PATHOGENICITY OF *RHIZOCTONIA* SPP. AND *SCLEROTIUM ROLFSII* ON WHEAT AND PEANUT AND GENETIC VARIATION AMONG *RHIZOCTONIA* ISOLATES

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CHAPTER I

INTRODUCTION

Peanut and wheat are important crops produced in Oklahoma. In the United States in 2005, Oklahoma was ranked second in winter wheat production and seventh in peanut production. In 2005, winter wheat was planted on 5.7 million acres in Oklahoma with an estimated production of 128 million bushels. Similarly, peanuts were planted on 35,000 acres with an estimated production of 105 million pounds. Together, these two crops had a combined production value estimated at 448 million dollars during 2005 (36).

Disease management is crucial for successful peanut and wheat production in Oklahoma. Along with foliar diseases, soilborne diseases are a major concern for the peanut and wheat industry through reductions in yield quantity and quality, and by lowering the overall value of the crop. Soilborne diseases such as southern blight and sclerotinia blight cause wide spread damage on peanut and other field crops (16). Southern blight was reported to cause a greater yield loss in peanut than any other disease (16). Similarly, wheat root and foot rots caused by several soilborne organisms such as *Bipolaris*, *Rhizoctonia, Pythium* and *Fusarium* are a common problem in Oklahoma (25).

Even though soilborne diseases are problematic on peanut and wheat, limited work has been done to identify the levels of pathogenicity, factors influencing the pathogenicity, genetic relatedness of the causal organism and sources of resistance. This is especially true with respect to southern blight on peanut and root rot and sharp eyespot caused by *Rhizoctonia* spp. on wheat.

Southern blight is reported to occur commonly on dicotyledonous crops such as peanut, but monocotyledonous crops are seldom affected by this disease (1). Moreover, the survival and growth of the causal organism, *Sclerotium rolfsii*, is favored by high temperature (1, 42). Hence this disease is not expected to cause major damage on a cool season crop such as winter wheat, which is widely cultivated in Oklahoma (36). However, seedling blight caused by *S. rolfsii* has reported from a field in Oklahoma, raising questions about the potential of this fungus to damage winter wheat in Oklahoma. No studies have reported on pathogenicity or the virulence of this pathogen on winter wheat cultivars commonly grown in Oklahoma.

Root rot or bare patch and sharp eyespot are a set of soilborne diseases that occur sporadically in winter wheat fields of Oklahoma. The causal organism, *Rhizoctonia* spp. is a widespread soilborne fungus with a wide host range that includes peanut (6, 13). The above set of diseases were earlier reported to be caused by *Rhizoctonia solani*, with root attacking strains causing root rot or bare patch and stem attacking strains causing sharp eyespot (11, 52). However, *Rhizoctonia cerealis*, a binucleate species of *Rhizoctonia* was identified to

cause a sharp eyespot on wheat and other Gramineae members (8, 10, 28). Hence these two soilborne diseases are currently studied and reported separately. Unlike *R. solani*, *R. cerealis* grows relatively slowly and produces superficial lesions that are observed at the stem base of both young and mature plants (10, 56). Significant yield losses from this disease have been reported from other parts of the world, including some regions of the United Kingdom where resistant wheat cultivars were identified to help alleviate yield losses (10, 24). The potential for sharp eyespot to cause major damage on Oklahoma winter wheat cultivars definitely exists, but characterizing the fungal isolates and testing the pathogenicity would help to provide quantitative information on the seriousness of this disease in Oklahoma.

Most wheat in Oklahoma is grown as a continuous monoculture. However, some rotation with various crops such as grain sorghum and soybean is occasionally practiced (55). In Central and Southwestern Oklahoma, wheat is occasionally rotated with peanut (personal communication with Dr. R.M. Hunger and Dr. H.A. Melouk). In such a rotation, wheat is usually grown for two years after a crop of peanut, but there is no understanding how such a rotation program affects the incidence and severity of soilborne diseases like southern blight and *Rhizoctonia* root rot.

In this regard, studies were initiated to address the following objectives:

1. Determine the characteristics and pathogenicity of *Rhizoctonia* spp. on winter wheat and peanut.

2. Determine the pathogenicity of *Sclerotium rolfsii* on peanut and winter wheat.
3. Determine the production of endo-polygalacturonase and oxalic acid by isolates of *Sclerotium rolfsii*.
4. Detect genetic variability of *Rhizoctonia* spp. isolated from peanut and winter wheat.

