ENVIRONMENTAL AND DEVELOPMENTAL ASPECTS OF SORGHUM DOWNY MILDEW WITH PARTICULAR EMPHASIS ON OOSPORES

A Dissertation

by

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ABSTRACT

Sorghum downy mildew (SDM), caused by *Peronosclerospora sorghi*, remains one of the primary constraints to sorghum production worldwide. Due to the fastidious nature of the pathogen, in particular the soilborne oospores, which form the major source of inoculum in most sorghum producing countries, studies of the basic biology of *P. sorghi* remain few. This study attempts to explore the environmental aspects affecting the incidence of SDM as well as expand the base of knowledge regarding the resistant reaction of sorghum seedling roots to infection by oospores of *P. sorghi*.

Soil moisture is an important environmental factor that affects soilborne plant pathogen. Soil moisture is often considered as the ability of the soil to release water, or matric potential (ψ_m). In this study the effect of ψ_m on the incidence of SDM was tested using the Haines pressure plate apparatus to control and maintain it. From the conditions tested in two soil types, a lower soil moisture of -33 kilo Pascales (kPa) was shown to be the most conducive condition for disease development as well as oospore germination when compared to the -20 (intermediate) and 0.0 (saturation) kPa.

Previous studies have suggested the use of bait crops to induce oospores, which must encounter a compatible host to survive, to germinate and therefore reduce inoculum potential in the field. This hypothesis was tested in a single field naturally infested with oospores of *P. sorghi* using seven different bait crops. In all treatments the incidence of SDM was significantly reduced compared to the fallow control.

A microscopic study of the infection structure and defense response of sorghum seedling against *P. sorghi* oospore derived germtubes remains largely undocumented. Roots of resistant and susceptible sorghum seedlings were inoculated with oospores and the stages of development were observed using fluorescence microscopy. In a separate experiment, the activity of plant defense-related enzymes in sorghum seedling roots inoculated with oospores was measured. Significantly increased activity levels of glucanase, peroxidase, and phenylalanine ammonia lyase were found in inoculated roots compared to untreated controls and compatible interactions. However, chitinase activity levels were similar among all treatments.

To my wife and family

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1. INTRODUCTION-LITERATURE REVIEW

1.1 INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is the fifth most globally important crop and is grown in the tropical and subtropical parts of the world for human consumption as well as animal feed, and, more recently, biofuels (Table 1). Sorghum produces significant biomass and yield in conditions unfavorable for other crops such as maize.

Table 1. Worldwide sorghum production statistics from 2013 (FAO, 2015)

Region	Area (x1000 ha)	Yield (kg/ha^{-1})	Production (x1000 MT)
World	42120.446	1457.4	61384.559
Africa	26539.118	968.6	25706.456
Asia	7841.579	1116.8	8757.759
South America	2155.936	3196.9	6892.362
USA	2642.6	3739.4	9881.788

Despite its hardiness, there several biological constraints to sorghum production, including sorghum downy mildew (SDM). SDM is caused by the oomycete *Peronosclerospora sorghi* (Weston and Uppal (C.G. Shaw)), present in most countries where sorghum is grown (Fig. 1) and can lead to significant yield losses under favorable conditions (Bock and Jeger 1996). Effective management strategies have been developed, but new pathotypes continue to evolve (Isakeit and Jaster 2005), which increases the need for both effective chemical treatments and new resistant genetic

material. Although the epidemiology and morphology of *P. sorghi* have been well studied, little research has been conducted to determine the factors affecting root infection by oospores of *P. sorghi,* as well as the development of disease *in planta* after infection through the roots.

1.2 TAXONOMY AND MORPHOLOGY OF *PERONOSCLEROSPORA SORGHI*

Peronosclerospora sorghi is classified as an oomycete in the kindom Chromista, order Oomycota, class Sclerosporales (Shaw 1978) and belongs to the group of downy mildews infecting graminaceous hosts such as sorghum and maize. *Peronosclerospora sorghi* was first described by Butler (1907) on sorghum, naming the isolate *Sclerospora graminicola var. Andropogonis-sorghi* due to its resemblance of the downy mildew disease of pearl millet. In 1932 Weston and Uppal, renamed this pathogen as *Sclerospora sorghi* based on morphology and host range. Later Shaw (1970) proposed the genus *Peronosclerospora* based on the mechanism of spore germination for several graminaceous downy mildews including *P. sorghi*.

The morphology of *P. sorghi* was described in 1932 by Weston and Uppal. Oospores of *P. sorghi* are reddish brown in color and have a textured surface. The range of dimensions for oospore diameters are 25-65 µm, averaging 33-40 µm. The sporangia are born on dichotomously branched sporangiophores. The sporangia are hyaline and ovoid and dimensions range in size from 15-32.5x15-26.9 µm and average 21-25x19-23 µm.

1.3 GEOGRAPHIC DISTRIBUTION

As the primary host of *P. sorghi* is sorghum, which originated in Africa, the pathogen most likely originated in there as well (Williams 1984). This has however been disputed by Shaw (1984) who argued that the greatest point of genetic diversity for the graminaceous downy mildews is Asia. Regardless of its origin, *P. sorghi* has spread throughout the tropics and sub-tropics. Butler (1907) first described SDM in India, and Toler et al. (1959) first reported the pathogen in the New World in Panama (Toler et al. 1959). Soon afterwards, SDM was observed in the USA in 1961 in College Station and Chillicothe, Tx (Reyes et al. 1964). Subsequently SDM has spread to many other states (Frederiksen 1980) as well as throughout Central and South America (Frederiksen and Renfro 1977). *P. sorghi* has been reported in all sorghum growing areas of the world (Williams 1984) (Fig. 1).

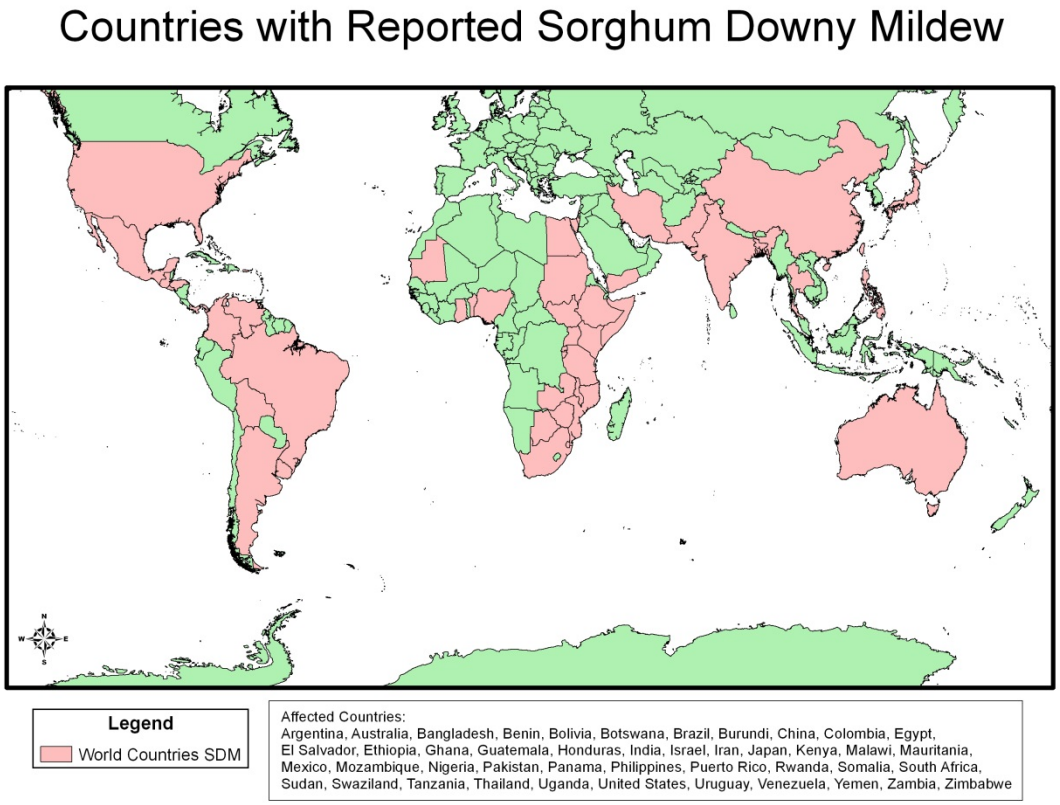

Fig. 1. Geographic distribution of sorghum downy mildew. Map generated by the author's spouse in ArcGIS (Environmental Systems Research Institute) using the list of countries according to William (1984).

1.4 BIOLOGY AND EPIDEMIOLOGY

Oospores are the sexual stage of oomycetes, formed from the fusion of oogonia and antheridia (Singh et al. 1993). Pawar (1986) attempted to determine the thallism of *P. sorghi* and concluded that *P. sorghi* is homothallic unlike the causal agent of pearl millet downy mildew, *Sclerospora graminicola* (Michelmore et al. 1982). They occur in between the vascular bundles of the leaves (Bock and Jeger

1996), and are the perennating propagule of *P. sorghi.*

Sporangia are the asexual stage of oomycetes. These propagules are derived from the vegetative stage of the pathogen and are ephemeral and short lived. Although they can be kept viable for a short time in cold conditions and even stored as frozen stocks, they often die a few hours after maturity (Jeger et al. 1998).

P. sorghi exhibits considerable genetic variability. Many different pathotypes have been discovered worldwide. This classification is based on an arbitrary system using established sorghum differentials. The first description of a new pathotype was in Texas (Craig and Frederiksen 1980). Subsequently, new pathotypes have been described in Latin America and Africa (Craig 1992) as well as several different pathotypes from the US. The pathotypes in the US have been referred to as 1, 3, and 6 (P1, P3, and P6). In 2005, Isakeit and Jaster reported the new pathotype P6 not only infected sorghum varieties at the time resistant to SDM, but was also resistant to the fungicide metalaxyl.

P. sorghi is an obligate biotroph that must infect and colonize a living host to complete its life cycle. In general, only young seedlings are infected by oospores or sporangia, establishing SDM as a monocyclic disease. Although this can vary due to environmental conditions (Schuh1986). In many areas that have defined growing seasons with unfavorable conditions occurring between the seasons, soilborne oospores are the main source of inoculum (Williams 1984). Infection of susceptible hosts by oospores results in plants that are systemically infected. The plants continue to develop and eventually oospores are produced and released into the soil. The oospores produced

in one season serve as inoculum for the next growing season, increasing the disease pressure.

The dispersal of oospores is thought to be through a combination of wind, water (Williams 1984), and transportation of crop debris and infested soil by humans and animals (Bock 1987). Infection of sorghum roots by oospores appears to be more highly influenced by environmental conditions such as soil sand content, moisture, and temperature than by oospore density (Pratt and Janke 1978). Schuh (1986) observed optimal conditions for disease development to be in soils of 80% sand content, with a matric potential of -20 kPa, and temperatures of 24-29ºC. Soil moisture is challenging to control and further efforts to study the effect of soil matric potential on infection of sorghum by *P. sorghi* remains to be accomplished. Oospores have been shown to germinate in the presence of host and non-host seedling roots, attributed to stimulation by root exudates (Pratt1978). Safeeulla (1976) observed penetration of sorghum seedling roots by germ tubes of *P. sorghi*. However, the development of disease *in planta* from root infection by oospores remains to be well documented.

Sporangia are airborne ephemeral propagules that generally only cause local lesion damage in areas with a defined dry season. In areas with high humidity, these spores can cause polycyclic disease cycles (Adenle and Cardwell 2000). Infection of older plants by sporangia has also been shown in some instances to result in systemically infected plants. These plants are referred to as local lesion systemics. Disease caused by local lesion systemics may contribute a significant percentage of total systemically infected plants on the Coastal Bend of Texas in

some hybrids (Odvody, *personal communication*). Sporangia are only produced in conditions of very high relative humidity (RH) on the abaxial side of the leaf. At 100% RH at 20ºC in the dark, 10,800 sporangia per cm² have been observed. This number is reduced to 3,600 sporangia per cm² at 85% RH, and no sporangia are produced at 80% RH (Shetty and Safeeulla 1981a). Sporangial production only occurs at temperatures ranging from 15-23ºC in the dark (Schmitt and Freytag 1974). Additionally, the formation of sporangia is photoperiod dependent as infected leaves must be exposed to a minimum of four hours of intense light (Schmitt and Freytag 1974). Sporangia produced on collateral hosts such as Johnson grass, *Sorghum halepense*, can contribute as a source of inoculum for young seedlings if environmental conditions are conducive.

1.5 SYMPTOMS AND CONTROL

Symptoms develop as early as two weeks after infection. Early systemic infection is characterized by leaf chlorosis and mosaic symptoms. Leaves with more advanced symptoms exhibit white longitudinal lesions, which necrotize, turn brown, and become the site of copious production of oospores. In the field, the leaves shred along the lesions and release oospores into the soil (Bock et al. 1997). Systemically infected plants are almost completely rendered sterile, resulting in a significant reduction of yield. Older plants exposed to sporangia can also develop local sporulating lesions. However, these lesions have little effect on yield (Williams 1984). The remission of symptoms in older plants has been observed, but remains an unexplained phenomenon (Singh and de Milliano 1989).

SDM has primarily been controlled through the use of the systemic fungicide metalaxyl. The mode of action of metalaxyl is disruption of the protein synthesis pathway (Williams 1984). Metalaxyl is most commonly applied as a seed treatment (Odvody and Frederiksen 1984a) and is effective 20-30 days after planting, which is sufficient to prevent infection of young seedling by soilborne oospores (Anahosur 1980). A foliar spray of metalaxyl has been shown to be effective, but is not labeled in the United States (Odvody and Frederiksen 1984b, Anaso et al. 1990). As the development of resistance to metalaxyl has been documented in Texas (Isakeit and Jaster 2005), there is compelling need for an integrated approach to disease management.

