cd	11.12 b
OKD2	-0.97	59.6 d	10.52 b
OKD2	-1.27	42.4 e	10.01 b
OKD2	-1.61	29.0 ef	9.96 b
OKD2	-2.00	19.2 f	8.94 b

[1]Mean conidia number per 1 ml produced by three isolates of PTR on CV8 amended to different matric potentials (ψ_m) using PEG 8000.

[2]Percentage of conidia germination.

[3] Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 6.2. Mean pseudothecia number and maturation percentage produced by three isolates of *Pyrenophora tritici repentis* on wheat straw treated with polyethylene glycol 8000 to create different matric potentials (ψ_m).

Isolate	Matric potentials in Mpa	Mean pseudothecia number[1]	Mature Pseudothecia (%)[2]
RBB6	-0.24	55.40 a[3]	9.18 a[3]
RBB6	-0.29	58.00 a	9.06 ab
RBB6	-0.39	42.80 a	6.22 ab
RBB6	-0.54	48.00 a	5.79 ab
RBB6	-0.73	53.00 a	5.57 ab
RBB6	-0.97	40.60 a	5.56 ab
RBB6	-1.27	48.60 a	4.57 ab
RBB6	-1.61	44.80 a	4.17 ab
RBB6	-2.00	40.20 a	4.06 b
OK-06-3	-0.24	82.60 a	13.51 a
OK-06-3	-0.29	72.00 ab	7.26 b
OK-06-3	-0.39	68.00 ab	6.22 b
OK-06-3	-0.54	59.60 bc	4.61 b
OK-06-3	-0.73	37.20 d	4.58 b
OK-06-3	-0.97	41.40 dc	4.41 b
OK-06-3	-1.27	38.20 d	4.03 b
OK-06-3	-1.61	14.60 e	3.33 b
OK-06-3	-2.00	10.40 e	2.91 b
OKD2	-0.24	81.60 a	2.95 a
OKD2	-0.29	73.80 a	1.75 a
OKD2	-0.39	66.60 ab	1.70 a
OKD2	-0.54	56.00 bc	1.54 a
OKD2	-0.73	43.80 cd	1.36 a
OKD2	-0.97	30.60 d	1.19 a
OKD2	-1.27	28.40 de	1.09 a
OKD2	-1.61	11.60 ef	0.96 a
OKD2	-2.00	7.00 f	0.76 a

[1]Mean pseudothecia number produced by RBB6, OK-06-3, and OKD2 of PTRon 5 pieces of wheat straws (80 mm each) treated with PEG 8000 to create different matric potentials (ψ_m).
[2]Percentage of pseudothecia maturation.
[3]Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

Table 7.2. Rating of hard red winter wheat ('TAM 105') to infection by three isolates of *Pyrenophora tritici-repentis* when water stressed using PEG 8000.

PTR Isolate	Water stress in Mpa	Disease rating[1]
RBB6	-0.00	3.85 d[2]
RBB6	- 0.05	4.05 d
RBB6	- 0.15	4.35 c
RBB6	- 0.30	4.40 bc
RBB6	- 0.49	4.30 c
RBB6	- 0.74	4.60 ab
RBB6	- 1.03	4.70 a
RBB6	- 1.37	4.75 a
RBB6	- 1.76	4.70 a
OK-06-3	-0.00	3.80 de
OK-06-3	- 0.05	3.65 e
OK-06-3	- 0.15	3.95 cd
OK-06-3	- 0.30	4.05 bc
OK-06-3	- 0.49	3.95 cd
OK-06-3	- 0.74	4.05 bc
OK-06-3	- 1.03	4.15 abc
OK-06-3	- 1.37	4.25 ab
OK-06-3	- 1.76	4.30 a
OKD2	-0.00	3.25 e
OKD2	- 0.05	3.25 e
OKD2	- 0.15	3.45 cde
OKD2	- 0.30	3.55 bcd
OKD2	- 0.49	3.40 de
OKD2	- 0.74	3.55 bcd
OKD2	- 1.03	3.65 abc
OKD2	- 1.37	3.75 ab
OKD2	- 1.76	3.80 a

[1]Disease reaction is the average value of rating 2 replicates on a scale of 1-5, where 1 = small dark brown to black spots without any surrounding chlorosis or tan necrosis, 2 = Small dark brown to black spots with very little chlorosis or tan necrosis (moderately resistant), 3 = Small dark brown to black spots completely surrounded by a distinct chlorotic or tan necrotic ring (lesions generally not coalescing), 4 = Small dark brown or black spots completely surrounded with chlorotic or tan necrotic zones (some of the lesions coalescing), 5 = most lesions consist of coalescing chlorotic tissues or tan necrotic zones.

[2]Two means in the same column and within the same level of isolate with the same letters are not significantly different at a 0.05 level of significance.

CHAPTER IV

A MULTIPLEX PCR FOR THE MOST IMPORTANT AGRICULTURAL ASSOSIATED SPECIES OF GENUS *SCLEROTINIA*

ABSTRACT

Sclerotinia homeocarpa F.T. Benn, *S. minor* Jagger, *S. sclerotiorum* (Lib.) de Bary, and *S. trifoliorum* Eriks are the most relevant plant pathogenic species within the genus *Sclerotinia* because of their large range of economically important hosts. Species identification based on morphological characteristics is challenging and time demanding, especially when one crop hosts multiple species. The objective of this study was to design specific primers compatible with multiplexing, for rapid, sensitive and accurate detection and discrimination among four *Sclerotinia* species. Specific primers were designed for the aspartyl protease gene (SSaspr) of *S. sclerotiorum*, the calmodulin gene (STCad) of *S. trifoliorum*, the elongation factor-1 alpha gene (SHef1) of *S. homeocarpa*, and the laccase 2 gene (SMLcc2) of *S. minor*. The specificity and sensitivity of each primer set was tested individually and in multiplex against isolates of each species and validated using genomic DNA of infected plants. Each primer set consistently amplified DNA of its target gene only.