CHAPTER II

LITERATURE REVIEW

Wheat (*Triticum aestivum* L.) is an annual monocotyledonous plant belonging to the grass family Gramineae. Wheats are broadly classified into diploids (2n), tetraploids (4n) and hexaploids (6n) based on their chromosome makeup. The first cultivated wheats were considered to be diploids with their origins traced to parts of Iran, Iraq and Turkey (14). Most of the modern cultivated wheats are hexaploids except for durum, which is a tetraploid.

Based on the texture of the endosperm, five basic classes of wheat are grown in the United States. They include hard red winter (HRW), hard red spring (HRS), soft red winter (SRW), white (winter or spring) and durum wheat. As of 2004, nearly 38% of the all wheat grown in the United States is of the HRW type followed by HRS and SRW (54). All wheat grown in Oklahoma is winter wheat, and is primarily of the HRW type (55).

The climatic conditions and rainfall distribution in Oklahoma are congenial for wheat production. Wheat is primarily grown as a dual-purpose crop in Oklahoma thereby allowing the crop to be used in the fall/winter for forage purposes and then harvested for grain in the spring (55). In Oklahoma,

planting wheat for grain purposes is usually scheduled from late September until the final week of October, but this varies by the region of the state. Wheat used for forage is planted two to six weeks earlier (55).

A healthy, mature wheat plant possesses a main stem with a head, tillers, nodes, internodes, leaves and roots. The main stem and tillers are formed by a succession of leaves enclosed around each other. Hence, the main stem is not a true stem but is called a pseudostem. Seminal or primary roots develop first as part of a seedling, and this is followed by the formation of crown or secondary roots on the main stem and tillers (12).

Development of wheat starts with the germination of seed under optimum temperature (12 to 25 C) (47) and moisture conditions followed by seedling and main stem growth, tillering, stem elongation, booting, inflorescence emergence, anthesis, milk development, dough development and ripening. These main stages are subdivided into different developmental stages, which have been designated using different scales. The Feekes' scale (1 through 11) is the most commonly used scale in the United States. A scale of 1 represents seedling emergence through the three-leaf stage and scale 11 represents the grain filling stage (47).

Peanut

Peanut (*Arachis hypogaea* L.) is a self-pollinated, annual, herbaceous legume and is considered to be a native of South America. The time and place of its introduction into the United States is not known (23). Commercial peanut

production in Oklahoma started in the 1930's and early 1940's due to an increased need for oil for different purposes.

Four market types of peanuts are grown in the United States. They include runner, spanish, virginia and valencia. The three southwestern states of United States comprising Texas, New Mexico and Oklahoma grow runner peanut mostly, followed by spanish peanut. Peanut is mostly grown on well drained, loose, sandy loam soils where optimum mean daily air temperatures are about 25°-30° C. Peanut in Oklahoma is generally planted during the month of May, but planting dates differ slightly for different market types. A soil temperature of 20°-35° C is optimal for successful germination of peanut seed (40).

Peanut has an indeterminate growth habit, wherein vegetative and reproductive growth occurs simultaneously. Flowering starts about 35-40 days after emergence and blooming is heavy during mid July. After successful pollination and fertilization, a peg develops downwards from the flower and takes about a week to ten days to penetrate completely into the soil. Under favorable soil and climate conditions, fruits mature 10-12 weeks after pollination (40).

Soilborne diseases in wheat

Soilborne diseases in wheat are usually described as either root diseases or stem base diseases. Root diseases in dry soils are primarily caused by *Cochliobolus sativus* (common root rot) and *Fusarium* spp. (*Fusarium* root rot),

whereas wet soils favor development of diseases like take-all caused by *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia* root rot and damping off of wheat caused by several *Rhizoctonia* spp., (usually *Rhizoctonia solani* AG-4, AG-5 and AG-8) and *Pythium* root rot caused by several *Pythium* spp. (13,18, 31, 57).

Eyespot, sharp eyespot and brown foot rot are the important stem base diseases in wheat (53). Eyespot is caused by *Tapesia* spp. (formerly, *Pseudocercosporella herpotrichoides*) whereas sharp eyespot is induced by *Rhizoctonia cerealis* (teleomorph: *Ceratobasidium cereale,* Murray & Burpee). *Microdochium nivale* and *Fusarium* spp. are usually associated with brown foot rot. The symptoms of these three stem base diseases are often confused, especially during early growth stages (20, 41).

Rhizoctonia root rot and sharp eyespot are usually grouped together since the causal agents for both these diseases are from *Rhizoctonia*. Symptoms of *Rhizoctonia* root rot in the field include scattered circular patches of diseased plants occurring at the same position for several years. Affected roots show brown rotting toward the root tips and intermediate portions of the roots. Additionally, numerous lateral roots arise leading to a highly branched root system. Characteristic symptoms of sharp eyespot are lens-shaped lesions with a sharper margin usually occurring on the lower leaf sheaths (7).