Crop rotation has been suggested as a method of cultural control based on the observations by Pratt (1978) and Tuleen et al. (1980) that oospores of *P. sorghi* germinate in the presence of non-host roots, although not all oospores will germinate. This management strategy has not been formally tested in the field. The use of bait crops to control pearl millet downy mildew has also been suggested by Thakur (1992). A bait crop is an alternative crop that can be used in between growing seasons or in some cases as an intercrop to control a pest, usually soilborne, on the main crop (Ahmed et al. 2011, Rod and Robak 1994). Both of these reports studied the effect of bait crops on stimulating the germination of the resting spores of *Plasmodiophora brassicae*, causal agent of club root of crucifers, to reduce the amount of inoculum in the field. Although the efficacy of crop rotation is not disputed as an effective form of control, little data have been collected on the effect of rotation or baiting on SDM incidence.

Additionally, a winter cover crop or fallow condition may sufficiently reduce the amount of inoculum in the field sufficiently to control the disease.

Planting dates have been shown to have a significant effect on SDM incidence. Tuleen (1980) observed a reduction in SDM incidence in sorghum fields planted March 15 versus those planted April 8 in Texas. This may have been due to soil moisture and soil temperature. In contrast, Balasubramanian (1974) observed an increase in SDM incidence in late planted sorghum in India.

Host plant resistance has been used with great efficacy to control the incidence of SDM (Williams 1984). Host resistance used in concert with chemical and cultural controls can present a durable and economic management strategy for controlling SDM, whereas the sole dependence on host plant resistance for disease management can result in the breakdown of resistance (Craig and Frederiksen 1980). The identification of resistance associated markers is an important component to continue the identification of genetic material resistant to SDM to use as parent in hybrid crosses.

Other methods of control that have been suggested but rarely implemented due to impracticality are deep tillage, in which oospores are displaced to reduce contact with sorghum seedling roots, and intercropping in which sorghum is planted simultaneously alongside another crop (Tuleen et al. 1980, Olanya and Fajemisin 1992). Research has been proposed to utilize naturally occurring parasites of *P. sorghi,* such as chytridious fungi. However, this option has not been fully developed (Kunene et al. 1990) as a viable form of control.

1.6 OBJECTIVES

There were four main objectives of this study. All of the objectives involved the interaction between sorghum seedling roots and oospores of *P. sorghi*. The first objective was to examine the effect of soil matric potential on the germination of *P. sorghi* oospores and the incidence of sorghum downy mildew under controlled experimental conditions. The second objective was to test the efficacy of bait cropping on reducing the incidence of SDM in a naturally infested field. The third objective was to observe the infection of sorghum seedling roots by *P. sorghi* oospores, and the development of the pathogen *in planta* using epifluorescence microscopy. The fourth objective was to study the sorghum seedling root defense response against infection by *P. sorghi* oospores.

2. THE EFFECT OF SOIL MATRIC POTENTIAL ON THE INFECTION OF SORGHUM ROOTS BY OOSPORES OF *PERONOSCLEROSPORA SORGHI*

2.1 INTRODUCTION

Soil moisture is an important factor affecting the interaction between soil microorganisms and plants. Soil moisture can affect pathogen spore germination, motility, and survival as well as influencing the susceptibility of plant hosts to root infection. Soil moisture influences soil water availability diffusion of host root exudates, and dissolved oxygen (Cook and Papendick 1972). Available soil moisture is measured using units of pressure, in this study kilopascals, and is referred to as matric potential (ψ_m). Matric potential is a component of water potential (ψ). Water potential, according to Tyree (2003), is the chemical potential of water expressed in pressure units. The other components of water potential are osmotic potential (ψ_o) and pressure potential (ψ_p). All three components are important variables when considering water potential in plants due to gradients between plant cells and the environment and plant height. However in soil, matric potential is nearly identical to water potential due to the uniform interaction between water and soil and the uniform movement of solutes in soil (Tyree 2003).

The environmental factors affecting oospore germination and infection by *P. sorghi* have not been clearly elucidated. Soil moisture and temperature have been shown to affect oospore germination and infection (Balasubramanian, 1974). Optimal soil temperature for infection was shown to be 26.6°C (Balasubramanian 1974). Available

soil moisture in the range of 44 to 47% was demonstrated by Balasubramanian (1974) to be conducive to the development of the disease while available soil moisture of 76 to 79% significantly reduced disease in unspecified soil, which makes this study of little use. Tuleen and Frederiksen (1981) observed a reduction in SDM incidence in a field that received rainfall 4-7 days after planting. Schuh (1986) reported that the most disease conducive ψ_m was -20 kPa in comparison with 0.0 kPa or -67 kPa. Disease incidence in soil at field capacity (-33 kPa) was not tested. However, these reports have been either based on observations in the field or in greenhouse experiments where soil moisture was not adequately controlled (Balasubramanian, 1974; Schuh, 1986). It would be interesting to investigate whether or not rain events could predictably reduce incidence of SDM Understanding the environmental factors that influence the development of the disease may be important for improving effective multifaceted management strategies.

The aforementioned studies did not adequately control soil moisture during the initial interaction between host and pathogen. There are approaches or devices that can be used to overcome this limitation. The Haines apparatus (Haines 1930) can be used experimentally to maintain constant matric potentials for the duration of the experiment, unlike other approaches, which cannot adjust for moisture losses from evaporation and transpiration. This apparatus has been used successfully to study other plant/pathogen systems (e.g. Shew 1983; Sidebottom and Shew 1985).

The Haines apparatus is based on the suction force of a column of water that counteracts capillary forces that hold water to soil. The soil is placed on a fritted disk (4-5.5 μm pore size) contained in a glass buchner funnel. The force exerted is

proportional to the distance between the disk and the water reservoir (Haines 1930). The Haines apparatus is not without limitations. One main limitation is that the maximum amount of soil that can be used is about 250 cubic centimeters. Another is that matric potentials lower than -33 kPa become impractical due to the vertical space required to raise the apparatus and achieve the desired lower matric potentials.

Although constant matric potentials are considered a synthetic environment except for constantly saturated soils, maintaining the ψ_m over a range of values provides an approach to determine soil moisture that is most conducive to the development of SDM in sorghum. Maintaining a constant ψ_m is a tool to identify the ψ_m that is significant in resulting on the infection of sorghum seedlings by *P. sorghi.* Both Schuh and Balasubramanian demonstrated that higher matric potentials inhibited disease (Schuh 1986, Balasubramanian 1974) but time interval required for soil to remain at high matric potentials to effectively inhibit disease is unknown. This information may allow growers to alter planting date or irrigation timing to suppress disease. Although planting date is usually adjusted to coincide with conducive temperatures, they can also be adjusted based on climatological precipitation forecasting. Sorghum is usually not irrigated just after planting, but a thorough understanding of disease-conducive soil moisture conditions may influence growers' decisions. Also, knowledge of the influence of soil moisture on SDM incidence may provide growers a predictive tool to estimate the amount of disease to expect when other factors are conducive to disease development, i.e. susceptible variety/hybrid and SDM resistant to metalaxyl. This predictive tool may be of great importance in organic operations that restrict pesticide

applications.

Additionally, the effect of different soil matric potentials on the germination of oospores of *P. sorghi* is unknown. The purpose of this experiment is to determine the effect of three matric potentials on the germination and infection of sorghum seedlings by oospores of *P. sorghi.* Further, the identification of the exposure duration of roots and oospores to a particular soil matric potential to suppress disease was sought. The hypotheses are the most conducive ψ_m in the range tested to disease will be -20 kPa and the most suppressive will be 0.0 kPa as reported by Schuh (1986). The soil matric potential of 0.0 kPa will inhibit *P. sorghi* oospores germination, and that an exposure of 4-7 days to soil matric conditions of 0.0 kPa will reduce the incidence of SDM.

2.2 METHODS AND MATERIALS

Two soils were used in this experiment: a Belk clay (BAA, 59% clay, 30% silt, 11% sand) collected from the Texas AgriLife Experiment Station, west of College Station, Texas, and a Houston black clay (HBC 49% clay, 36% silt, 15% sand) collected from the Stiles Farm near Thrall, Texas. The oospores of *P. sorghi*, pathotype 3 (P3) from Texas used for this experiment were extracted from systemically infected sorghum leaves collected from greenhouse plants. The leaves were air dried at approximately 25°C for five days, then cut into three-cm sections and pulverized in a Waring blender using two 30-second pulses. This material was then suspended in distilled deionized water and filtered through a 2-ply cheesecloth. After 10 minutes, the supernatant was decanted and the sediment was passed through a 37-μm sieve and then air-dried overnight at approximately 25°C. Sorghum hybrid Pioneer 84G62, susceptible to this

strain, was used in this experiment.

Dried oospore powder (approximately 400,000 oospores/g, determined by serial dilution and extrapolation) was mixed with each soil to obtain a concentration of approximately 100 oospores per gram of air-dried soil. The Buchner funnel (diameter 8 cm, disk porosity 4-5.5 µ) assembled to function as a Haines apparatus was filled with approximately 250 cm^3 of infested soil and planted with 40 seeds. The Haines apparatuses were then equilibrated to 0.0 kPa, -20 kPa, or -33 kPa ψ_m by raising the Haines apparatuses to 0 m, 2 m, or 3.3 m, respectively, above the water reservoirs. The seedlings were grown under fluorescent lighting at 25°C with one of the three ψ_m for two weeks. After two weeks, the seedlings were transplanted into half gallon pots containing Baccto Premium potting mix (64% reed sedge peat, 10% sand, 6% perlite, lime to pH 5.5-6.5, charge at 4 lbs per cubic yard), to allow for symptom development. Plants were grown for two weeks in a greenhouse and disease incidence was then evaluated by counting total diseased versus healthy plants. This was determined visually by counting those plants expressing mosaic symptoms as diseased. The experiment was repeated 5 times with 3 replicates per treatment.

An experiment was conducted to determine the effect of a two-day exposure at 0 kPa $\psi_{m,}$ occurring at different time intervals after planting, on subsequent disease development. This is based on the report by Schuh (1986) that soil saturation conditions inhibit disease development. Haines apparatuses containing HBC soil infested with oospores were planted with sorghum as previously described. Each apparatus was equilibrated to -20 kPa ψ_m. At two-day intervals beginning two days

after planting, soil was brought to 0 kPa ψ_m for two days and then returned to -20 kPa ψ_m by raising and lowering the Haines apparatus. The control soil remained at -20 kPa ψ_m for the duration of the experiment, which was 14 days. Five different intervals were evaluated in addition to the control. This experiment included three replicates per treatment and was repeated three times. SDM incidence was evaluated as previously described. Since disease incidence followed a binomial distribution, data was analyzed using logistic regression (Madden et al. 2007). Logistic regression was performed using JMP Statistical Software (SAS Institute, Cary, North Carolina). Analysis of variance tests and mean separations were performed using SAS Statistical Software.

To test the effect of three soil matric potentials on *P. sorghi* oospore germination in the presence of host roots, oospores were sealed between polycarbonate membranes (pore size 0 .4 µm) (Pratt 1978). These membranes were buried ~1cm in HBC or BAA placed in Haines apparatuses. About 40 sorghum seeds were planted in each apparatus in direct contact with the membranes to ensure oospore germination. The matric potentials evaluated were 0 kPa, -20 kPa, and -33 kPa ψ_m. After 1 week of incubation the membranes were recovered, stained with phenolic Rose Bengal, and the proportion of germinated oospores was determined. There were three replicates per treatment, and the experiment was repeated three times. Mean separation was performed using Fisher's Protected Least Significant Difference Test.

2.3 RESULTS

Disease incidence was highest at -33 kPa, followed by -20 kPa and then 0 kPa. There was a significant ($P<0.01$) difference in disease incidence among the three soil

matric potentials for both soil types (Fig. 2). A constant -33 kPa ψ_m was most conducive to disease development, followed by -20 kPa ψ_m and then 0 kPa ψ_m. There was no significant interaction between matric potential and soil type (P=.9509) (Fig. 2). There was a significant difference in disease incidence between the two soil types at both -33 kPa and -20 kPa ψ_m ($P<0.01$), but not at 0 kPa ψ_m (P=0.6616) (Fig. 3). Overall, HBC was significantly more conducive to disease development than BAA (Fig. 3).

Disease incidence was significantly lower in soil brought to saturation from -20 kPa ψ_m four days after planting, compared with soils saturated at other intervals after planting, or kept constant at -20 kPa ψ_m. The mean disease incidence in soil brought to saturation four days after planting was 10%, while disease incidences in all other treatments ranged from 21.8% to 30.7% (Fig.4).

In HBC soil, oospore germination was significantly ($P<0.05$) affected by matric potential. A mean separation test indicated that the greatest germination occurred at -33 kPa and least at 0.0 kPa ψ_m for both soils (Fig.5). The oospore germination in BAA at -33 kPa ψ_m was significantly ($P<0.05$) higher than at 0.0 kPa ψ_m, while -20 kPa ψ_m was intermediate (Fig 5). The mean oospore germination levels in HBC were higher than in BAA, but differences between the soils were not statistically significant ($P<0.05$).

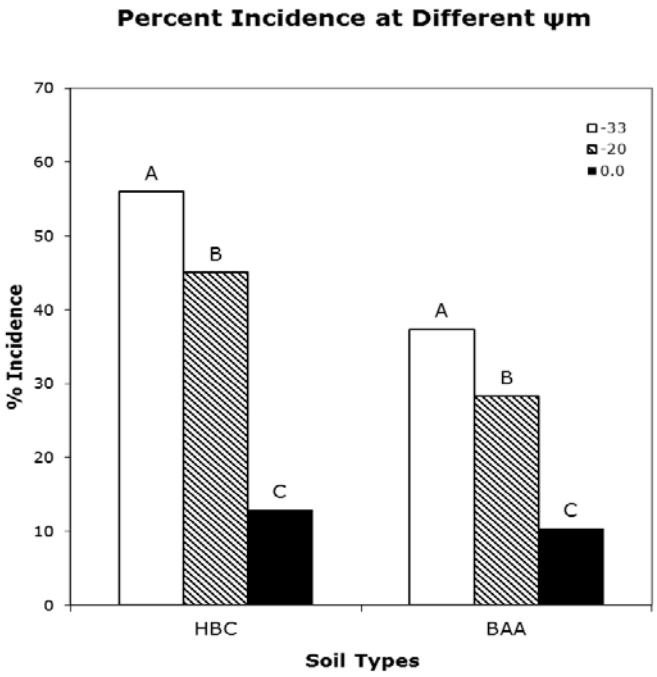

Fig. 2: Mean incidence of SDM at -33 kPa, -20 kPa, and 0.0 kPa ψ_m in Houston black clay (BAA) and Belk clay (BAA). Letters indicate significant differences ($P<0.01$) based on logistic regression analysis.