Four DNA fragments of different sizes were amplified: a 264 bp PCR product for *S. minor*, a 218 bp product for *S. homeocarpa*, a 171 bp product for *S. sclerotiorum*, and a 97 bp product for *S. trifoliorum*. Primer sets differed in their lower sensitivity limits: SMLcc2= 1 pg/µl; SHelf1=0.1 pg/µL; SSaspr, and STCad=10 pg/µL. These primer sets can be used individually for verifying the identity of isolates of a particular species or in a multiplex assay. The multiplex assay developed has a lower sensitivity limit of 0.0001 pg/µL of each species. The multiplex assay developed is an accurate and rapid tool to differentiate between the most relevant plant pathogenic *Sclerotinia* species in a single PCR reaction.

INTRODUCTION

Sclerotinia species are destructive and cosmopolitan plant pathogens that cause stem and crown rot on various agronomic and horticultural crops and wild species (Andrew and Kohn, 2009). *Sclerotinia* spp. belongs to the *Sclerotiniaceae* an important family of the class Ascomycotina (Willetts and Wong, 1980). The distribution of these fungi is cosmopolitan but they are most common in temperate regions (Reichert, 1958). Two hundred forty-six species of *Sclerotinia* have been reported (Andrew and Kohn, 2009). The main species of phythopathological interest in the genus *Sclerotinia* are *S. sclerotiorum* (Lib.) de Bary, *S. minor* Jagger, *S. trifoliorum* Erikss., *Sclerotinia homoeocarpa* F.T. Bennett (Bennett, 1937) and the undescribed species *Sclerotinia* species 1 (Winton *et al*. 2006).

Sclerotinia sclerotiorum is a destructive and cosmopolitan plant pathogen that causes white mold and watery soft-rot diseases in a wide variety of agricultural, ornamental, and wild plants in the families Solanaceae, Cruciferae, Compositae,

Chenopodiaceae, and Leguminosae. Under favorable environmental conditions the fungus probably infects many more (Boland and Hall, 1994). *S. minor* has a similar but somewhat narrower host range than *S. sclerotiorum* (Willetts and Wong, 1980). *S. minor* infects important crops such as lettuce, sunflower, spinach, tomato, pepper,and peanut (Melzer *et al*. 1997). *S. trifoliorum* was reported to cause crown and stem rot of forage legumes such as alfalfa (*Medicago sativa* L.), red clover (*Trifoliorum pratense* L.), and white clover (*Trifoliorum repens* L.), as well as several other legumes (Njambere *et al*. 2010). Recently *S. trifoliorum* was reported to cause severe losses on chickpea (*Cicer arientinum* L.) (Njambere *et al*., 2010). Some early articles reported *S. trifoliorum* on sunflower, lettuce, bean and tomatoe (Brooks, 1953), and on cauliflower (Henderson, 1962). Diseases caused by the three species are generally known as "white molds" (Abawi and Grogan, 1979).White molds are easily identified by the characteristic white cottony mycelia that grow on the surfaces of infected tissues (Abawi & Grogan, 1979). *S. homeocarpa* causes dollar spot of turfgrass (Smiley *et al*. 1993).

S. homeocarpa is a major pathogen of turfgrass worldwide and causes tremendous annual losses in the U.S.A. (Smiley *et al*., 1993). Currently, the dollar spot pathogen is classified as *Sclerotinia homoeocarpa*. However, this classification is under revision, and once completed, the fungus may be reclassified as *Lanzia*, *Moellerodiscus*, or *Rutstroemia*. Reasons for the suggested reclassification of the fungus are *S. homoeocarpa* does not form sclerotia which is a characteristic of *Sclerotinia* spp.; apothecial morphology of *S. homoeocarpa* differs from that of other *Sclerotinia* spp.; electrophoretic protein patterns and ribosomal DNA of *S. homoeocarpa* are similar to those of *Lanzia*, *Moellerodiscus*, and *Rutstroemia* (Rotter *et al.* 2009). However, *S. homeocarpa* still

listed under Sclerotiniaceae in (www.mycobank.org) and (http://www.indexfungorum.org).

Sclerotinia species produce apothecia and sclerotia but lack an obvious conidial stage (Bardin and Huang, 2001). The main criteria used to distinguish between these species have been size and general characteristics of the sclerotia, host range, and dimensions of ascospores and asci (Bardin and Huang, 2001). Identification studies on fungi are often complicated by the relatively few stable characters available for comparison. Multigenic involvement and responses to environment may lead to variability within each character and result in overlap between species (Cruickshank, 1983). No differences in the structure of hyphae have been reported between *S. sclerotiorum*, *S. trifoliorum*, and *S. minor* (Willets and Wong, 1980). It is not always accurate and rapid to separate *S. sclerotiorum*, *S. trifoliorum*, *S. minor* and *S. homeocarpa* into distinct species based on traditional morphological traits such as cultural characteristics, sclerotial size, ascus and ascospore dimensions, time of apothecial development in the field, host association and disease symptoms. *S. sclerotiorum* produces large smooth sclerotia, *S. trifoliorum* produces large irregular sclerotia, while *S. minor* produces smaller rough sclerotia (Morrall *et al*. 1972). There is an overlap in sclerotia size and shape under certain circumstances and this character is not reliable for identification (Willets and Wong, 1980). Reports in the literature suggest that mycelial characteristics of theses fungal species do not show distinctive differences and are of only very limited use for identification purposes (Willets and Wong, 1980).

Analyses based on sequences of the 18S rDNA or the ITS region, common methods for the identification of filamentous fungi and yeasts (Freeman *et al.* 2002),

revealed that the Sclerotiniaceae have almost identical sequences and are not useful for species identification. Other gene loci, e.g. the glyceraldehyde-3-phosphate dehydrogenase, heat-shock protein 60, or DNA-dependent RNA polymerase subunit II have been used for identification instead (Staats *et al*. 2004). Hirschhäuser and Fröhlich (2007) reported novel laccase 2 (lcc2) sequences localized in the genome among different Sclerotiniaceae for a fast and novel detection and identification of *S. sclerotiorum* and *S. minor*. They suggested that this gene could be used for the identification of more members of the Sclerotiniaceae.