Sclerotium wilt or southern blight on wheat caused by *Sclerotium rolfsii* causes pre-or post-emergence damping off of seedlings, if the infection occurs

early in crop cycle. Further development of the disease may result in rotted culms and crowns and eventual death of the plant.

Unlike *Cochliobolus*, *Fusarium,* and *Pythium*, which are considered as common pathogens of wheat, *Sclerotium rolfsii* is usually considered a nontraditional soilborne pathogen with its occurrence most commonly related to tropical environment (17, 46, 48). Hence, the disease is seldom a problem in temperate wheat growing areas.

Although wheat is listed as a host of *S. rolfsii* (through artificial inoculation) (1), little is known about its pathogenicity. In 1915, *S. rolfsii* was reported to cause blighted heads in wheat in certain parts of the United States. Symptomatology involved appearance of brown lesions on the crown and lower portions of the culms. Heads were completely devoid of grain and ripened prematurely (19). Seedling blight caused by *S. rolfsii* was reported in Bangladesh, with seedlings appearing yellowish during initial stages of infection and later become blighted. In 1922, a serious disease of winter wheat was reported to cause by *Sclerotium rhizodes* in Idaho resulting in considerable losses (26). The disease was reported to occur in spots, killing the majority of plants in that area.

Soilborne diseases in peanut

Soilborne diseases cause significant problems in the entire peanut producing areas of the United States. The most common and important soilborne diseases affecting the peanut plant include *Aspergillus* crown rot, *Rhizoctonia* limb, pod, and root rot, *Sclerotium* stem rot (southern blight),

Cylindrocladium black rot, *Sclerotinia* blight, *Verticillium* wilt, *Pythium* pod rot and *Thielaviopsis* black hull (32).

Rhizoctonia induced diseases can occur on all parts and at all growth stages of the peanut. *Rhizoctonia solani* Kuhn is the primary pathogen in the *Rhizoctonia* group, affecting the seeds (seed decay), seedlings (damping off), leaves (foliar blight), roots (root rot), limbs (limb rot), peg (peg rot) and pods (pod rot) (6, 15, 32). This fungus usually causes decaying of germinating seed and forms light to dark brown sunken lesions on infected hypocotyls, branches, roots, pegs and pods. Disease losses caused by *Rhizoctonia* are difficult to assess due to its association with other soilborne and foliar pathogens (6).

Southern blight is found in almost all peanut growing areas of the world, and peanut probably sustains a greater annual loss from this disease than any other commercially grown crop (1). Even though many names of the disease caused by *Sclerotium rolfsii* are in usage, white mold, southern blight, southern wilt and southern stem rot are the three most commonly used names for the field phases of this disease in the United States (1).

Southern blight occurs in Oklahoma mainly in mid- to late season. Initial symptoms of the disease include yellowing and wilting of a branch or the whole plant. Infection is characterized by white rope-like mycelial growth towards the base of the stem that imparts a white washed appearance (16). As the disease progresses, this fungus produces light to dark brown lesions on the branches and pegs. Rotting of infected pods occurs without any aboveground symptoms (32).

The pathogens

***Rhizoctonia solani* Kuhn [teleomorph: *Thanetephorus cucumeris* (Frank) Donk]**

The genus *Rhizoctonia* was first described by de Candolle in 1815 (38). The species concept of *Rhizoctonia solani* primarily evolved from the work of Duggar during 1915 (38). Some of the common characteristics of isolates of *R. solani* include pale to dark brown hyphal pigmentation, multinucleate cells in young hyphae, branching near the distal septum of actively growing hyphae, constriction of branch hyphae and formation of branch near the point of origin (38, 49). Relationships among the isolates of *Rhizoctonia* spp. are usually assessed through hyphal fusion (anastomosis), morphology of hyphae, and culture characteristics (49). Currently, *R. solani* is subdivided into eleven anastomosis groupings (AG-1 to AG-10, and AG-BI). Some of these groupings are further subdivided based on pathogenicity and nutritional requirements (9). Evidence from previous studies suggests that variation exists among the isolates belonging to the same anastomosis group (35). More recently, anastomosis groupings of isolates of *R. solani* were investigated using molecular techniques such as RAPD-PCR, RFLP-PCR of the r DNA ITS region (18, 39).