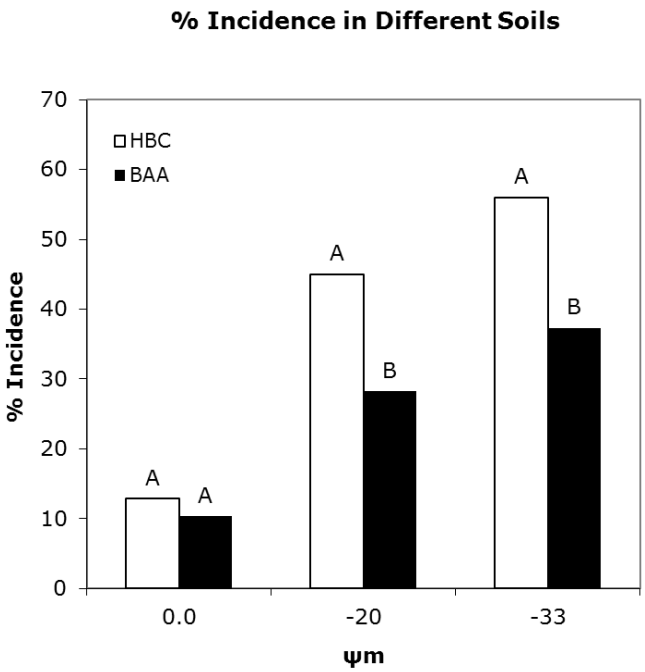

Fig. 3: Mean incidence of SDM in Houston black clay (HBC) and Belk clay (BAA) at 0.0 kPa, -20 kPa, and -33 kPa ψ_m . Letters indicate significant ($P<0.01$) differences, based on logistic regression analysis.

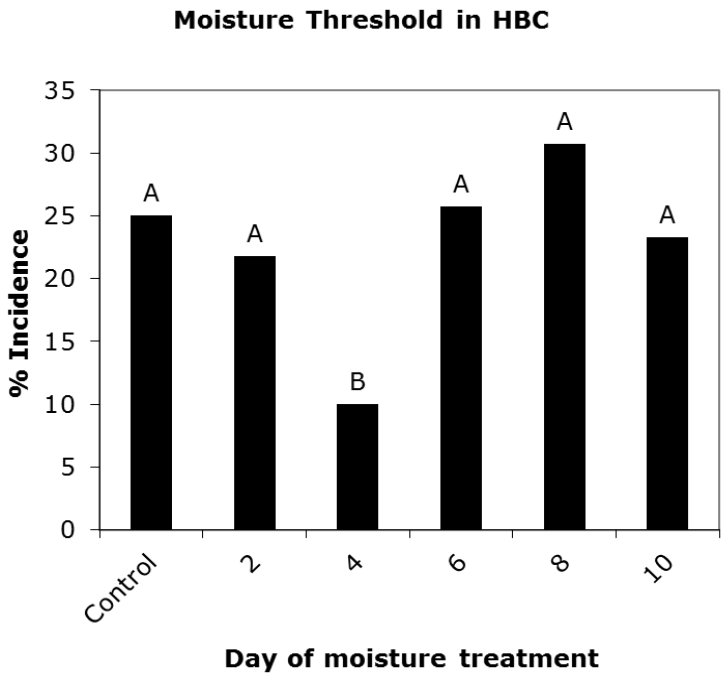

Fig. 4: Mean incidence of SDM following two days of incubation of oospore-infested HBC at saturation, occurring at different intervals after planting. Letters inidicate significant ($P<0.01$) differences, based on logistic regression analysis.

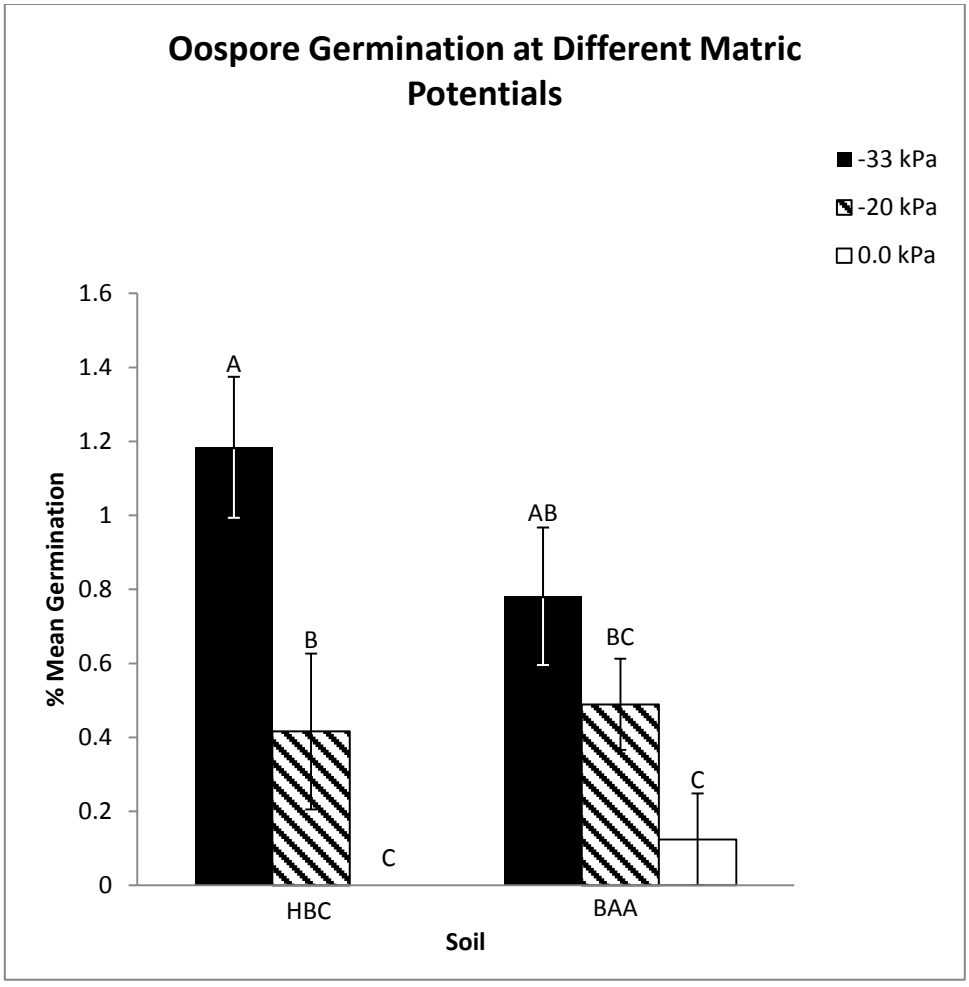

Fig. 5: Mean *P. sorghi* oospore germination (%)ψ_m in Houston black clay (HBC) and Belk clay (BAA). at -33 kPa, -20 kPa, and 0.0 kPa Letters indicate significant ($P<0.05$) differences using Fisher's Protected Least Significant Difference Test.

2.4 DISCUSSION

In this study, the greatest incidence of sorghum downy mildew occurred in soil maintained at -33 kPa ψ_m (field capacity), followed by -20 kPa ψ_m, and then 0.0 kPa ψ_m (saturation). Additionally, exposure to saturation 4 days after planting reduced disease incidence in comparison with earlier or later saturation exposures. Oospores of *P. sorghi* incubated with sorghum seedlings showed reduced levels of germination corresponding with increasing matric potential. These data support the findings by Pratt (1978) and Schuh (1986) that higher available soil moisture results in reduced disease. However this is the first report studying the effect of soil moisture on SDM where matric potential is controlled using a (Haines) apparatus. The use of the Haines apparatus allows for long-term control of matric potential to accurately measure the effects of soil moisture in a system. Pratt (1978) reported a significant reduction in the germination rates of *P. sorghi* oospores that were watered daily in the presence of host roots, suggesting an inhibitory effect of high soil moisture. Additionally, Balasubramanian (1974) reported that available soil moisture levels of 76-79% inhibited the incidence of sorghum downy mildew (SDM) compared with 44-47 % available soil moisture. In his experiments, Schuh (1986) amended soil with the appropriate amount of water to achieve the desired matric potential. Inevitably, soil moisture changes very quickly after planting due to surface evaporation and the evapotranspiration (Orgaz et al. 2005). Schuh (1986) determined that the maximum infection occurred at -20 kPa ψ_m. In this study, the lower soil moisture of -33 kPa ψ_m (field capacity), was significantly more conducive than -20 kPa ψ_m to disease. These results were consistent

when tested in two different soil types. In general, soilborne fungal and oomycetes pathogens grow and develop more successfully in environments of higher available moisture although there are exceptions (Cook and Papendick 1972). Incidence charcoal rot of sorghum caused by *Macrophomina phaseolina,*for example, is favored by drier conditions. However this is mainly attributed to the physiological effect that the water stress has on the host rather than the effect on the pathogen (Diourte et al. 1995). Extreme cases of fungi favoring low available-water environments include those of the genus *Xeromyces.* In this case the ability to thrive at very low water potentials seems to be an adaptation to be able to exploit very sugar rich nutrient sources (Leong et al. 2011). Chytrid fungi have been observed to mycoparasitize and have been proposed as a form of biocontrol to control SDM although this option has never been extensively studied (Kunene et al. 1990). Chytrids which often favor moist conditions may reduce the germination and subsequent incidence of SDM and therefore contribute to the observed reduction in SDM incidence at higher ψ_m (Freeman et al. 2009). Fungicide treated oospores may help to verify this.

The incidence of SDM was previously shown to be greater with sorghum grown in sandy soils compared with clay soils (Schuh 1986). One soil used in this study, Houston black clay (HBC 49% clay, 36% silt, 15% sand), has less clay content than the second soil, Belk Clay (BAA 59% clay, 30% silt, 11% sand). The disease incidence was less in BAA than in HBC with incubation at the same matric potentials. Similar effects on *Pythium spp.* population diversity and incidence by different soil textures have been reported by Broders et al. (2009). Additionally, higher rates of root

colonization of *Juniperus procer* by arbuscular mycorrhiza fungi were observed in soils with less clay content (Al-Ghamdi and Jais 2013). Clay particles release water less readily than silt or sand. Therefore the availability of water from less clay soils versus more clay soils may have an effect on the ability of *P. sorghi* to successfully infect host roots. This effect may also be due to sandy and silty soils having better aeration (Saif 1981) than clay soils. Sandy and silty soils also have larger pore sizes, which may have an effect on the ability of hyphae to develop. Thus, the observed effect of a matric potential of 0.0 kPa being less conducive to SDM incidence may be attributed to poor soil aeration and limited pore sizes as a result of high clay content. There may also be chemical or biological differences that resulted in increased incidence in HBC.

Tuleen and Frederiksen (1981) reported that a rain event of 4-7 days after planting reduced the incidence of SDM. This coincides with the timing of oospore germination (Safeeulla 1976). Rain after planting was simulated using the Haines apparatus by equilibrating soil to -20kPa ψ_m and then bringing them to 0.0 kPa ψ_m every 2 days to simulate a rain event. Matric potential was re-adjusted to -20 kPa ψ_m after 2 days. The results of these experiments showed a significant reduction in SDM incidence at the 4 day saturation treatment. The work reported here and that of Tuleen and Frederiksen (1981) provide strong evidence that conditions of high matric potentials ~4 days after planting may effectively reduce incidence of SDM. To further validate these findings, additional SDM incidence data following rain events need to be collected over the course of several years and locations. This information may then be used to make SDM management recommendations for altering planting dates or in

response to climatic events or irrigation.

To determine whether the reduction in SDM incidence was due to reduced oospore germination, oospores were incubated with sorghum roots in both soils and germination determined. As matric potential increased from -33 kPa ψ_m to 0.0 kPa ψ_m, the germination of oospores decreased significantly. These data indicate that ψ_m affects infection of sorghum roots by *P. sorghi*. This effect may be due to a dilution of germination stimulants such as root exudates by excessive moisture (Stevenson et al. 1995) or inhibition of oospore germination in response to depleted oxygen at high soil moisture levels (Odvody 1992). This effect of moisture on oospore germination should be further analyzed to incorporate soil moisture into a SDM disease prediction models such as the sorghum yield loss simulation model developed by Tuleen and Frederiksen (1981). This information can also be used to update current management recommendations by taking into account climatological data into planting date decisions (Balasubramanian, 1974). Perhaps disease control may be obtained through management of soil water potential as suggested by Cook and Papendick (1972). They suggest that in the case of charcoal rot of sorghum, an appropriately timed irrigation shortly after bloom may be used to control it. In the case of charcoal rot of sorghum, drought stress is a predisposition factor. Alleviating drought stress results in reduced incidence of charcoal rot. Of course the feasibility of this in sorghum growing regions may be limited since sorghum is often grown on marginal lands with little or no irrigation capabilities.