PCR-based methods have been developed to detect *S. sclerotiorum* (Yin *et al*. 2009; Rogers *et al*. 2009; Freeman *et al*. 2002). Also, Njambere *et al.* (2010) developed microsatellite markers for *S. trifoliorum*. A multiplex PCR was developed by Hirschhauser & Frohlich, (2007) to discriminate some fungal members of the *Sclerotiniaceae* but *S. trifoliorum* or *S. homeocarpa* were not included in their study. A single nucleotide polymorphism (SNPs) protocol was developed by (Andrew and Khon, 2009) to identify *S. sclerotiorum, S. minor, S. trifoliorum* and the undescribed species *Sclerotinia* species 1. Their protocol, however, is time consuming and costly if compared to multiplex PCR and it requires costly equipment.

Methods for use in diagnostics and detection of plant pathogens need to be quick, simple, reliable, and cost effective. In spite of the importance of the agriculture associated species of *Sclerotinia* spp, there is no rapid and accurate procedure for routine detection of these pathogens. Developing a sensitive multiplex PCR for the detection of the most common four species of the genus *Sclerotinia* is crucial for accurate and fast diagnostics.

MATERIAL AND METHODS

Fungal isolates and growth conditions

All isolates (Table 1) were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for 3 to 5 days and were then transferred into standing-culture potato dextrose broth (PDB; Difco Laboratories, Detroit, MI) for 7 days. On both solid and liquid media, cultures were grown at ambient room temperature (20 to 22°C).

Artificial inoculation

Asymptomatic seedlings of peanut Okrun at 5 weeks old were each inoculated with one species of the four species used in this study. Mycelial plugs (5 mm) taken from 2-day-old PDA cultures were placed on the pocket between the third petiole and the main stem at the vertical midpoint of the stem mycelial side touching the surface of the stem. Five seedlings were inoculated per fungal species. Plants were then placed in humidity chambers (150 x 60 x 60 cm) built from PVC pipe and clear plastic. Temperature was maintained at 19±2 $^{\circ}$C at night and 26±2 ºC during the day and relative humidity was maintained at 95 to 100%. The chamber provided adequate light (13.5 µmol/s/m^2) to sustain healthy plants throughout experiments. Inoculated plants were watered thoroughly every other day for the duration of the experiments (Faske *et al*., 2006).

Fungal, infected and healthy plants genomic DNA extraction

DNA samples were obtained using the protocol described by Sambrook and Russell (2001). Lyophilized mycelia, infected and healthy plant tissues were freshly frozen in liquid nitrogen and grounded with a mortar and a pestle. One gram of crushed mycelia per sample and 600 µl of genomic extraction solution (1 ml 0.5 M EDTA, 1 ml 10% SDS and 8 ml dH_2O) were added into a 1.5-ml microcentrifuge tube and incubated at 68C for 10 minutes. The tubes were centrifuged at 13.000 rpm for 10 minutes and the

supernatant was transferred into fresh 1.5-ml microcentrifuge tubes. Then, 40 µl of 5M potassium acetate was added and mixed by inversion and the tubes were placed on ice for 10 minutes. The samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred into fresh tubes. The DNA was precipitated with 2.5X volume of 95% ethanol, centrifuged at 13,000 rpm for 10 minutes, and the supernatant discarded. The pellet was washed twice with 70% Ethanol and air dried.

Finally, the DNA was dissolved in 100 µl TE buffer and RNase (Grand Island, NY) was added (1 µg). Extracted DNA was quantified using a NanoDrop ND1000 spectrophtometer (NanoDrop Technologies, Wilmington, DE) and stored at -20 ºC until use.

Sequence analysis and design of PCR primers

The Nucleotide Sequences Search program provided by the National Center for Biotechnology Information (NCBI) (http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD) was used to retrieve several *Sclerotinia* spp genes sequences. Retrieved nucleotide sequences from the GenBank were aligned using the program CLUSTAL-X 2.0.12 (http://www.clustal.org/) (Thompson *et al*., 1997) and were examined for the conserved regions of the different sequences. Percent identity matrices and nucleotide sequence alignments for each species were generated using GeneDoc (Nicholas and Nicholas, 1997). Specific nucleotide regions were selected to design the multiplex PCR primers for detection of the four *Sclerotinia* species. All the primers were designed based on specificity, stability and compatibility. Different specific primers with similar annealing temperature were designed subsequently using the program primer3 (http://frodo.wi.mit.edu/primer3). Internal structures, hairpins, self and hetero dimers

were detected by the MFOLD program (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). Three primer sets for each species were designed and of the three, only one primer set produced a single band for each species when tested individually or in multiplexing. Compatible PCR primers designed are listed in (Table 2).

PCR reactions

To determine the specificity of the designed primers, each primer set was tested separately on DNA of all isolates of the four species of *Sclerotinia* spp. individually, the closely related species *Monilinia fructicola*, and healthy peanut, sunflower, and alfalfa plants (Table 1). Assays were performed as follows. Each 25 µl simplex PCR reaction contained 1.4 µl $MgCl_2$ 25 mM (Promega), 5 µl of 5X Green GoTaq Flexi Buffer (Promega), 2 µl of dNTP (Promega), 0.16 µl of 0.5 U of Taq polymerase (Promega), 2 µl of each forward and reverse primer (Table 2) , 1 µl of DNA template (25 ng/µl), and 11.47 µl of dH_2O.

The amplification process involved an initial denaturation of 2 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 61 ºC for 20 sec and extension at 72ºC for 40 sec. The final extension was at 72 ºC for 3 min. Primer sets were tested on all isolates collected from different geographical areas of each species.

Multiplex polymerase chain reactions were performed in a total volume of 50 µl, each containing 10 µl of 5X Green GoTaq Flexi Buffer (promega), 3 µl of 25 mM $MgCl_2$, 4 µl of 25 mM dNTPs, 5 µl of the four primer sets mixture, 1 µl of DNA of each fungal species, 0.5 µl of Go Taq DNA polymerase (5U/µl), and 23.5 µl of dH_2O. The multiplex PCR amplification process consisted of an initial denaturation at 95 ºC for 3

min, followed by 35 cycles of denaturation at 95 ºC for 20 sec, annealing at 60 ºC for 90 sec and extension at 72 ºC for 90 sec. The final extension was at 72 ºC for 7 min.