***Rhizoctonia cerealis* [teleomorph: *Ceratobasidium gramineum* (syn: *C. cereale, Corticium gramineum*]** *Rhizoctonia cerealis* is one among several binucleate species of *Rhizoctonia*. Isolates of binucleate *Rhizoctonia* spp. were subdivided into 17 anastomosis groups (AG-A to AG-Q), which are sometimes referred to as Japanese groups (50). Isolates of North America were assigned to seven

anastomosis groups (CAG 1 to CAG 7) and most of them correspond to Japanese groups with few exceptions (50). Isolates of *R. cerealis* belong to AG-D and were shown to anastomose with members of AG-D and CAG-1 (50). Colonies of this fungus were described to be white to light brown on potato dextrose agar with no zonation (50). Sullivan described *R. cerealis* (CAG-1) colonies to be a pale color when cultured on potato dextrose marmite agar (37). Apart from conventional characterization, attempts were made to identify markers common to all the isolates using marker systems like RAPD assays (34).

***Sclerotium rolfsii* Sacc [teleomorph**: ***Athelia rolfsii* (Curzi) Tu and Kimbrough]** *Sclerotium rolfsii* was first observed in the United States by Peter Henry Rolfs in 1892 on tomato (1). The fungus is a Deuteromycete that is characterized by the presence of white silky mycelia and brown to black, round sclerotia. Hyphae are hyaline and sparsely septate when young. Sclerotia start to form when growth is restricted due to nutrient stress and other factors (42). The fungus does not produce any asexual spores. The appearance of the teleomorphic stage is very rare. The formation of basidia is infrequent in nature and is influenced by the isolate, nutrient composition of the substrate and the age of culture (43). Basidiospores have been shown to be pathogenic to host tissue under controlled conditions. *S. rolfsii* sometimes causes aerial leaf blights and leaf spots, and in such conditions basidiospores are suspected to be a source of inoculum (33, 45). Host range of this fungus is extensive and includes many

crops of great economic value (1). The fungus is usually distributed in tropical and subtropical regions of the world where warm temperatures prevail.

Infection process in *Rhizoctonia* spp.

Elucidation of the infection process is critical to understanding the pathogenicity of these fungi. The activities of *Rhizoctonia solani* prior to penetration of host tissue are complex (29), and include frequent formation of convoluted hyphal structures called infection cushions. *R. solani* forms infection cushions on peanut hypocotyls that facilitate penetration of the fungus through epidermal and cortical cells and cause death of the tissues (6). *R. solani* can form tenacious infection cushions either by a single hypha, by an aggregation of short hyphal branches, by proliferation of hyphal branches, or by the aggregation of several hyphae (27). *R. solani* also can produce numerous infection cushions on synthetic films like cellophane (30, 51).

Infection process in *Sclerotium rolfsii*

Phytotoxins such as oxalic acid and cell wall degrading enzymes play a key role in the infection of a host (5, 42) and *S. rolfsii* produces a multi-enzyme system for the degradation of different polysaccharides in the host tissue (21). In one early study (2), polygalacturonase was reported to be the primary component of the "macerating enzyme" in diseased bean tissue, but the enzyme was reported to hydrolyze the calcium pectate in the host cell wall only in the presence of oxalate ions (3). This led to a hypothesis that oxalic acid and polygalacturonases are produced simultaneously in infected tissue by *S. rolfsii,*

and that this simultaneous production enables them to act synergistically to degrade calcium pectate in cell walls. Bateman and Beer (5) proved that simultaneous production and synergistic action of oxalic acid and polygalacturonase is necessary for rapid destruction of the host tissue by *S. rolfsii*. In a later study (44), simultaneous production of endo-polygalacturonase and oxalic acid along with the rapid mycelial growth was shown to be critical for the infection process by *S.rolfsii*. A cellulase system was also shown to play a key role in pathogenesis of *S. rolfsii* (4). Other enzymes produced by this fungus are hemicullulolytic enzymes including xylanase and mannanase, pectin methylesterase, cutinase, phosphotidase, arabanase, galactanase and β-glucosidases (22, 42).

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CHAPTER III

CHARACTERISTICS AND PATHOGENICITY OF *RHIZOCTONIA* SPP. FROM WINTER WHEAT AND PEANUT

ABSTRACT

Rhizoctonia spp. cause important root and shoot diseases in peanut and winter wheat, which are important crops grown in Oklahoma. A study was conducted to characterize the isolates of *Rhizoctonia* spp. and to determine their pathogenicity on peanut and winter wheat cultivars of Oklahoma under greenhouse conditions. Cultural morphology, nuclear condition, anastamosis grouping and growth rates were determined on four *Rhizoctonia solani* isolates obtained from diseased peanut pods (G-24, JY-1, RS-00-07 and RSP) and four isolates from wheat culms with sharp eyespot lesions (Fellers, Marshall, Altus and Lahoma). Pathogenicity of isolates G-24 and Fellers were determined on three winter wheat cultivars (Jagger, 2137 and 2174) and four peanut genotypes (Okrun, Tamspan 90, Southwest Runner and C 209 6-60). Fungal isolates were grown on oat seed which was used for inoculation, and disease severity was measured on a 1-6 scale (1=no disease; 6=complete death of the host). Data on shoot height and root and shoot fresh weights were also collected. Degree of