3. THE EFFECT OF BAIT CROPPING ON THE INCIDENCE OF SORGHUM DOWNY MILDEW IN A FIELD NATURALY INFESTED WITH OOSPORES OF *PERONOSCLEROSPORA SORGHI*

3.1 INTRODUCTION

A bait crop is used to reduce the inoculum potential of pathogen propagules in infested soil by stimulating propagule germination in the absence of a host plant (Ahmed et al. 2011). This is most likely to be effective against pathogens that have a narrow host range such as *P. sorghi*. To complete its life cycle *P. sorghi* requires a living compatible host. Once oospores germinate, the germ tubes must find a host or the tube will die. Pratt (1978) showed that *P. sorghi* oospores germinate in the presence of wheat, oats, cotton, and soybean seedling roots. Later, Gowda and Bhat (1986) expanded this list to include pearl millet (*Pennisetum glaucum*), mustard (*Brassica juncea*), and green gram (*Vigna radiata*). These authors suggested that using non-hosts as bait crops to reduce inoculum potential in the fields may be an effective component of a management strategy. In 1980 Tuleen et al. demonstrated, that naturally- infested soil sown with several non-hosts 15 day prior to sorghum seeding reduced disease incidence (Tuleen et al., 1980). This experiment was performed in a greenhouse over 32 days. The list of non-hosts tested in the greenhouse included wheat (*Triticum aestivum*), but did not include rice (*Oryza sativa*), soybean (*Glycine max*), or hairy vetch (*Vicia villosa*). These three crops are cultivated in Texas and may represent feasible options in alternative control strategies. Growers may value information that allows them to reduce inoculum without the use of chemical controls. This study examines the efficacy of using bait

cropping as a management alternative to reduce incidence of SDM in naturally infested field. The hypothesis is that all bait crops tested will effectively reduce incidence of SDM compared to a non-baited control in a naturally infested field.

3.2 METHODS AND MATERIALS

The non-hosts tested to induce germination of oospores were wheat, rice, and hairy vetch. Sorghum was included as a positive control. Seeds were surface disinfected in 70% ethanol for 5 minutes and 1% NaClO for 2 hours, rinsed in sterile water and germinated on a moist filter paper in a Petri dish at 35°C. As soon as germination occurred, the seedlings were transferred to a second Petri dish that contained nine , $1cm^2$ polycarbonate membranes onto which an unquantified oospore suspension has been placed. Each plate was moistened with 1.5 mL of sterile water and incubated at 25°C in a sealed plastic box in the dark. Membranes were sampled after 3, 4, and 5 days incubation. Each membrane was stained for at least 30 minutes in phenolic Rose Bengal and destained in water. The proportion of germinated oospores was counted using a compound microscope.

The efficacy of these bait crops in reducing inoculum potential was examined in a field naturally infested with *P. sorghi* near Palacios, Texas. The experiment was designed as an 8x8 Latin Square to account for the unknown bidirectional variation of distribution of inoculum in the field (Table 1). Each plot was two rows, six feet long, with one foot of spacing between plots. Each plot was planted on March 20 with the appropriate bait crop except for the untreated control. These crops were grown for 3 weeks. After 3 weeks the bait crops were removed by hoeing and all blocks were

planted with Pioneer sorghum hybrid 84G62, which is susceptible to both P3 and P6 pathotypes of *P. sorghi*. The incidence of systemically infected plants was evaluated after one month. An analysis of variance was performed using SAS Statistical Software (SAS, Cary, North Carolina). The experiment was performed once.

Table 2. Latin square design of bait cropping experiment

Rep	1	2	3	4	5	6	7	8
Row#	6 ft	6 ft	6 ft	6 ft	6 ft	6 ft	6 ft	6 ft
1	A	F	C	G	B	E	H	D
2	B	C	D	E	F	H	A	G
3	C	H	E	B	G	F	D	A
4	D	G	B	F	E	A	C	H
5	E	D	H	C	A	G	F	B
6	F	E	G	A	H	D	B	C
7	G	B	A	H	D	C	E	F
8	H	A	F	D	C	B	G	E

Treatment
A-Sorghum
B-Wheat
C-Rice
D-Soy
E-Vetch
F-Fallow
G-Corn
H-Cotton

3.3 RESULTS

All bait crop seedlings were able to induce oospore germination. Germination was observed at four and five days after exposure to roots. % Mean oospore germination rates and standard errors are presented in Table 2.

Table 3. Percent mean germination of *P. sorghi* oospores in the presence of host and non-host roots

Treatment	4 days	5 days
Sorghum	1.26±1.26	3.67±2.65
Wheat	2.18±0.31	0.93±0.48
Rice	0.5±0.26	3.3±1.21
Vetch	0	0.29±0.29
Control	0	1.71±0.07

In the field experiment, disease incidences with treatments were lower than the untreated control ($P<0.01$) (Fig. 6).

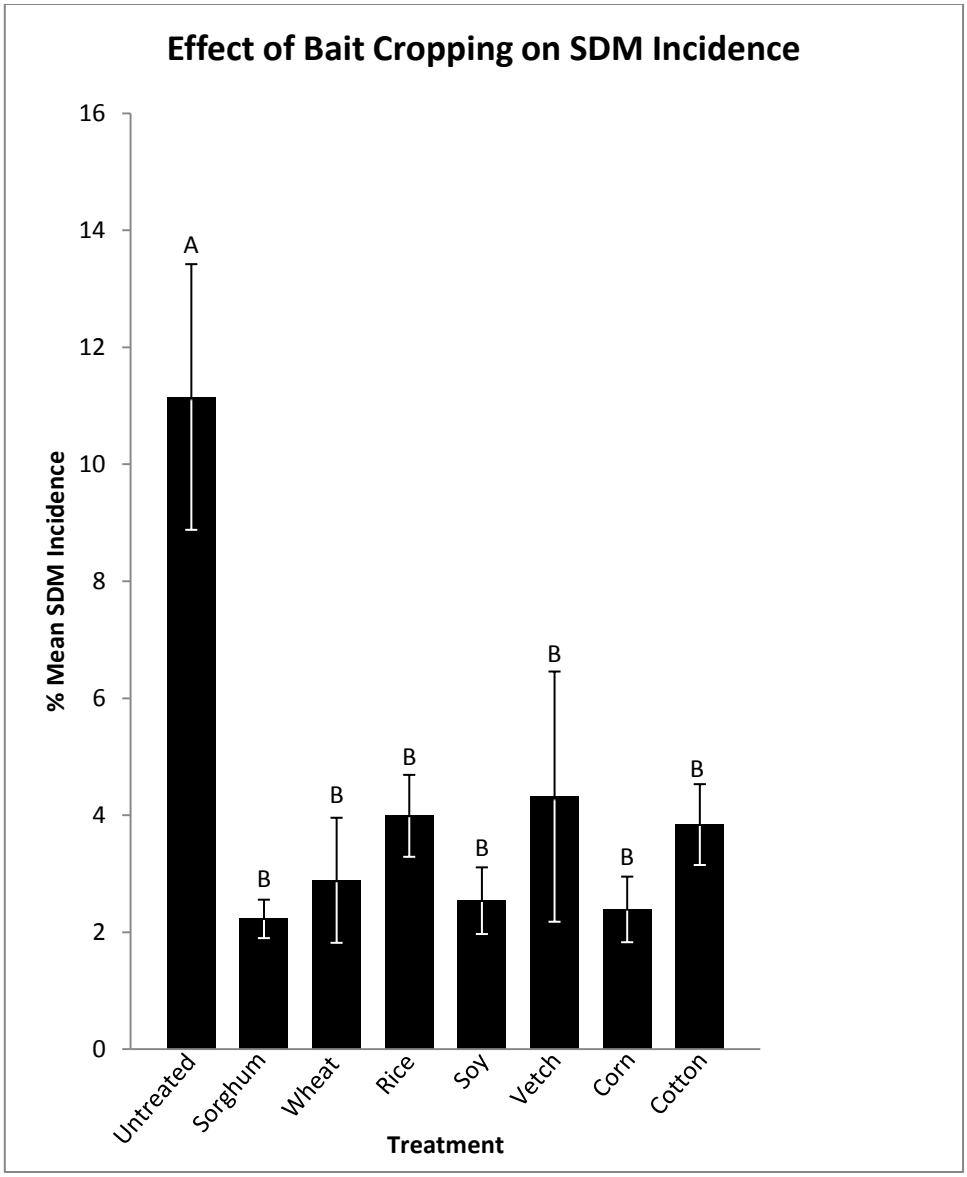

Fig. 6. Percent mean SDM incidence in a naturally infested field sown with various bait crops. Letters assigned using Fisher's Protected Least Significant Difference test ($P<0.01$).

3.4 DISCUSSION

Crop rotation is a well-known component of integrated pest management but bait cropping is a control method that has not been evaluated in many plant-pathogen systems. Often the principle of disease control by crop rotation relies on not allowing pathogens to complete their life cycles, thus preventing an increase of inoculum (Bullock 1992). In the case of SDM, oospores of *P. sorghi* are induced to germinate in the presence of host or non-host roots (Pratt 1978). *P. sorghi* is an obligate biotroph and must infect a compatible host to complete its life cycle. The hypothesis that inoculum levels of *P. sorghi* can be reduced by baiting with non-host roots was tested in this experiment. Baiting here is defined as the stimulation of oospore germination by non-host roots, which does not allow *P. sorghi* to complete its life cycle. Oospores were tested to confirm that germination occurs in the presence of non-host roots. The rates of germination were inconsistent as reported by Pratt (1978) who observed germination rates in the presence of non-host roots ranging from 0.00 % to 1.31% at 4 days. Tuleen et al. (1980) expanded upon this study by performing a greenhouse experiment to test the efficacy of bait cropping in pots. The results of Tuleen's study indicate that bait cropping may be a viable addition to current management recommendations for SDM. In the current study a field naturally infested with SDM was sown with various bait crops and subsequently seeded with sorghum. The results of this experiment further support the hypothesis that crop rotation or bait cropping can reduce inoculum potential of SDM in the field. Further experiments over time and location are required before bait cropping can be suggested as a possible management

recommendation. Monitoring oospore levels in the soil as well as repeating the experiment over several seasons and locations would help to understand the actual effect of bait cropping and crop rotation on *P. sorghi* oospores. Additionally it would be very interesting to test the effect of bait cropping versus crop rotation on the reduction of inoculum potential of SDM. Baiting with commonly used cover crops between growing seasons may significantly reduce inoculum in the field. Experimentally-derived minimum temperatures that oospores can be stimulated to germinate would greatly assist to determine if an over-winter non-host baiting could effectively reduce inoculum present in the soil. One of the obvious limitations to baiting is the costs involved in purchasing seed and planting seed that does not yield a profitable harvest. For this reason a cover crop or forage that can be used as a bait crop may present a more feasible alternative. Hairy vetch is a nitrogen fixing legume that has been shown to be beneficial as a green manure in increasing nitrogen uptake in sorghum when used as a winter cover crop (Choi and Daimon, 2008). It has also been shown to suppress *Fusarium oxysporum* and *Thievaliopsis basicola* when used as a soil amendment (Candole and Rothrock 1997) (Zhou and Everts 2004). Despite that sorghum growers do not currently practice bait cropping as a way to control SDM, determining if a short term bait cropping cycle or over-winter fallow could work instead of a full year crop rotation would be useful.

4. STUDY OF THE MICROSCOPIC INTERACTION BETWEEN SORGHUM SEEDLING ROOTS AND *PERONOSCLEROSPORA SORGHI* AND THE DEVELOPMENT OF THE PATHOGEN FROM ROOT TO SHOOT

4.1 INTRODUCTION

Knowledge of the developmental events of a pathogen during infection may be important to develop effective management strategies for the disease. Although the infection process of sorghum leaves by sporangia has been described, oospore derived root infection remains only briefly studied (Yeh and Frederiksen 1979). Germ tubes extending from oospores penetrate roots and intercellularly colonize the host (Safeeulla 1976). However, there is no clear description of the colonization nor the time required for *P. sorghi* to become fully systemic. Pawar (1986) suggested that all leaves after the first leaf become systemically colonized, but the reason that the first leaf remains uncolonized is not known although it is possible that *P. sorghi* may have difficulty colonizing differentiated tissue (Dernoeden and Jackson 1980).

Gametogenesis in *P. sorghi* has been studied (Safeeulla and Thirumalachar 1955, Pawar 1986). Oospores differentiate from hyphal tips in the intercellular spaces in systemically-infected leaves. Both oogonia and antheridia are derived from these invasive hyphae (Safeeulla and Thirumalachar 1955). Pawar (1986) was unable to identify mating types and concluded that *P. sorghi* is likely homothallic. Despite these studies, high-quality images of the infection and colonization process of SDM remain few (Pawar, 1986). The objective of this study was to observe and document the colonization and the compatible (susceptible) and incompatible (resistant) interactions

between sorghum and *P. sorghi*. The hypothesis of this study is that the infection of susceptible sorghum seedling roots by oospore-derived hyphae will progress by distinct stages of colonization whereas resistant sorghum seedlings will either not be infected or prevent colonization.

4.2 METHODS AND MATERIALS

Approximately 1cm² samples from the midsection of sorghum leaves were taken at different symptomatic as well as asymptomatic stages. The samples were excised from 30 day old, laboratory-infected sorghum plants of the Pioneer hybrid 84G62 grown in a green house. Plants were inoculated according to Craig (1976). Tissue was also taken from uninfected sorghum plants of the same hybrid and age. Samples were prepared according to Hood and Shew (1996), but modified by using aniline blue as a fluorochrome. Aniline blue binds to β-1,3-glucan, which is present in the cell walls of oomycetes. The samples were decolorized in 1M KOH, placed in a boiling water bath for 5 minutes, and then rinsed three times in tap water. The prepared samples were then mounted in 5% aniline blue/.067M K_2HPO_4 at pH 9.0 and examined using epifluorescence microscopy (Olympus U-MNU2 filter at an excitation wavelength of BP360-70, emission BA420, and dichromatic mirror 400).

To examine root infection and development of *P. sorghi* in resistant and susceptible sorghum seedlings, seeds of Pioneer hybrid 84G62 (susceptible to P3/resistant to P1) and Pioneer hybrid 82P75 (resistant to P3) were surface disinfected by treatment with 95% ethanol for 5 minutes and 10% bleach for an additional 2 hours. Seeds were then germinated on moistened filter paper for 2 days at 35°C. Seedling

roots were coated in oospores by dredging in oospore powder and incubated in moist chambers at 25°C. The experimental design is depicted in table 4. Moist chambers were Petri dishes with sterile filter paper moistened with 1.5 ml autoclaved ddH2O. Oospore powder was prepared from greenhouse collected systemically-infected leaves exhibiting necrotic striping as previously mentioned in Chapter 2. Samples were collected 2, 6, and 8 days after inoculation (dpi). Later samples of the 84G62/P3 interaction were taken at 10, 12, and 14 dpi.