Sensitivity tests of the simplex and multiplex PCR assays.

To determine primer sets sensitivity, 10-fold serial dilutions (2.5 x 10 -10^{-8} ng/µl) were made from each *Sclerotinia* species DNA in nuclease-free water. Sensitivity assays were performed under the PCR conditions described above for simplex and multiplex PCR. PCR products were resolved by horizontal gel electrophoresis in 1.5% agarose gels in 0.5x (Tris-borate EDTA) (TBE) buffer at 100 V cm^{-1} for 45 min. Multiplex PCR products were resolved in 2.5% agarose gels in 0.5x TBE buffer, at 80 V cm^{-1} for 3 hours. Gels were pre-stained with ethidium bromide (0.5 µg mL^{-1}) digitally visualized and photographed by Gel Doc-lttm Imaging System (UVP-LLC, CA, USA).

RESULTS

Under standardized PCR conditions, each primer set was highly specific and amplified only target sequences when tested on DNA of *S. minor*, *S. homeocarpa*, *S. sclerotiorum* and *S.trifoliorum* in simplex and multiplex reactions (Table 3). SMLcc2, SHelf1, SSaspr and STCad amplified only the target gene sequences *Lcc2*, *Ef1-α*, *Aspr* and *Cad* with the distinct product sizes, i.e. 264 bp for *S. minor*, 218 bp for *S. homeocarpa*, 171 bp for *S. sclerotiorum*, and 97 bp for *S. trifoliorum*, respectively. No amplification products were obtained from DNA of closely related species *M. fructicola* and *B. cinerea* and DNA of healthy peanut, sunflower, bent grass and alfalfa. Multiplex PCR assays using fungal or infected plant DNA, amplified target gene sequences with no evident competition between them, and no nonspecific products or primer dimers (Fig 1).

The detection limits of the individual specific primer sets for *S. minor*, *S. homeocarpa*, *S. sclerotiorum* and *S. trifoliorum* were 10^{-3}, 10^{-4}, 10^{-2} and 10^{-2} ng/µl template DNA, respectively (Fig 2). In multiplex PCR, all primers were able to detect down to 10^{-7} ng/µl template DNA from lyophilized mycelia and infected plants (Fig 3).

DISCUSSION

PCR diagnostic techniques are faster than morphological identification following isolation of the pathogens (Martin *et al*., 2000). Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction Traditional methods of pathogens detection are time consuming, labor intensive and subjective since they rely on culture-based techniques (Freeman *et al*., 2002). Generally, there are no available accurate, sensitive, and cost effective molecular tools to detect and discriminate between *S. homeocarpa, S.minor, S. sclerotiorum* and *S. trifoliorum*; the most common species of the genus Sclerotinia on agricultural crops. Freeman *et al*. (2002) reported a PCR assay for detecting ascospores of *S. sclerotiorum* that could be applied to air samples. Although the primers were designed specifically to detect *S. sclrotiorum*, they were also identical to sequences from *S. minor* and *S. trifoliorum* and were able to detect these species as well (Freeman *et al*., 2002). Njambere *et al*. (2007) designed primers for the ITS region of *S. trifoliorum* but after sequencing the PCR products some of the sequences were identified as *S. sclerotiorum*, therefore, the primers were not specific to detection of *S. trifoliorum*. Andrew and Kohn (2009) developed single nucleotide polymorphism (SNPs) to identify *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and the undescribed species *Sclerotinia* species 1. SNPs assays require expertise, time consuming and expensive if compared to the multiplex PCR.

Robust and cheap protocols to extract DNA and/or RNA from infected plant tissues and pathogen are needed for practical application of PCR detection and diagnostic methods (Martin *et al*., 2000). The DNA isolation protocol that has been reported in this study is a cheap and fast protocol. The required chemical can be prepared in short time with low cost since it does not require any special kits.

The multiplex PCR assay reported does not require isolation of the pathogens. Therefore the identification process takes comparatively short time compared with methods based on morphological characteristics. When the fungal agent infecting a crop is suspected to belong to one of the most common four species of the genus *Sclerotinia*, the multiplex PCR reported in this study can be used as a qualitative method to further identify the species among the four species. The multiplex PCR method developed herein proved to be a sensitive, accurate and reliable technique for the detection of the most important agricultural associated fungal species of the genus *Sclerotinia*. The multiplex PCR reported here successfully detected DNA mixture of *S. minor*, *S. homeocarpa*, *S. sclerotiorum* and *S. trifoliorum* simultaneously using a single PCR reaction.

For individually amplified genes, the annealing temperature had significant impact on the specificity and yield of PCR products. Increasing or decreasing the annealing temperature (61 ºC) visibly decreased the amplification of target genes, even though we tried to compensate using a longer annealing time. For multiplex PCR, both the annealing temperature and time were crucial for the sensitivity and specificity of the primers mix. We found that annealing temperature of 60ºC for 90 S was the best combination for target genes amplification (Fig 1 and 2). When 61ºC was the annealing temperature some primer dimers have been formed. When 62 ºC was the annealing

temperature there was uneven amplification. Lowering the annealing temperature was required for the co-amplification of target genes in multiplex. These observations are in agreement with (Henegariu *et al*., 1997).

Our results show that multiplex PCR requires longer time for extension and final extension than simplex PCR to obtain higher yields of amplicons. In multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all products. The detection sensitivity of the multiplex PCR was higher than the simplex PCR (Fig 4). The annealing and extension times used in multiplex PCR are longer than that used in simplex PCR and this could enhance the efficiency and sensitivity of the four primer sets (Henegariu *et al*., 1997).

We found that in multiplex PCR amplifications, the Promega kit produced better results than other available kits. This may be because that the Promega buffer contains a balanced mix of salts and additives that enhance the efficiency of the annealing and extension of the primers mixture.