pathogenicity of the isolates was determined by quantifying the formation of infection cushions on cellophane membrane in response to stimulation by roots of peanut and winter wheat. All *R. solani* isolates from peanut had dark colored mycelia, multinucleate cells, faster growth rate, and belonged to anastamosis group AG 4. Isolates of *Rhizoctonia* spp. from eyespot lesions of wheat were confirmed as *R. cerealis* based on yellow-white to light tan colored mycelia, binucleate cells, slow growth and belonged to the CAG 1 anastomosis group. Isolate G-24 from peanut was the most virulent on all peanut and winter cultivars and caused the greatest reduction in shoot height and shoot fresh weight as compared to the *R. cerealis* isolate Fellers. Averaged across isolates, no significant differences in disease severity were observed among peanut cultivars. However, within winter wheat cultivars, seedlings of Jagger received a significantly lower disease severity rating compared to 2137 and 2174. In the infection cushion study, G-24 formed significantly more infection cushions per 2 cm^2 of cellophane compared to Fellers. Southwest Runner and 2137 had significantly less infection cushions among peanut and winter wheat cultivars, respectively. Results suggest that quantification of infection cushions can be a useful technique for screening cultivars for resistance to infections caused by *Rhizoctonia*.

INTRODUCTION

The genus *Rhizoctonia* causes disease on a wide range of host plant species (16). It comprises a diverse group of fungi with their teleomorphs separated into three genera of the sub-division Basidiomycotina: *Thanatephorus* (anamorph=*R. solani* Kuhn), *Ceratobasidium* (anamorph=binucleate *Rhizoctonia*) and *Waitea* (anamorph=*R. zeae* Voorhees) (2). The multinucleate *R. solani* is the most studied species within the genus (16). *R. solani* infects plants representing 142 species from cycadopsida to the monocotyledonae (16). Eleven anastamosis groups have been described for this species some of which are pathogenic with a worldwide distribution (2, 16). The binucleate *Rhizoctonia cerealis* occurs worldwide and some of the pathogenic isolates are known to cause serious diseases in many crops including cereals such as wheat (2). Isolates of *Rhizoctonia cerealis* are assigned to the anastamosis group CAG-1 (1).

Rhizoctonia solani causes important root and shoot diseases in peanut and winter wheat, which are important crops grown in Oklahoma (11, 20). In peanut, the fungus attacks pods and limbs causing pod rot and limb rot, respectively, that cause significant annual losses (6, 5, 19). In winter wheat, the fungus usually causes root rots and preemergence damping off resulting in circular patches of chlorotic or dead plants that produce little or no grain (10, 13, 15).

R. cerealis is another important species that causes sharp eyespot in winter wheat (3, 4, 7, 8, 9). Sharp eyespot was earlier reported to be caused by *R. solani*

(18); however, in 1977, Van der Hoeven reported a new species, *R. cerealis* Van der Hoeven, causing sharp eyespot lesions on wheat in Netherlands (9). Hence, two different fungi were reported to cause sharp eyespot in wheat. The disease is most commonly found in temperate regions of the world and generally does not incur major losses (9, 12, 14, 17).

Since peanut and wheat are occasionally used in rotations in Oklahoma and since the pathogenicity of isolates of *Rhizoctonia* spp. on peanut and winter wheat cultivars of Oklahoma is unknown, this research was initiated to address the following objectives:

1. Identify and characterize isolates of *Rhizoctonia* spp. obtained from peanut and winter wheat.
2. Determine the pathogenicity of isolates of *Rhizoctonia* spp. on peanut and winter wheat cultivars through artificial inoculation in the greenhouse.
3. Quantify formation of infection cushions by *Rhizoctonia* spp. on cellophane membranes in response to stimulation by roots of peanut and winter wheat and assess the utility of this technique for studying the response of peanut and winter wheat cultivars to inoculation with *Rhizoctonia* spp.

MATERIALS AND METHODS

Collection and maintenance of *Rhizoctonia* isolates

Isolates of *Rhizoctonia* were obtained from different sources in 2004 (Table 1). Peanut isolates were maintained on SPDA [Potato Dextrose Agar containing streptomycin sulfate at 0.14 g/L]. Isolates from wheat were obtained from wheat collected from northern and southwestern Okalahoma that exhibited characteristic sharp eye spot symptoms. Small sections of the infected tissue having the sharp eye lesion were cut and surface sterilized for 3 minutes with 2% sodium hypochlorite, rinsed in sterile distilled water, plated on 2% water agar and incubated at room temperature (24° C). After 48 h, fungal colonies with mycelia characteristics of *Rhizoctonia* spp. were transferred and maintained on SPDA.