Table 4. Experimental design for compatible/incompatible interactions

Hybrid/Pathotype	P3	P1	No oospores
84G62	compatible**	incompatible*	Control
82P75	incompatible*	not done	Control

4.3 RESULTS

In 30 day-old systemically-infected leaves, *P. sorghi* had a compact hyphal morphology and was restricted between the vascular bundles of the leaf (Fig. 7A). Similar morphology was observed by Dernoeden and Jackson (1980) during the colonization of *Poa pratensis* by *Sclerophthora macrospora* and was referred to as robust secondary polymorphic hyphae. Although it was reported that oospore-producing leaves are no longer able to produce sporangia (Safeeulla 1976), leaves with brown striping were observed that exhibited both oospores and compact sporangia

hyphal patterns (Fig. 7B). The compact hyphal patterns may be a remnant of previous sporangial production. In older, systemically-infected leaves with striping that indicates oospore development (Safeeulla 1976), there was a dense, prolific hyphal colonization of the intervascular tissues (Fig. 7C). Oospore development can be clearly seen (Fig. 7C) and appears to be derived from invasive hyphae as previously described (Safeeulla and Thirumalachar 1955). There was no detectable colonization in non-symptomatic leaves (Fig. 7D).

In the seedling infection experiment, no oospore germination was visible at 2dpi in any of the compatible or incompatible treatments (Fig. 8). Compatible plant/pathogen interactions result in disease and a limited or no defense responses from the plant. Incompatible plant/pathogen interactions result in limited or no disease/colonization and elevated defense responses from the plant.

Samples of the incompatible interactions were prepared and observed until at 2, 6, 8 10 and 12 dpi. In the incompatible interactions, oospore germination and the development of appressoria-like structures were observed by 6 dpi (Fig. 9A). Despite the development of these appressoria-like structures the pathogen was never able to colonize the host (Fig. 10C). No further pathogenic development was observed during the incompatible interactions.

Samples of the compatible interaction between sorghum seedlings and *P. sorghi* were taken at the same time points as those described with and additional sample at 14 dpi. During the compatible interaction germination and infection was visible by 6dpi (Fig. 9). Infection during the compatible interaction progressed

longitudinally and intercellularly (Fig. 11B). This type of growth was referred to by Dernoeden and Jackson (1980) as narrow primary extension hyphae when observed during the colonization of *Lolium perenne* by *Scerophthora macrospora.* The pathogen was present in the root tips at 8dpi (Fig. 10B). At 10 dpi haustoria were clearly visible as brightly fluorescent structures in the leaves (Fig. 11A). At 12 dpi the seedling was systemically infected, and the morphology of the colonization in the leaf was consistent with the compact hyphal morphology morphology of leaves able to produce sporangia (Fig. 11C). Complete root (Fig. 12C) and second leaf tip (Fig. 12B) colonization occurred at the root tips by 14dpi while the first leaf remained uncolonized throughout the 14 days (Fig. 12D).

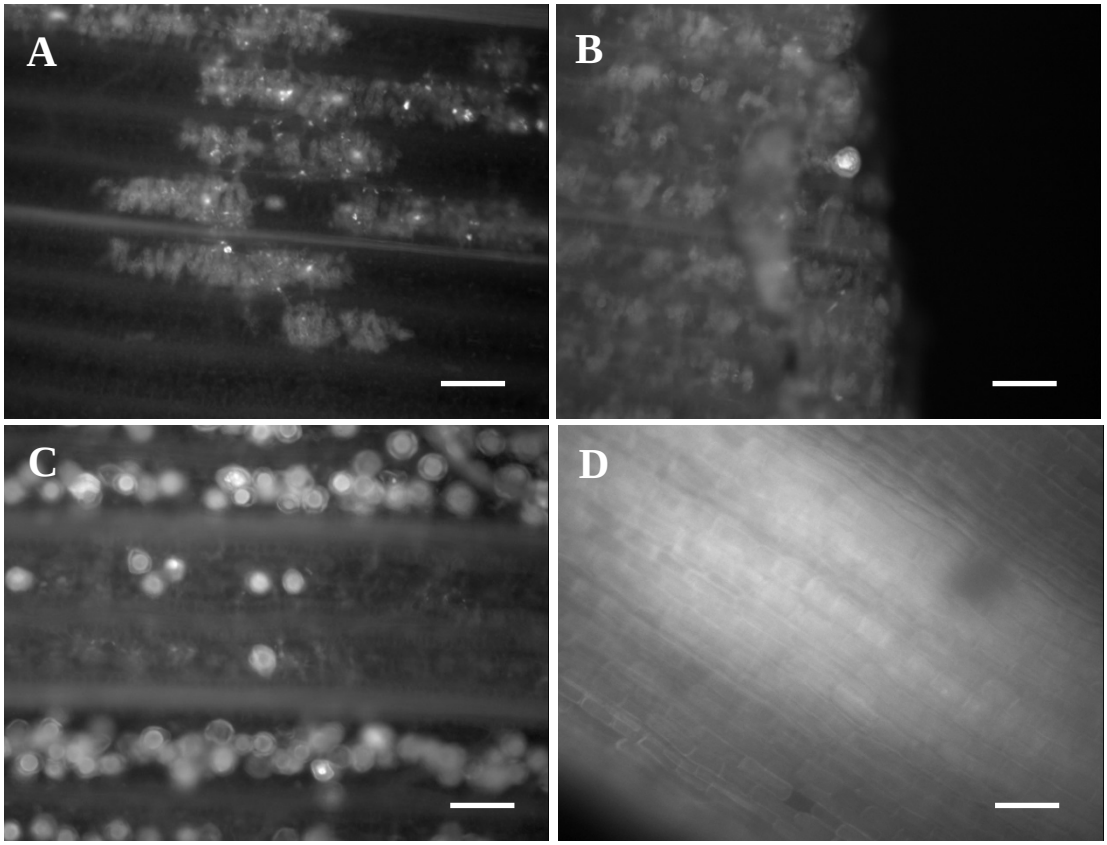

Fig. 7. Fluorescent microscopy images of *P. sorghi* morphology at different symptom stages, prepared using the KOH/aniline blue staining method 10x magnification, scale bars = 100 µm. **A**, Hyphal colonization pattern in a young, systemically-infected leaf. Typical pattern seen in infected tissue prior to sporangia production; **B**, A single oospore formed in a young systemically infected leaf; **C**, Oospores separated by vascular bundles in a systemically infected leaf exhibiting leaf striping symptoms; **D**, Non-symptomatic control.

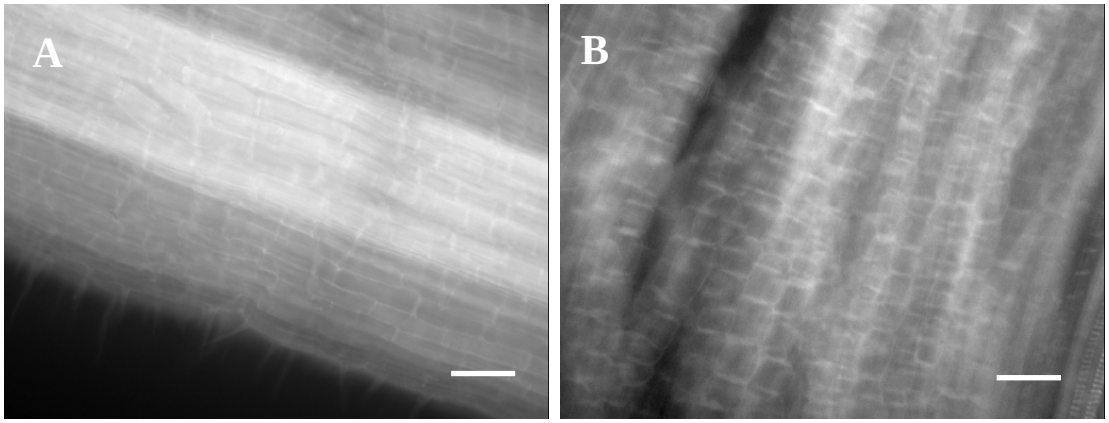

Fig. 8. Fluorescent microscopy images of sorghum hybrid Pioneer 84G62 inoculated with oospores of P3, 2 dpi, 10x magnification, scale bar =100µm. **A,** Uncolonized root tissue; **B,** Uncolonized leaf tissue.

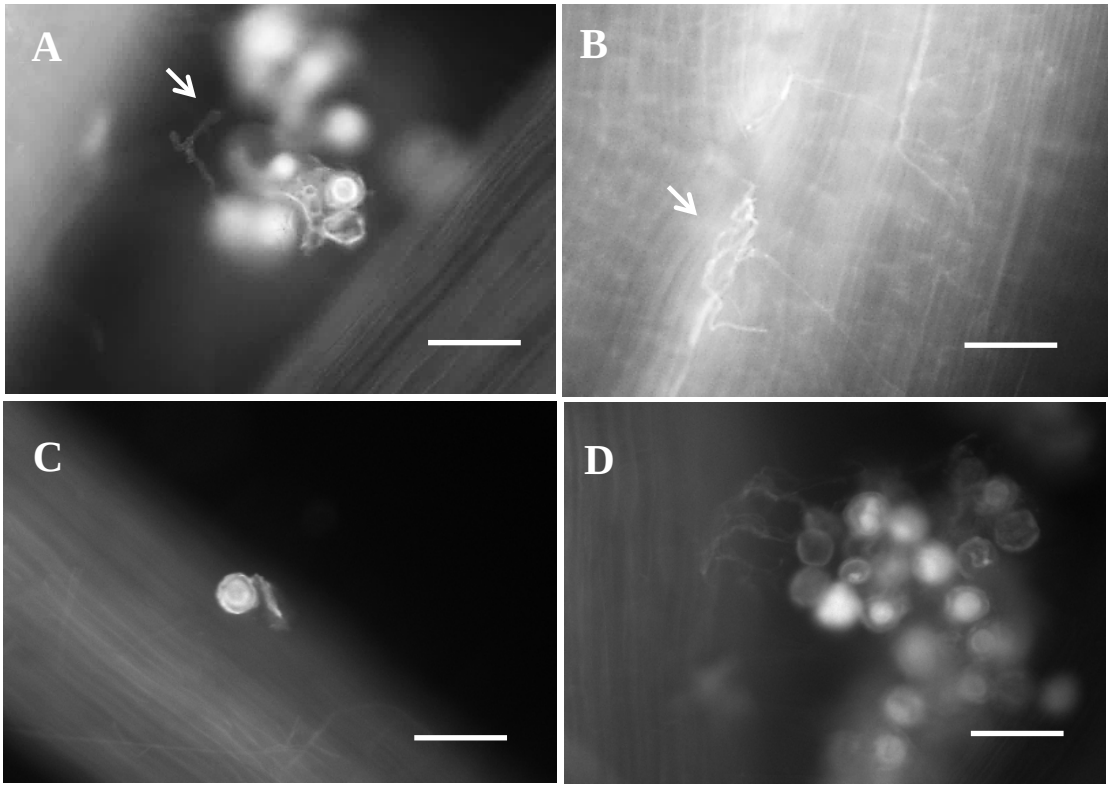

Fig. 9. Fluorescent microscopy images of *P. sorghi* morphology at 6 dpi, prepared using the KOH/aniline blue staining method 10x magnification, scale bars = 100 µm, *=incompatible interaction, **=compatible interaction. **A**, Oospore germination and appressoria-like structure (arrow): 84G62+P1*; **B**, Initial root colonization (arrow): 84G62+P3**; **C**, Oospore germination, no penetration: 82P75+P3*; **D**, Oospore germination: 84G62+P3**.

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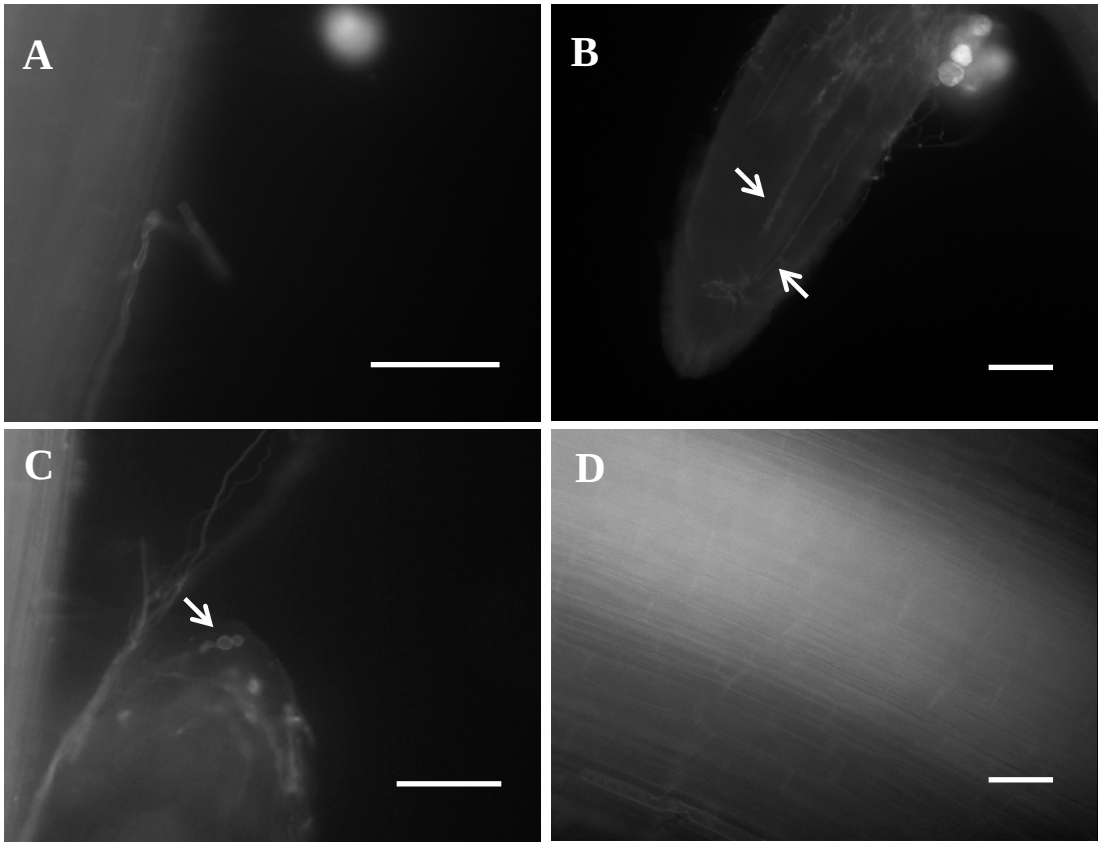

Fig. 10. Fluorescent microscopy images of *P. sorghi* morphology at 8 dpi, prepared using the KOH/aniline blue staining method 10x magnification, scale bars = 100 µm, *=incompatible interaction, **=compatible interaction. **A**, Germ tube on the surface of root, but no penetration 84G62+P1*; **B**, Root tip colonization (arrows): 84G62+P3**; **C**, Appressoria-like structure (arrow): 82P75+P3*; **D**, No leaf colonization: 82P75+P3*.