In conclusion, the multiplex PCR assay described is a reliable, rapid, sensitive, specific and cost-effective diagnostic for the most common agricultural associated species of the genus *Sclerotinia*. It should be useful in detection and discrimination of *S. mino*r, *S. homeocarpa*, *S. sclerotiorum* and *S. trifoliorum* for application in diagnostics and rapid screening of infected plants to enhance monitoring resistance in breeding programs and in plant certification. The multiplex PCR technique provides the basis for the future development of a quantitative and more sensitive PCR method using real-time PCR technology.

LITERATURE CITED

1- Abawi, G. S and Grogan, R.G. 1979. Epidemiology of diseases caused by *Sclerotinia* spp. *Phytopathology* 69:899-904.

2- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

3- Andrew, M. and Kohn, L.M. 2009. Single Nucleotide Polymorphism-Based Diagnostic System for Crop-Associated *Sclerotinia* Species. *Applied and environmental microbiology* 5600-606.

4- Bardin, S. D. and Huang, H. C. 2001. Research on biology and control of Sclerotinia diseases in Canada. *Canadian Journal of Plant Pathology*. 23, 88-98.

5- Bennett, F. T. 1937. Dollar spot of turf and its causal organism *Sclerotinia homoeocarpa* N.SP. *Annals of applied Biology* 24, 236-257.

6- Boland, G. J. and Hall, R. 1994. Index of plant hosts of Sclerotinia sclerotiorum. *Canadian Journal of Plant Pathology* 16, 93-108.

7- Brooks, F.T. 1953. *Plant Diseases*. Geoffrey Cumberlege (ed.). Oxford University Press, London and New York. 457 pp.

8- Cote, M-J., Tardif, M-C. and Meldrum, A.J. 2004. Identification of *Monilinia fructigena, M. fructicola, M. laxa,* and *Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant Disease 88*, 1219–1225.

9- Cruickshank, R. H. 1983. Distinction between Sclerotinia species by their pectic zymograms. *Transactions of the British Mycological Society* 80 (1), 117-19.

10- Faske, T., Melouk, H. A. and Payton, M. E. 2006. Comparison of *Sclerotinia minor* inocula for differentiating the reaction of peanut genotypes to Sclerotinia blight. *Peanut Science* 33: 7-11.

11- Freeman, J., Ward, E., Calderon, C. and McCartney, A. 2002. A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. *European Journal of Plant Pathology* 108, 877–886.

12- Graf, F. and Schumacher, T. 1995. *Sclerotinia glacialis* sp. nov., from the alpine zone of Switzerland. *Mycological Research* 99, 113–17.

13- Henderson, R. M. 1962. Some aspects of the life cycle of the plant pathogen *Sclerotinia sclerotiorum* in Western Australia. *Journal Proceedings of the Royal Society* 45, 133-35.

14- Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H. and Vogt, P. H. 1997. Multiplex PCR: critical parameters and step by step protocol. *BioTechniques* 23,504-511.

15- Hirschhäuser, S. and Fröhlich, J. 2007. Multiplex PCR for species discrimination of Sclerotiniaceae by novel laccase introns. *International Journal of Food Microbiology* 118, 151–157.

16- Kohn, L. M. 1979. A monographic revision of the genus *Sclerotinia*. *Mycotaxon* 9:365–444.

17- Kohn, L. M. 1979. Delimitation of the economically important plant pathogenic *Sclerotinia* species. *Phytopathology* 69, 881-86.

18- Martin, R. R., Delano, J. and Levesque, C.A. 2000. Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology* 38, 207-239.

19- Melzer, M. S., Smith, E. A. and Boland, G. J. 1997. Index of plant hosts of Sclerotinia minor. *Canadian Journal of Plant Pathology* 19, 272–80.

20- Morrall, R. A. A., Duczek, L. J. and Sheard, J. W. 1972. Variations and correlations within and between morphology, pathogenicity, and pectolytic enzyme activity in *Sclerotinia* from Saskatchewan. *Canadian Journal of Botany* 50, 767-86.

21- Nicholas, K. B. and Nicholas, Jr. H. B. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. Available from: www.psc.edu/biomed/genedoc.

22- Njambere, E. N., Chen, W., Frate, C., Temple, S. R., Wu, B. M. and Muehlbauer, F. J. 2007. Cultural and DNA-based identification of *Sclerotinia trifoliorum* infecting chickpea in the U.S. *PISUM Genetics* 39, 30-32.

23- Njambere, E. N., Vandemark, G. and Chen, W. 2010. Development and characterization of microsatellite markers of the fungal plant pathogen *Sclerotinia trifoliorum*. *Genome* 53, 494-500.

24- Reichert, I. 1958. Fungi and plant diseases in relation to biogeography. *Transactions of the New York Academy of Sciences*. 20, 333-39.

25- Rogers, S. L., Atkins, S. D. and West, J. S. 2009. Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology* 58, 324-331.

26- Rotter, D., Amundsen, K., Bonos, S. A., Meyer, W. A., Warnke, S. E. and Belanger, F. C. 2009. Colonial bentgrass genetic linkage mapping. In: Yamada T, Spangenberg G, eds. *Molecualr Breeding of Forage and Turf*. Springer Science, 309-322 .

27- Sambrook, J. and Russell, D.W. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press

28- Smiley, R.W., Dernoeden, P. H. and Clarke, B. B. 1993. *Compendium of Turfgrass Diseases*. APS Press, Minneapolis-St. Paul, MN. 98 p.

29- Staats, M., van Baarlen, P. and van Kan, J. A. L. 2004. Molecular phylogeny of the plant pathogenic genus Botrytis and the evolution of host specificity. *Molecular Biology and Evolution* 22, 333–346.

30- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24, 4876–4882.

31- White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNAgenes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, Hite TJ (Eds.), *PCR Protocols A Guide to Methods and Applications*. Academic Press Inc, pp. 315–322.

32- Willetts, H. J. and Wong, J. A. L. 1980. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. *The botanical review* 46, 101-65.

33- Winton, L. M., Leiner, R. H. and Krohn, A. L. 2006. Genetic diversity of Sclerotinia species from Alaskan vegetable crops. *Canadian Journal of Plant Pathology* 28, 426-34.