Isolates from peanut and wheat were maintained on autoclaved oat seeds. Oat seed (25 g) was mixed with 25 ml of water and autoclaved for 20 minutes in glass petriplates (9 cm dia.). After autoclaving, plates were inoculated with ten mycelial plugs (0.5 cm dia.) from 4-day-old cultures and incubated at room temperature for 7days and dried in open air for a week.

Cultural characteristics

Mycelial plugs (5 mm dia) of *Rhizoctonia* isolates were excised from the outer margin of 4 day old cultures actively growing on SPDA and transferred to SPDA in 100 X 15 mm plastic Petri plates. These plates were then incubated at 24^{o}

C for two weeks in the dark and then observed for colony color, sclerotial color and zonation.

Nuclear staining and anastomosis grouping

The nuclear number of the isolates was determined by staining with 0.5% aniline blue in lactophenol. Isolates from peanut were known to belong to anastomosis group 4 (AG-4). Hence anastomosis of these isolates was not studied in detail. However, isolates collected from wheat were tested for their anastomosis using tester strains of *R. solani* (AG-4) and *R. cerealis* (CAG-1). Autoclaved glass slides were coated with 2% water agar and 0.5 cm hyphal discs of the isolate and the tester were placed in pairs, 2 cm apart. The glass slides were incubated at 25^{o} C in the dark until the hyphae from opposite discs overlapped. Overlapped hyphae were stained with 0.5% aniline blue in lactophenol and examined for hyphal fusion using a light microscope at 100X.

Hyphal extension

Hyphal extension of the isolates was measured by transferring 5 mm mycelial plugs excised from outer margin of actively growing culture onto SPDA plates and incubating at 25 C. Radial growth was measured after 24, 48, 72 and 96 hrs. Each treatment was replicated three times. The data were analyzed using PROC MIXED (SAS 9.1, SAS Institute Inc., Cary, NC).

Pathogenicity tests

Our preliminary tests has revealed that isolates G-24 and Fellers were considerably more pathogenic among *R. solani* and *R. cerealis* isolates respectively. Hence these two isolates were used for further pathogenicity tests.

Isolates, *R. solani* (G-24) and *R. cerealis* (Fellers) were tested for their pathogenicity on four peanut entries (Okrun, Tamspan 90, Southwest Runner and C209-6-60) and three winter wheat cultivars (Jagger, 2137 and 2174) under greenhouse conditions. Experiments on peanut and winter wheat were conducted separately. None of the above genotypes were previously tested for their reaction to *Rhizoctonia* spp.

R. solani isolate G-24 was obtained from Dr. Terry Wheeler (TAES Research Plant Pathologist, Lubbock, TX) and was originally isolated from peanut pods showing symptoms of pod rot collected from Yoakum County, TX in 2003. *R. cerealis* isolate (Fellers) was isolated in 2004 from a sharp eyespot lesion on wheat stem collected from a grower's field in Northwestern Oklahoma by Dr. Robert Hunger (OSU Extension Plant Pathologist, Stillwater, OK).

Preparation of inoculum

In a 9-cm dia. glass petri plate, 25 ml of deionized water was added to 25 g of oat seed and autoclaved for 20 minutes (121 C and 1 kg/cm^2). After autoclaving and subsequent cooling, plates were inoculated with ten plugs (0.5-cm dia) excised from the outer margin of 4-day-old *Rhizoctonia* cultures. Inoculated oat seed were then incubated at 25 C for 7days. To avoid clumping,

seeds were mixed with a sterile needle once every two days. Seeds were then dried in the open air for a week, transferred to coin envelopes and stored at 4 C in a refrigerator until further use. To test for contamination, ten seeds from each plate were plated onto SPDA and observed for the growth characteristic of *Rhizoctonia*.

Wheat study

Wheat pre-emergence test

Three hard red winter wheat cultivars, Jagger, 2137 and 2174, which are commonly cultivated in Oklahoma, were used in this study. Ten certified wheat seeds of each cultivar were planted at a depth of 2 cm in 12-cm dia. plastic pots containing a mixture of sand, soil and shredded peat moss (2:1:1; v/v/v). Each healthy wheat seed was planted with an oat seed infested with each of the *Rhizoctonia* isolates. The pots were kept in the greenhouse at 25-30 C for fourteen days. The combination of two isolates (G24 and Fellers) and the non-inoculated control and three cultivars (Jagger, 2137 and 2174) resulted in nine treatments. Treatments were arranged in a randomized complete block design with four replications. The experiment was conducted twice.