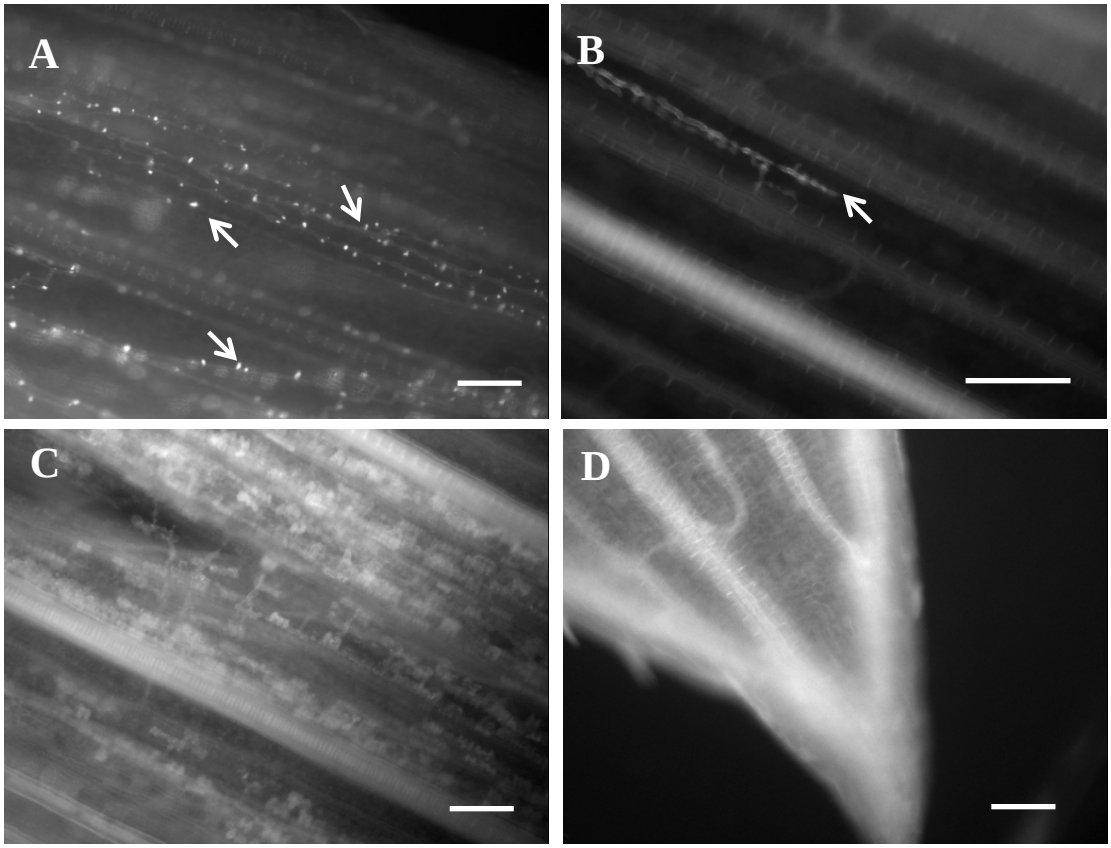

Fig. 11. Fluorescent microscopy images of *P. sorghi* morphology at 10 and 12 dpi, prepared using the KOH/aniline blue staining method 10x magnification, scale bars = 100 µm, *=incompatible interaction, **=compatible interaction. **A**, Leaf colonization 10 dpi, haustoria visible (arrows): 84G62+P3**; **B**, Invasive hypha (arrow) 10 dpi, no haustoria: 84G62+P3**; **C**, Leaf colonization 12 dpi, consistent with sporangia production: 84G62+P3**; **D**, Leaf tip not colonized 12 dpi: 84G62+P3**.

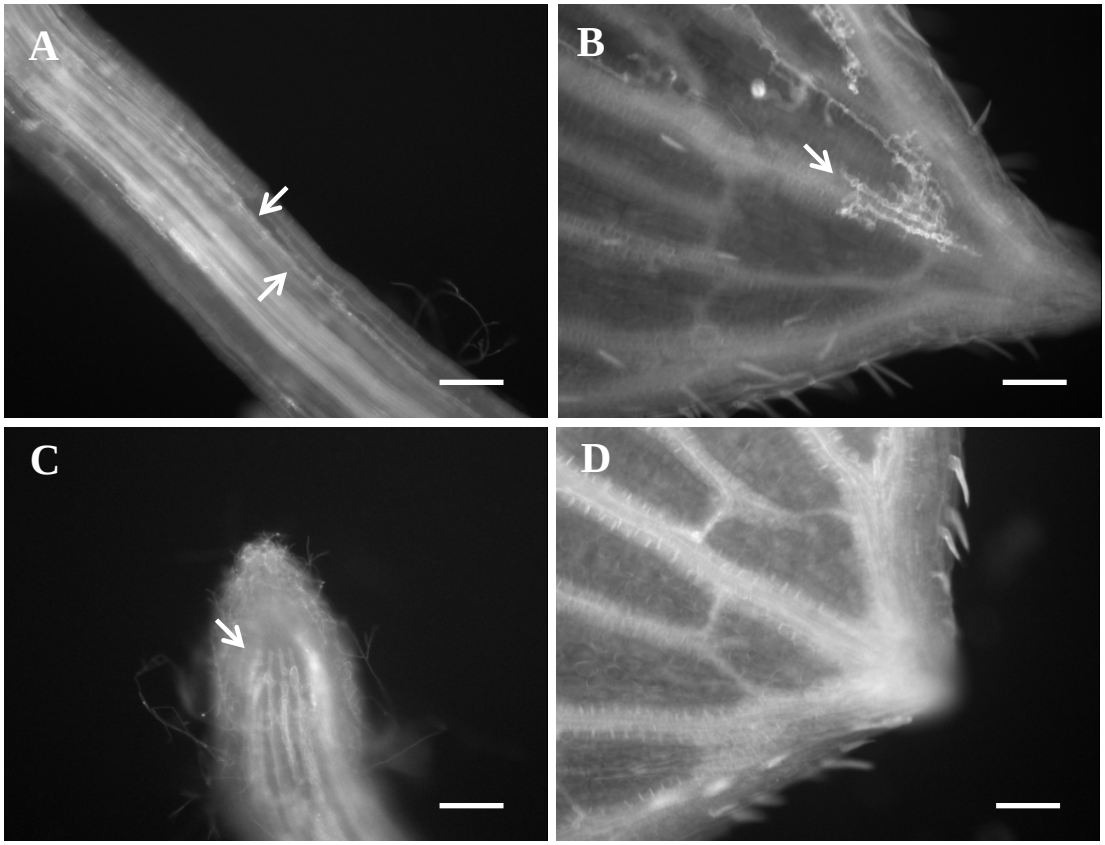

Fig. 12. Fluorescent microscopy images of *P. sorghi* morphology at 14 dpi, prepared using the KOH/aniline blue staining method 10x magnification, scale bars = 100 µm, *=incompatible interaction, **=compatible interaction. **A**, Extensive root colonization: 84G62+P3**; **B**, Tip of the 2nd leaf colonized by invasive hyphae: 84G62+P3**; **C**, Root tip colonized by invasive hyphae: 84G62+P3**; **D**, Leaf tip of 1st leaf not colonized: 84G62+P3**.

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4.4 DISCUSSION

There have been several studies (Mauch-Mani et al. 1989, Safeeulla 1976, Yeh and Frederiksen 1979) that describe the germination of *P. sorghi* sporangia. The sporangia germinate shortly after being produced on the abaxial side of systemically infected sorghum leaves. Sporangia placed on sorghum leaves can be observed germinating by a single germ tube. This germ tube then penetrates the leaf through the stomata. Electron microscopy has shown hyphal colonization of sorghum leaves resulting from sporangia derived infection (Mauch-Mani et al., 1989).

Oospores however are the primary of inoculum for SDM and the microscopic colonization of sorghum tissues by oospore derived infection remains poorly studied. Safeeulla (1976) reported appressoria-like structures at the infection point on sorghum roots inoculated with *P. sorghi* oospores. In the present study swelling of the hyphal tips may indicate the formation of penetration structures as suggested by Subramanya et al. (1983) (Fig. 9A). Similar structures have been observed during the infection of *Medicago truncatula* by the root infecting oomycete, *Phytophthora palmivora* (Rey at al. 2015). Oospore germination studies in the proximity of sorghum seedling roots have not revealed the development of these structures indicating that this hyphal swelling only occurs in contact with roots. Similar structures have been observed in germ tubes derived from sporangia penetrating sorghum leaves (Mauch-Mani et al. 1989). However, further studies are required to confirm the hypothesis that hyphal swelling is associated with root penetration.

This study also clearly shows colonization, not only of the aerial organs of

the seedling but also of the roots. Indeed, root tip colonization is observed before the colonization of the leaves (Fig. 10B). The highly fluorescent structures observed in the leaves, which resemble haustoria (Fig.11A), are not observed in the roots. This indicates that, as in many other biotrophic systems, the pathogen likely sequesters plant derived sugars from its host (Wahl et al. 2010), although haustoria can also form in roots such as in the interaction between *M. truncatula* and *P. palmivora* (Rey et al. 2015).

A time course over the different stages of infection reveals distinct phases of pathogen colonization. In neither the case of compatible nor incompatible interactions does colonization of the first leaf occur (Fig. 12 D). A similar effect was observed during colonization of both *Lolium perenne* and *Poa pretensis* by *Sclerophthora macrospora* (Dernoeden and Jackson 1980). This effect may be due to the inability of some pathogens to biotrophicaly colonize differentiated tissue. By the time the infection progresses from the root to leaf tissue the first leaf has already differentiated. This may indicate why older sorghum plants are considered resistant to infection by oospores of *P. sorghi.* The development of systemic infection by local lesions caused by *P. sorghi* sporangia then remains an unexplained phenomenon. Oospore germination occurs 4-5 days post inoculation (dpi) in the presence of both resistant and susceptible roots. Following germination is the penetration stage. Hyphae swell to resemble appressoria-like structures. These are visible on the surfaces of both susceptible and resistant sorghum varieties. In the case of the P3/82P75 incompatible interaction, colonization does not occur. Infection of 84G62 by P3 progresses both

through leaf and root tissue until the plant is systemically colonized. Haustoria can clearly be seen and likely provide nutrients to invasive hyphae, which continue to grow towards the distal portion of the leaves. This is in contrast to haustoria observed from sporangia derived infection on leaves, which exhibit finger-like haustoria. The fungal structures reported by Mauch-Mani et al. (1989) do not depict the progression or morphology of oospore-derived systemic colonization. Once the plant is systemically infected, morphologies develop that correspond to the reproductive stages of *P. sorghi*. Further studies are required to identify more clearly the invasive structure of *P. sorghi*. Previous studies have reported invasive structures of *P. sorghi* inside of sorghum leaves derived from sporangia-derived leaf infection (Mauch-Mani et al. 1989, Yeh and Frederiksen 1979). However the present study represents the first time that oospores-derived root infection and systemic colonization has been observed. The use of the KOH/aniline blue method results in high quality images that clearly show the colonization of sorghum tissues by *P. sorghi*. Prolific hyphal colonization and oospore production are clearly visible, indicating that this is a viable method for future studies involving the development of *P. sorghi*.

5. STUDY OF THE ENZYMATIC DEFENSE RESPONSE IN SORGHUM SEEDLING ROOTS ELICITED BY INOCULATION BY *PERONOSCLEROSPORA SORGHI* OOSPORES

5.1 INTRODUCTION

Information regarding the molecular interactions between sorghum and *P. sorghi* is scarce, primarily due to the challenges associated with this system including extremely low oospore germination and inconsistent infection rates (Craig 1983). The lack of basic research may also be due in part to the relative ease in deployment of resistant hybrids.

The resistance response of sorghum to *P. sorghi* oospore infection is a poorly studied facet of the SDM/sorghum interaction. In contrast, other sorghum/pathogen interactions have been well studied and characterized (Basavaraju et al. 2008). Grain mold disease of sorghum, for example, has been shown to be affected by the expression of phytoalexins and defense related proteins (Katile 2007).