34- Yin, Y., Ding, L., Liu, X., Yang, J. and Ma, Z. 2009. Detection of *Sclerotinia sclerotiorum* in planta by a real-time PCR assay. *Journal of Phytopathology* 157, 465-469.

Table 1.3. List of studied *Sclerotinia* spp isolates.

Species	Organism code	Source
S homeocarpa	S 18	Pennsylvania State University
S. homeocarpa	S 30	Pennsylvania State University
S. homeocarpa	OKC-OSU	Oklahoma State university
S. homeocarpa	99$	University of Massachusetts Amherst
S. homeocarpa	SD20	University of Massachusetts Amherst
S. homeocarpa	Logan	University of Massachusetts Amherst
S. homeocarpa	Spot 06	University of Massachusetts Amherst
S. homeocarpa	Homeo RCC10	University of Massachusetts Amherst
S. homeocarpa	Homeo RCC11	University of Massachusetts Amherst
S trifoliorum	CF 6	UC DAVIS
S. trifoliorum	CF 18	UC DAVIS
S. trifoliorum	CF 24	UC DAVIS
S. trifoliorum	CF 31	UC DAVIS
S. trifoliorum	CF 34	UC DAVIS
S. trifoliorum	Trif A	UC DAVIS
S. trifoliorum	Trif B	UC DAVIS
S. trifoliorum	Trif C	UC DAVIS
S. sclerotiorum	Peanut	Oklahoma State University
S sclerotiorum	Pumpkin	Oklahoma State University
S. sclerotiorum	UF15	University of Florida
S. sclerotiorum	UF28	University of Florida

S. sclerotiorum	321 DB2	Oklahoma State University
S. sclerotiorum	44 Ea1	Oklahoma State University
S. sclerotiorum	44 B17	Oklahoma State University
S. sclerotiorum	44 Bb6	Oklahoma State University
S. sclerotiorum	Maj 70	Oklahoma State University
S. sclerotiorum	Peanut	Oklahoma State University
S minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
S. minor	Peanut	Oklahoma State University
Monilinia fructicola	MF	Oklahoma State University

Table 2.3. Primers codes designed for the four species of the genus *Sclerotinia*

Pathogen	Target gene	Gene code	Primer code	Primer Sequence (5'-3')	Product size (bp)
S minor	laccase 2	*Lcc2*	SMLcc2 F SMLcc2 R	CCCTCCTATCTCTCTTCCAAACA TGACCAATACCAATGAGGAGAG	264
S. homeocarpa	elongation factor-1 alpha	*Ef1-α*	SHelf1 F SHelf1 R	CGGTATGACTTCTCCACCTTTC GAACCCTTTCCCATCTCCTT	218
S. sclerotiorum	aspartyl protease	*Aspr*	SSaspr F SSaspr R	CATTGGAAGTCTCGTCGTCA TCAAACGCCAAAGCTGTATG	171
S. trifoliorum	calmodulin	*Cad*	STCad F STCad R	TCCTAGATCGACTCTCCTCCTTT CGTGTTATTGCCTCCTTGTTG	97

Table 3.3. Details of inclusivity and exclusivity panels

DNA samples used	*S. minor* SMLcc2	*S. homeocarpa* SHelf1	*S. sclerotiorum* SSaspr	*S. trifoliorum* STCad
Peanut	-	-	-	-
Sunflower	-	-	-	-
bentgrass	-	-	-	-
Alfalfa	-	-	-	-
Monilinia fructicola	-	-	-	-
S. minor	+	-	-	-
S. homeocarpa	-	+	-	-
S. sclerotiorum	-	-	+	-
S. trifoliorum	-	-	-	+

- = no amplification; + = amplification

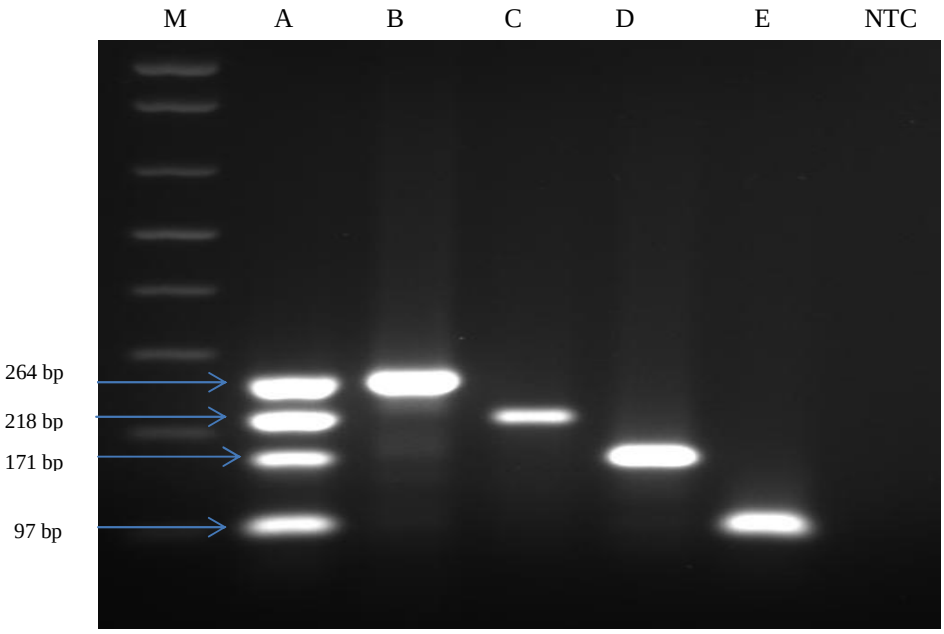


Fig 1. Multiplex PCR assay with gDNA isolated from mechanically inoculated peanut plants using selected four isolates of *Sclerotinia* species. (A) gDNA from all four infected plants, (B) gDNA from infected plants with *S. minor* G170, (C) gDNA from infected plants with *S. homeocarpa* 99$, (D) gDNA from infected plants with *S. sclerotiorum* 44B17 and (E) gDNA from infected plants with *S. trifoliorum* CF24. Lane M; 1kb ladder (Invitrogen), NTC; non template control (water).