Seedling emergence was recorded 7 days after planting (DAP). At 14 DAP, seedlings were removed from the pots keeping the roots of seedlings intact and then washed in running tap water to remove soil debris. Infection from *Rhizoctonia* was rated on a scale of 1 to 6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4=

rotting at the base of the stem; 5=damping off or yellowing; 6=no emergence). Plant height was measured from the base of pseudostem to the end of second leaf and fresh root and shoot weights were determined. To fulfill Koch's postulates, *Rhizoctonia* was re-isolated on to SPDA from infected tissues. All data were analyzed using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC).

Wheat post-emergence test

In this test, pathogenicity of *Rhizoctonia* isolates G24 and Fellers ,was tested by inoculating 6-week old winter wheat plants. Certified wheat seeds of each cultivar (Jagger, 2137 and 2174) were planted in 12 cm diameter plastic pots at a depth of 2 cm containing a mixture of sand, soil and shredded peat moss (2:1:1; v/v/v). At 4 DAP, plants in each pot were thinned to 10 healthy seedlings. At 6 weeks after planting, a single oat seed infested with *Rhizoctonia* was placed at a 2 cm depth close to each healthy wheat plant. The combination of two isolates (G24 and Fellers) and the non-inoculated control and three cultivars (Jagger, 2137 and 2174) resulted in nine treatments. Treatments were arranged in a randomized complete block design with four replications and the experiment was conducted twice.

At 14 days after inoculation, plants were removed from the pots keeping the roots intact and were washed in running tap water to remove soil debris. Plants were rated for infection from *Rhizoctonia* on a scale of 1 to 6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf

sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=complete death). Fresh root and shoot weights were also determined. To fulfill Koch's postulates, *Rhizoctonia* was re-isolated on to SPDA from infected tissues. All data were analyzed using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC).

Peanut study

Peanut seeds were germinated in 9-cm dia. Petri plates lined with water moistened Whatman # 1 filter papers in an incubator in dark at 30 C for 48 h. Germinated seeds with uniform radical were transferred to 15 cm x 30 cm pots containing a 2:1:1 (v/v/v) mixture of sand, peat moss and topsoil, with four germinated seeds per pot. The combination of two isolates (G-24 and Fellers) and the non-inoculated control and four genotypes resulted in 12 treatments. Treatments were arranged in a randomized complete block design with four replicates and the experiment was conducted twice.

Inoculum of the *Rhizoctonia* spp. was prepared using oat seeds as described previously for wheat. Peanut seedlings at the V-1 to V-2 growth stage were inoculated by placing 2 oat seeds 2 cm below the soil level close to the main stem. After inoculation, pots with inoculated seedlings were placed in a greenhouse for 2 weeks at 24-27 °C, which is a temperature range conducive for *Rhizoctonia* growth and infection of hypocotyls. After 2 weeks, seedlings were uprooted and roots were separated from hypocotyls. After careful washing, hypocotyls were rated on a 1 to 6 scale developed by the Cotton Seed Treatment

Committee of the National Cotton Council (5) with modifications as follows: 1= no symptoms, 2= discoloration and/or small pinpoint lesions, 3 = small, distinct necrotic lesions, 4 = large necrotic lesions, 5 = girdling lesion, and 6 = dead seedling. Seedling height and fresh weight were recorded. All data were analyzed using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC).

Quantification of infection cushions

Growth chamber studies were conducted to quantify the formation of infection cushions by isolates of *Rhizoctonia* spp. on cellophane membrane in response to stimulation by root system of peanut and winter wheat. Experiments were conducted separately on peanut and winter wheat. Mycelial inoculum of *Rhizoctonia* was prepared as follows: Three hyphal plugs of 0.5 cm diameter taken from four day old culture of *Rhizoctonia* were transferred to a sterile (121 C for 20 min) 250 ml Erlenmeyer flask containing 100 ml of 2% potato dextrose broth. The inoculated flasks were then placed on a Lab-Line Orbit shaker (Lab-Line Instrument Inc., Melrose Park, IL) set at 150 rpm for 7 days. After 7 days, the contents of a flask were passed through a Whatman #1 filter paper disk under vacuum. Contents were under vacuum until no moisture was detected on the filter paper disk. A 0.5 g of fresh mycelium was then homogenized in 50 ml of deionized water by using a Tekmar II Tissuemizer MarkII (Tekmar Co., Cincinnati, OH) set at 10,000 rpm for 45 sec. Formation of infection cushions by two isolates of *R. solani* (G-24 and RSP) and an isolate of *R. cerealis* (Fellers) was studied on four peanut genotypes (Okrun, Tamspan 90, Southwest Runner and