Under pathogen attack, plants can respond with several defense-related proteins. Cui et al. (1996) documented the gene expression of phenylalanine ammonia lyase (PAL) and chalcone synthase in sorghum leaves inoculated with *P. sorghi* sporangia, but mRNA expression levels are not a measure of enzyme activity. The present study evaluates actual enzymatic activity. Deepak et al. (2007) examined the enzymatic activity of PAL, peroxidase, catalase, β-1,3-glucanase, and chitinase in pearl millet plants inoculated with *Sclerospora graminicola*, downy mildew of pearl millet and found an increase in the levels of PAL, peroxidase, and β-1,3-glucanase

activity but not an increase in catalase or chitinase activity. The PAL pathway is important for generating phenolic precursors to defense response products such as flavonoids and lignins (Tanaka et al. 1989). Plant derived chitinases and glucanases act directly on the structural components of fungi and oomycetes, respectively (Mauch et al. 1988). Chitinases cleave chitin into the structural component, N-acetyl glucosamine while glucanase activity on glucans yield glucose. In addition to the PAL pathway and the production cell wall degrading enzymes, plants also generate reactive oxygen species (ROS) such as hydrogen peroxide in response to pathogen attack. Peroxidase may play a role in the production of ROS (Liu et al. 2010) and ROS production is often an important part of plant defense responses (Shetty et al. 2008).

Understanding the molecular interaction between plants and their pathogens is critical not only for the advancement of fundamental knowledge of a disease but, important for identifying defense markers for molecular breeders, targets for chemical treatments that may induce defense related compounds, and to unravel the traits in currently used varieties that make them either resistant or susceptible to SDM. This study aims to test whether the expression of certain defense-related compounds can easily be measured and used as markers to breed for resistance.

5.2 METHODS AND MATERIALS

Experimental design was the same as described in chapter 4 (Table 4). Sorghum seeds of Pioneer hybrids 84G62 and 82P75 were surface disinfected with 70% ethanol for 5 minutes followed by 2 hours in 1% NaClO. The seeds were

germinated on sterile filter paper moistened with sterile distilled water in Petri plates at 35°C for 2 days. Seedlings were then dredged in the oospore preparation as previously described in chapter 4. Oospores of two different *P. sorghi* pathotypes, pathotype 1 (P1) and pathotype 3 (P3) were used examine the expression of four defense related enzymes during susceptible (compatible) and resistant (incompatible) interactions. The treatments were: 84G62+P1 oospores*, 84G62+P3**, 84G62 untreated, 82P75+P3*, and 82P75 untreated (*=incompatible interaction, **=compatible interaction). Seedling roots were harvested at 6 days post inoculation, ground in liquid nitrogen in a mortar and pestle and extracted using the appropriate extraction buffer depending on the enzyme being assayed according to standard protocols described by Deepak (2007). The tissue was extracted at a concentration of 100mg tissue/1mL of buffer. Total proteins were determined using the bicinchoninic acid (BCA) method (Smith 1985).

Chitinase activity was evaluated as a control since *P. sorghi*, as an oomycete that does not have chitin as a cell wall component, should not elicit strong chitinase activity. To measure chitinase activity, seedlings were extracted in 0.05 M sodium acetate buffer, pH 5.2. The samples were centrifuged at 12,000g for 30 minutes. The supernatant was removed and used as a crude enzyme extract. Chitinase activity was determined using the dimethylaminobenzaldehyde (DMAB) method for N-acetyl glucosamine (Reissig et al. 1955). Colloidal chitin in 0.05 M sodium acetate buffer, pH 5.2 was used as a substrate at a concentration of 1%. The reaction mixture consisted of 25 μL of enzyme extract and 25 μL of colloidal chitin. The mixture was incubated for 2 hours at 37°C.

After incubation 10 μL of 0.8 M potassium tetraborate pH 9.1 was added to the reaction mixture and boiled for 3 minutes. The samples were then cooled on ice and 300 μL of DMAB was added. This was incubated for 20 minutes at 37°C to allow for color development. Absorbance was read at 585 nm. Chitinase activity was expressed as μmol N acetyl glucosamine/μg protein.

β1,3 glucanase activity was determined using the same extraction protocol as for chitinase activity. The substrate used was 0.1% laminarin in 0.05 M sodium acetate buffer, pH 5.2. The reaction mixture consisted of 10 μL of enzyme extract in 90 μL of substrate buffer. The reaction was incubated for 30 minutes at 35°C. After incubation, released glucose was measured using the dinitrosalicylic acid (DNS) reagent method (Isaac et al. 1982). 200 μL of DNS was added to each sample and samples were boiled for 5 minutes. After boiling, samples were cooled and absorbance was read at 540 nm. β1,3 glucanase activity is expressed as μg glucose/μg protein.

To determine phenylalanine ammonia lyase (PAL) activity, root samples were extracted in 25 mM sodium borate buffer, pH 8.8 and 32mM β-mercaptoethanol. The samples were centrifuged at 20,000g for 20 minutes and the supernatant was used a crude enzyme extract. 50mM L-phenylalanine in 100 mM sodium borate buffer, pH 8.8 was used a substrate. The reaction mixture consisted of 33μL of enzyme extract in 1 mL of substrate buffer. The reaction was incubated for 5 minutes at 35°C. Absorbance was read at 290 nm. PAL activity is expressed as μmol transcinnamic acid/μg protein (Lisker et al. 1983).

Peroxidase activity was determined by extracting the samples in 0.2 M Tris/HCl buffer pH 8.0. The samples were centrifuged for 15 minutes at 12,000g. The supernatant was used as a crude enzyme extract. The reaction buffer consisted of 0.25% (v/v) guaiacol and 10mM hydrogen peroxide in 10mM potassium phosphate buffer, pH 6.9. The reaction mixture was 8.3 μL enzyme extract in 1 mL reaction buffer. Absorbance was measured for every 15 seconds for 2 minutes at 25°C at 470 nm. Peroxidase activity is expressed as the change in absorbance at 470 nm (Δ470 nm), Δ470 nm/μg protein (Hammerschmidt et al. 1982).

Each enzymatic assay was performed separately and the experiment was done three times using three replicates per treatment. Analysis of variance and Fisher's Least Significant Difference means separation test (LSD) were performed using SAS Statistical Software (SAS Institute, Cary, North Carolina).

5.3 RESULTS

There was differential activity in all enzymes tested except for chitinase (Fig. 13). Glucanase, PAL, and peroxidase activity was elevated during the incompatible defense response of sorghum against *P. sorghi* versus the compatible interaction and the control (Fig.s 14-16).

There was no significant difference in chitinase activity between the incompatible interactions and the non-infected controls ($P<0.01$). Likewise there was no significant difference in chitinase activity between compatible and incompatible interactions (Fig. 13).

There was a significant difference in β-1,3 glucanase activity between the

incompatible interactions and the uninoculated control. Similarly there was a significant difference in β-1,3 glucanase activity between compatible and incompatible interactions in which the incompatible resulted in a higher activity of β-1,3 glucanase activity. The trend observed was an elevated level of β-1,3 glucanase activity in the defense response in sorghum seedling roots inoculated with *P. sorghi* oospores as comparted to the compatible interaction and the untreated control. Levels of β-1,3 glucanase activity in the untreated control of 84G62 versus the compatible interaction of 84G62 and P3 were not significantly different ($P<0.05$) (Fig. 14).

Activity of phenylalanine ammonia lyase (PAL) during the incompatible interaction was significantly higher than during the untreated control. Likewise the activity of PAL in the incompatible interaction versus the compatible interaction was significantly higher in the incompatible interaction. The trend observed was an elevated level of PAL activity in the defense response in sorghum seedling roots inoculated with *P. sorghi* oospores. Levels of PAL activity in the 84G62/P3 compatible interaction were not significantly different when compared to the untreated control. ($P<0.01$) (Fig. 15).

There was a significantly higher mean level of peroxidase activity during the incompatible interactions versus the untreated control. The trend observed was an elevated level of peroxidase activity in the defense response in sorghum seedling roots inoculated with *P. sorghi* oospores. However, there was no significant difference in the level of peroxidase activity between the compatible and incompatible interactions. ($P<0.05$) (Fig. 16).

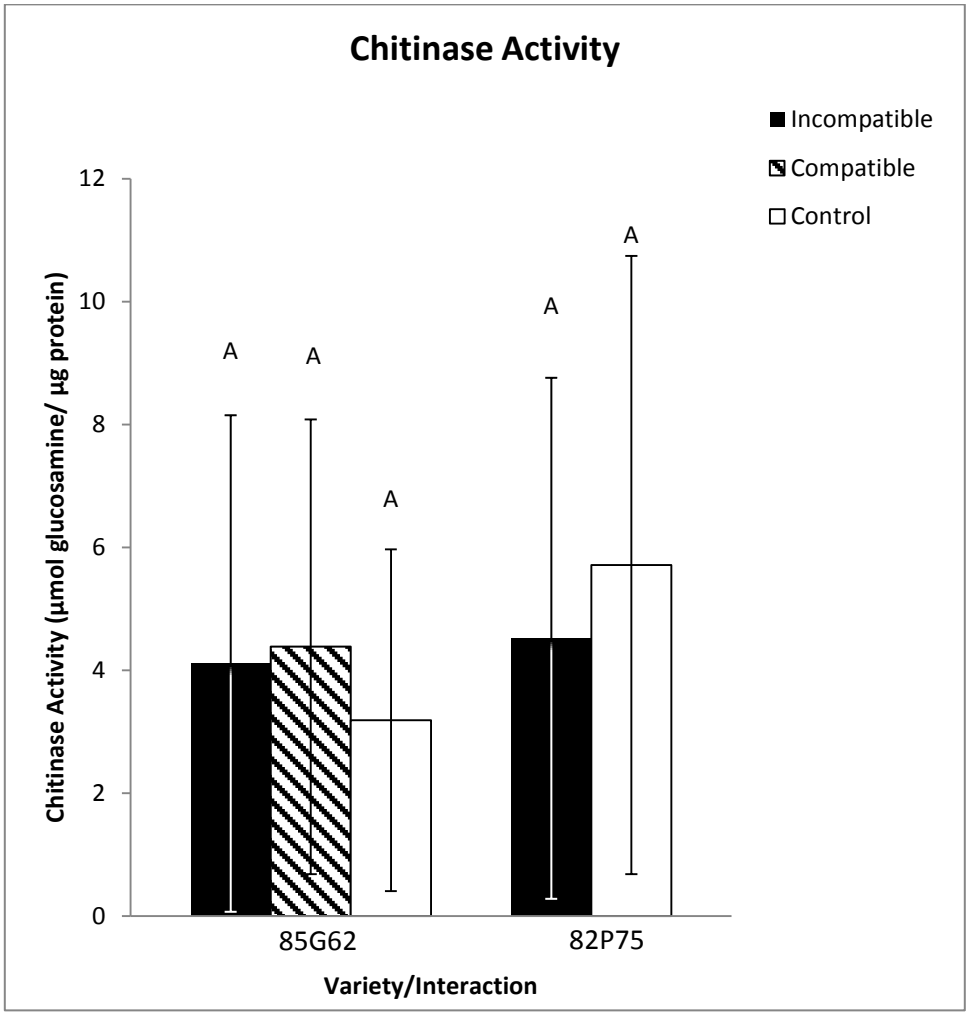

Fig. 13. Chitinase activity in incompatible (■), compatible (▨), and uninoculated (□) controls. Chitinase was assayed 6 days post inoculation. Enzyme activity is expressed as µmol glucosamine/µg protein. Values are the means of the enzymatic activity levels of three independent experiments. Letters assigned using significance P=0.01 based on Fisher's Protected Least Significant Difference Test.

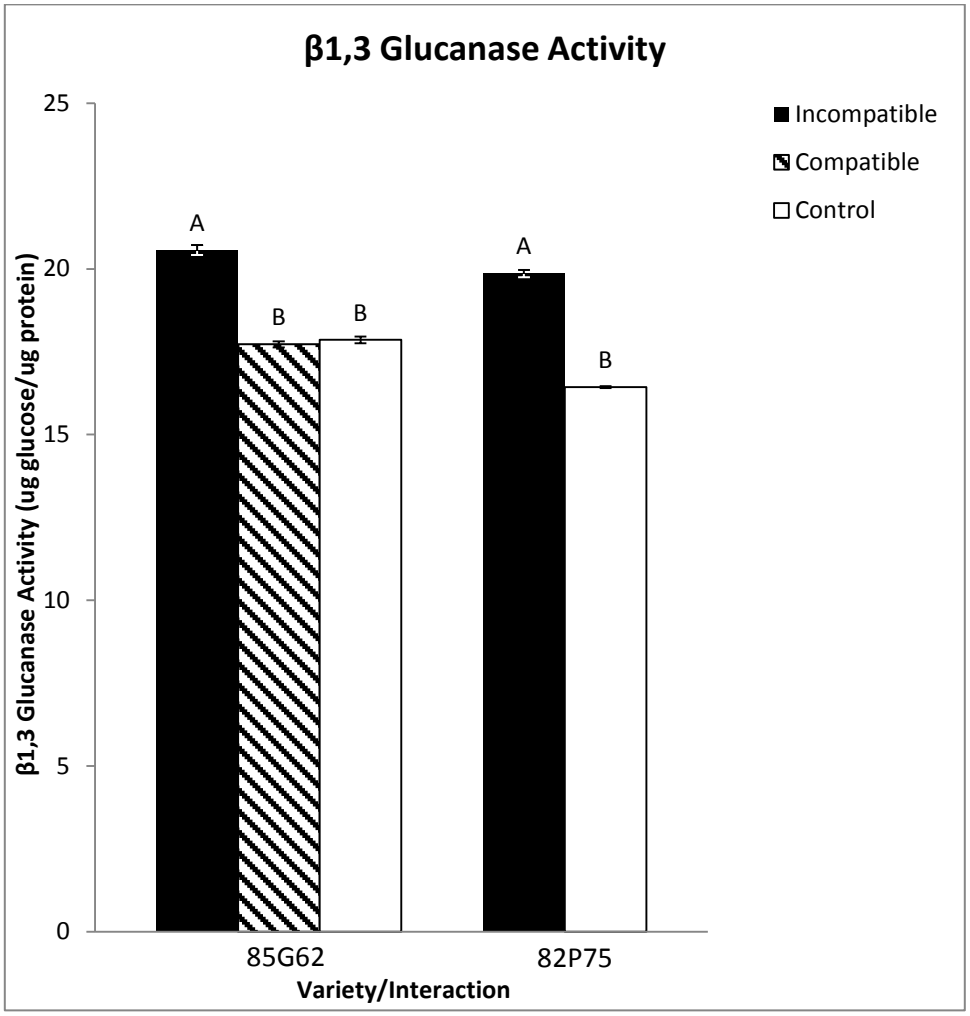

Fig. 14. β1,3 glucanase activity in incompatible (■), compatible (▨), and uninoculated (□) controls. β1,3 glucanase was assayed 6 days post inoculation. Enzyme activity is expressed as µg glucose/µg protein. Values are the means of the enzymatic activity levels of three independent experiments. Letters assigned using significance P=0.05 based on Fisher's Protected Least Significant Difference Test.