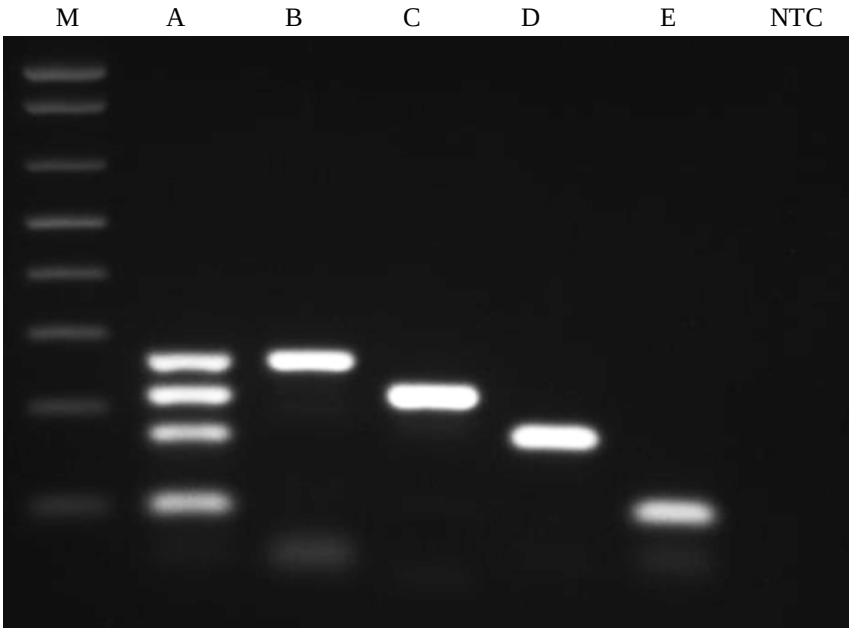


Fig 2. Multiplex PCR assay with fungal gDNA of selected four isolates of *Sclerotinia* species. (A) gDNA of all four *Sclerotinia* species (B) *S. minor* G170, (C) *S. homeocarpa* pot06, (D) *S. sclerotiorum* 44BP6 and (E) *S. trifoliorum* CF18. Lane M; 1kb ladder (Invitrogen), NTC; non template control (water).

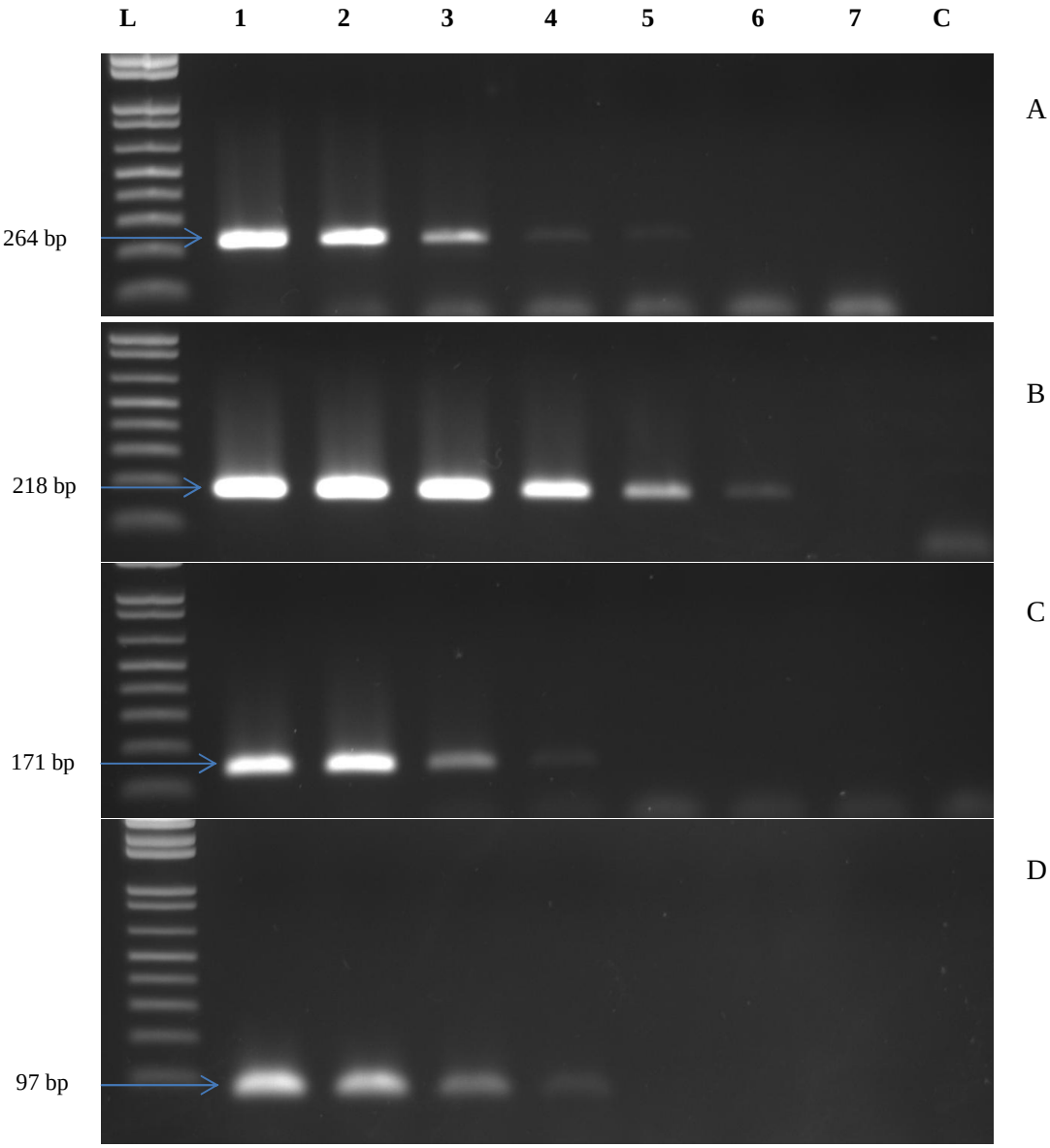


Fig 3. Sensitivity assays of end point PCR using 10 fold serial dilution of gDNA (A) *S. minor* using primer set SMLac2 (B) *S. homeocarpa* using primer SHelf1, (C) *S. sclerotiorum* using primer set Ssaspr, (D) *S. trifoliorum* using primer set STCad. Lane L: 1kb ladder. Initial DNA concentration was 25 ng/µl. 2= 25 ng/µl; 3= $2.5x10^{-1}$; 4= $2.5x10^{-2}$; 5= $2.5x10^{-3}$; 6= 2.5×10^{-4}; 7= 2.5×10^{-5}; 8= negative control.

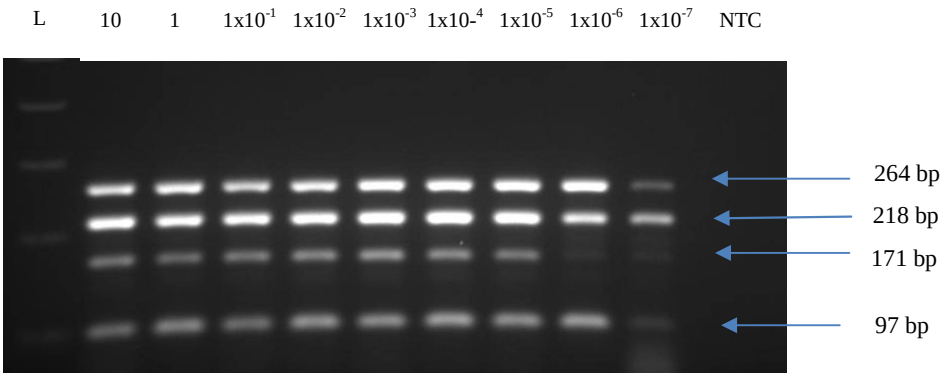


Fig 4. Multiplex PCR sensitivity assay using a 10-fold serial dilution of each *Sclerotinia* species gDNA starting from concentrations in10 ng/µl of template DNA. Lane L; 1 kb ladder, Lane NTC; non template control.

.

VITA

AHMED ABD-ELMAGID

Candidate for the Degree of

Doctor of Philosophy

Thesis: WATER POTENTIAL INTERACTION WITH HOST AND PATHOGEN AND DEVELOPMENT OF A MULTIPLEX PCR FOR *SCLEROTINIA* SPECIES

Major Field: PLANT PATHOLOGY

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in July, 2012.

Completed the requirements for the Master of Science in Plant Pathology at Assiut University, Faculty of Agriculture, Assiut, Egypt in 2003.

Completed the requirements for the Bachelor of Science in Agriculture at Assiut University, Faculty of Agriculture, Assiut, Egypt in 1999.

Experience:

Teaching Assistant, Faculty of Agriculture, Assiut University, Egypt, 2000 to 2003. Research Assistant, Faculty of Agriculture, Assiut University, Egypt, 2003 to 2006. Research Assistant, Faculty of Agriculture, Western Australia University, Perth, Australia, 2006 to 2007. Graduate Research Assistant, Department of Entomology and Plant Pathology, Oklahoma State University, USA, 2007 to 2012.

Professional Memberships:

American Phytopathological Society.

Name: Ahmed Abd-Elmagid Date of Degree: July, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: WATER POTENTIAL INTERACTION WITH HOST AND PATHOGEN AND DEVELOPMENT OF A MULTIPLEX PCR FOR *SCLEROTINIA* SPECIES

Pages in Study: 116 Candidate for the Degree of Doctor of Philosophy

Major Field: Entomology and Plant Pathology.

Scope and Method of Study: Soil water potential (Ψ) is an important parameter that affects the development of plant diseases. Sclerotinia blight of peanut caused by *S. minor* and *S. sclerotiorum* has been chosen as a model for soil-borne diseases. To study the effect of Ψ on Sclerotinia blight, the following objectives have been addressed: 1) the effect of Ψ_s and Ψ_m on the vegetative growth in vitro and sclerotia viability of the pathogens. Mycelia were grown on Potato Dextrose Agar (PDA) media amended to different Ψ_s and Ψ_m. The Area under Mycelial Progress Curve (AUMGPC), sclerotia number and germination were recorded; 2) Study the pathogenicity of mycelial inocula of the pathogens produced at various Ψ_s and Ψ_m. Seedlings of "Okrun" cultivar were infected by mycelia produced at various Ψ_s and Ψ_m. Lesions lengths were recorded daily; 3) Determine the effect of water stress on the infection of the cv. Okrun by the two pathogens. Seedlings received PEG 8000 and on the sixth week, plants were infected by the inocula of the pathogens. Lesions lengths were recorded daily. Tan spot of wheat has been chosen as a model for foliar diseases. The following objectives have been addressed: 1) Determine the role of Ψ on mycelial growth, conidia production and germination of *Pyrenophora tritici-repentis* (PTR); 2) Determine the effect of Ψ on initiation and maturation of pseudothecia of PTR on wheat straw in vitro; 3) Study the impact of water stress on the infection of wheat "TAM105" cultivar by PTR. In a separate project a Multiplex PCR for the most important species of the Genus *Sclerotinia* was developed.

Findings and Conclusions: Mycelial growth of *S. minor* and *S. sclerotiorum* tolerated a wide range of Ψ_s and Ψ_m, up to -4.0 and -3.5 MPa, respectively. Sclerotial formation and germination of the two pathogens occurred at Ψ_s and Ψ_m lower than those at which most crops seeds germination is curtailed -1.4 to -2.0 MPa. Pathogens can retain their virulence under high levels of Ψ_s and Ψ_m.Okrun stressed plants had smaller lesions. In tan spot system, low Ψ decreased vegetative growth, conidia production and germination, pseudothecia production and maturation of PTR on wheat straws. Increasing water stress predisposed TAM 105 to tan spot. The developed multiplex PCR was rapid, sensitive and specific to separate between *S. minor, S. homeopcarpa, S. sclerotiorum* and *S. trifoliorum*.

ADVISER'S APPROVAL: Dr. Hassan Melouk