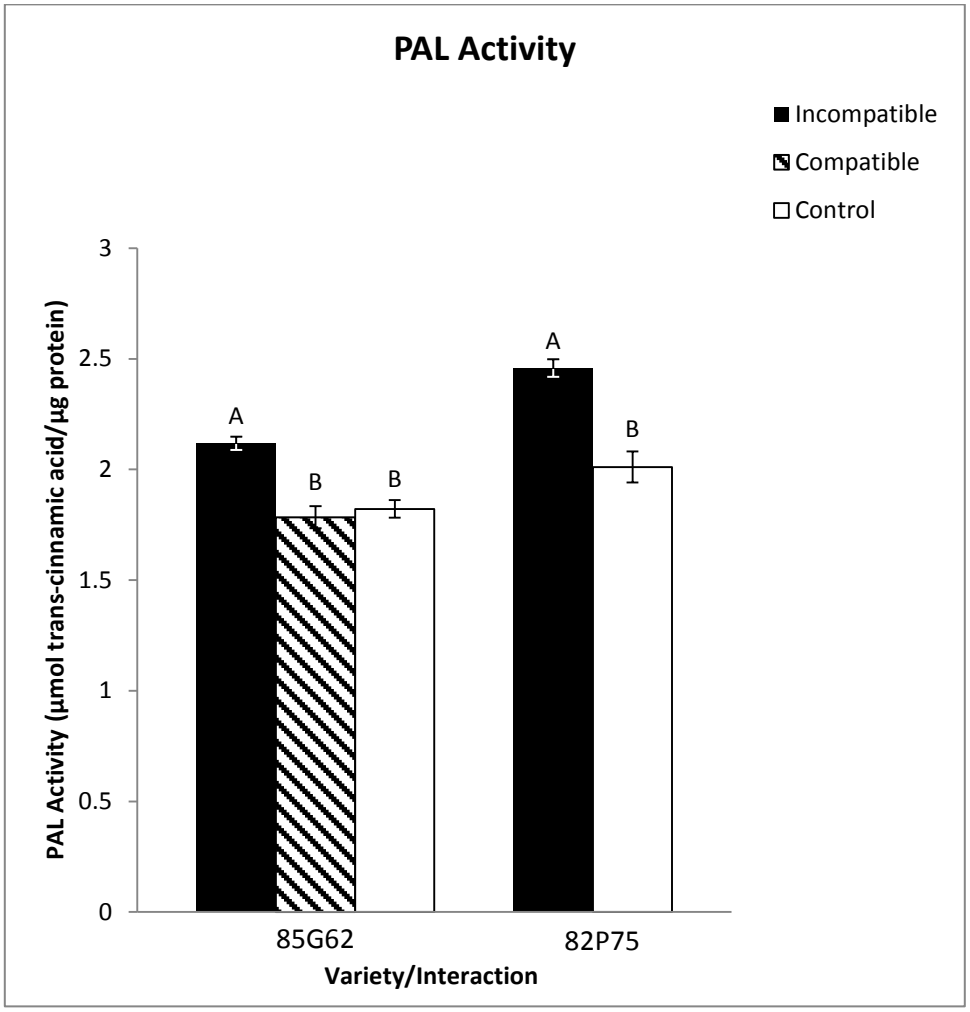

Fig. 15. PAL activity in incompatible(■), compatible(▨), and uninoculated (□) controls. PAL was assayed 6 days post inoculation. Enzyme activity is expressed as µmol trans-cinnamic acid/µg protein. Values are the means of the enzymatic activity levels of three independent experiments. Letters assigned using significance P=0.01 based on Fisher's Protected Least Significant Difference Test.

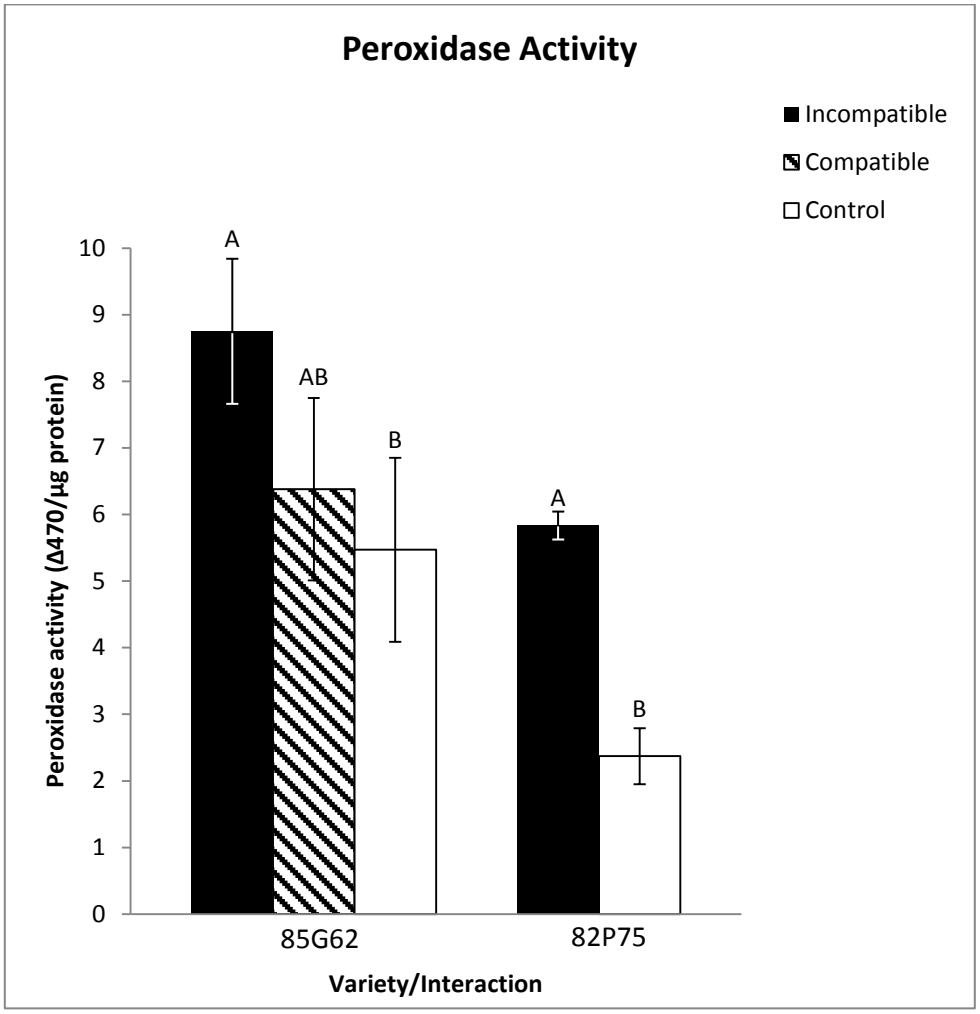

Fig. 16. Peroxidase activity in incompatible (■), compatible (▨), and uninoculated (□) controls. Peroxidase was assayed 6 days post inoculation. Enzyme activity is expressed as Δ470nm/µg protein. Values are the means of the enzymatic activity levels of three independent experiments. Letters assigned using significance P=0.05 based on Fisher's Protected Least Significant Difference Test.

5.4 DISCUSSION

Although many defense responses are well documented in other systems, the defense response of sorghum to root infection by *P. sorghi* measuring enzyme activity levels has remained undocumented until now. In 1996 Cui et al. reported upregulation of mRNA transcripts encoding phenylalanine ammonia lyase (PAL) and chalcone synthase, two plant defense related enzymes in leaves infected with *P. sorghi* sporangia. However, most disease is a result of root infection by soilborne oospores (Bock 1995). Due to the low oospore germination rates and inconsistent incidence resulting from oospore infection, this important facet of the biology of *P. sorghi* remains understudied (Craig and Frederiksen, 1983).

This study represents the first time that sorghum seedling root derived plant defense enzymes have been studied using *P. sorghi* oospores as a source of inoculum. These results are correlated with mRNA expression studies that report an increase in PAL gene expression (Cui et al. 1996) in sorghum seedling leaves infected with *P. sorghi* sporangia. These data also reflect the expression of pearl millet derived defense related enzymes against zoospore infection of *S. graminicola*. In other plant/pathogen systems differential defense responses have been shown between roots and leaves infected with the same pathogen (Chen et al. 2014). Pioneer sorghum hybrid 84G62 is resistant to P1 of *P. sorghi*, but susceptible to P3. Therefore an elevated activity of defense related enzymes would be expected during the resistant response or incompatible interaction. In this case the incompatible interaction is between P1 and 84G62, and P3 and 82P75. Chitinase has been shown to be produced in response to

fungal infection in many plants including sorghum (Kasprzewska 2003) (Huang and Backhouse 2006). However *P. sorghi* is an oomycete, thus its primary cell wall components are glucans (Fry and Niklaus 2010). This studied showed no difference in the activity of plant derived chitinases, but a significant difference in the activity of β-1, 3 glucanases. Pioneer sorghum hybrid 82P75 is resistant to P3. An elevated level of β-1, 3 glucanase activity was observed during the incompatible interactions of 84G62 inoculated with P1 and 82P75 inoculated with P3. The level of β-1, 3 glucanase activity in the untreated controls and 84G62 inoculated with P3 were not significantly different. These results implicate β-1, 3 glucanases, but not chitinase, in the defense response of sorghum to *P. sorghi*. A similar pattern of enzymatic activity was observed in the PAL experiment. Levels of PAL activity during the incompatible 84G62/P1 and 82P75/P3 interactions were significantly higher than the compatible 84G62/P3 treatment and the untreated controls, respectively. This is not unexpected as the PAL pathways is an important precursor for several phytoalexins, phenolic compounds, and structural changes. Peroxidases are involved in the production of reactive oxygen species (ROS) as well as cell wall crosslinking and lignification (Almagro et al. 2009) (Young et al. 1995). Although the mean levels of peroxidase activity indicated a higher level of enzymatic activity in the incompatible 84G62/P1 interaction than the compatible 84G62/P3 treatment, they were not significantly different. However the level of peroxidase activity during the 84G6/P1 incompatible interaction and the untreated control was significantly different. During the 82P75/P3 incompatible interaction the level of peroxidase activity was significantly higher than in the untreated control. These

results indicate that peroxidase activity as well as PAL and β-1, 3 glucanase activities are implicated in the defense response of sorghum to *P. sorghi*. Other studies are needed to further elucidate the role of other defense response related pathways. Plant defense response pathways often involve complex cross talk between plant signaling hormones. Resistance to downy mildews in other systems is correlated with the expression of pathogenesis- related (PR) genes and activation of the phenylpropanoid pathway (Perazzolli et al. 2012). In many cases the defense response against biotrophic pathogens such as the downy mildews has been shown to involve salicylic acid (SA) signaling, which often is inversely correlated with the jasmonic acid (JA) signaling pathway (Halime et al. 2006)). In more recent studies it has been shown that this inverse regulation between SA and JA pathways is not always the case. In some instances there is synergistic activity of both JA and SA pathways (Clarke et el. 2000). Indeed the JA pathway has been implicated in plant defense responses against downy mildews of both grape and pearl millet (Deepak et al. 2007, Perazzolli et al. 2012). There can also be differential responses between defense response of roots and aerial plant organs (Chen et al. 2014). In addition to well described plant signaling pathways and defense responses, other, independent defense mechanisms may play a role in downy mildew resistance (van Damme et al. 2009). Determining the defense response is important to facilitate future molecular breeding efforts. This can only be accomplished if the details of the defense reaction from the hormonal signaling pathways to the expression of PR proteins are determined, as well as localization of the defense reaction. It may be possible that sorghum screening trials against SDM, which are carried out using sporangia

inoculation of leaves, do not always indicate the resistance of a line or hybrid against *P. sorghi*. However these tests have been shown to have a positive correlation in greenhouse and field trials (Craig 1981). This method of screening continues to be the most efficient and high throughput method of screening for resistance (Radwan et al. 2011).

6. SUMMARY

Sorghum downy mildew continues to be a disease that affects sorghum production on the Coastal Bend of Texas as well as in other sorghum producing parts of the world. However, due to the fastidious nature of the causal organism, *Peronoscerospora sorghi*, as well as to the relative ease in which resistant sorghum varieties are generated, studies on the environmental factors affecting disease development as well as studies on the development of the disease through soilborne oospores, remain few. The present study addresses soil moisture as an environmental factor affecting disease development, the use of baiting using non-hosts in order to reduce inoculum potential in the field, a study on the microscopic development of the disease from root to shoot in both compatible and incompatible interactions, and a study on defense related enzymes produced by sorghum during the defense response against *P. sorghi.*

In this study it was found, using a Haines apparatus to control matric potential, that soil matric potentials near field capacity represent the most conducive environment for disease development versus two higher soil moisture treatments.

The experiment evaluating the effect on reducing inoculum in the field by baiting revealed a statistically significant reduction in disease in the baited treatments versus the fallow control. Further experiments over years and locations are required to continue to validate this as a viable form of control.

During the microscopic study of the colonization of sorghum seedling by *P. sorghi* it was observed that infection follows distinct stages of germination, penetration,

and colonization. This study represents the first time that the development of oospore-derived colonization has been observed and reported using high quality imaging.

Two defense related enzymes were shown to be elevated during the incompatible interaction between sorghum seedling roots and *P. sorghi* versus compatible interactions and the uninoculated control. These two defense related enzymes β1,3 glucanase and phenylalanine ammonia lyase can potentially be used as markers during breeding for sorghum resistance against *P. sorghi.*

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