C209-6-60). In peanut study, seeds were germinated in Petri plates lined with moist Whatman # 1 filter papers in an incubator at 30 C for 48 h. Germinated seeds with good radical growth were transferred to 7 cm diameter pots containing 2:1:1 (v/v/v) mixture of sand, peat moss and topsoil with one seed per pot. Plants were maintained in a greenhouse for 2 weeks, at which time the plants were uprooted, washed with clean water, and the root system was enclosed in a tube-like pouch made from cellophane membrane dialysis tubing (Sigma-Aldrich, St. Louis, MO) with a molecular wt cut off at 12,000. Each pouch with the root system was then transferred into a styrofoam cup (360 ml) filled with perlite and mixed with 50 ml of fragmented mycelial suspension of *Rhizoctonia*. Four days later, plants were uprooted and the cellophane pouch around the roots was removed carefully. Eight squares of cellophane (2 cm^2 each) were cut from each pouch, placed on a glass slide (2 squares per slide), stained with 0.5% aniline blue in lactophenol and observed for infection cushion formation under a light microscope at 100X.

In the wheat study, ten certified seeds of each cultivar (Jagger, 2137 and 2174) were planted in 12 cm plastic pots at a depth of 2 cm containing a mixture of sand, soil and shredded peat moss (2:1:1; v/v/v). Plants were maintained in a greenhouse for six weeks, after which time the plants were uprooted, washed with clean water and root system was enclosed in a tube-like pouch made from cellophane membrane as described for peanut. Each pouch with the root system was then transferred into a styrofoam cup (360 ml) filled with perlite and mixed

with 50 ml of fragmented mycelial suspension of *Rhizoctonia*. Four days later, plants were uprooted and the cellophane pouch around the roots was removed carefully. Ten squares of cellophane (2 cm^2 each) were cut from each pouch, placed on a glass slide (2 squares per slide), stained with 0.5% aniline blue in lactophenol and observed for infection cushion formation under a light microscope at 100X.

RESULTS

Morphological characters of *Rhizoctonia* cultures

Isolates of *R. solani* from peanut were clearly distinct from isolates of *R. cerealis* isolated from wheat culms with sharp eyespot lesions. *R. solani* isolates from peanut (G-24, JY-1, RS-00-007 and RSP) produced light to dark brown mycelium and showed no zonations. Except for isolate JY-1, aerial hyphae were sparse. All *R. solani* isolates produced irregular, darkly pigmented sclerotia randomly throughout the colony. Hyphae of all *R. solani* isolates were comparatively thicker than *R. cerealis* isolates.

R. cerealis isolates from wheat (Fellers, Marshall, Altus and Lahoma) produced yellow-white to light-tan colored mycelium with a concentric pattern of growth. Mycelial pigmentation increased with age and aerial hyphae were not observed. None to very few sclerotia developed on the edges of the agar surface after prolonged incubation. Sclerotia, when present, were dark brown and irregular in shape.

Nuclear staining and anastamosis grouping

The rapid staining technique with 0.5% aniline blue in lactophenol was effective in determining the nuclear condition of the *Rhizoctonia* isolates. *R. solani* isolates from peanut had hyphal cells that were multinucleate, whereas isolates of *R. cerealis* from wheat culms with sharp eyespot lesions had binucleate cells.

R. solani isolates from peanut were known to belong to the anastomosis group AG-4 and hence, no tests of hyphal anastamosis were made on these isolates. Vegetative hyphae of *R. cerealis* isolates from wheat (Fellers, Marshall, Altus and Lahoma) anastomosed with the CAG-1 tester isolate of *R. cerealis* but failed to anastamose with the AG-4 tester isolate of *R. solani* (Table 2).

Growth rates

The hyphal growth rates of *R. solani* isolates were significantly higher than those of *R .cerealis* isolates and ranged from 19.3-32.6 mm/24 hr (Table 2). Among the *R. solani* isolates, RSP had the slowest hyphal extension rate (19.3 mm/24 hr) whereas G-24 had the fastest (32.6 mm/24 hr) (Table 2). Even though significant differences in growth rates were observed among *R. solani* isolates, no such differences occurred among *R. cerealis* isolates with the range varying only between 15.2-15.5 mm/24 hr (Table 2).

Pathogenicity tests

Wheat pre-emergence test

Disease severity was significantly affected by *Rhizoctonia* isolate and variety and there was no significant interaction between the two factors (Tables 3, 4 and 5). Non-inoculated seedlings of all varieties received the lowest disease severity rating (Table 3). *R. solani* isolate G-24 (from peanut)-inoculated seedlings of all varieties had significantly ($P \leq 0.05$) higher disease severity as compared to the *R. cerealis* isolate Fellers (from wheat) and the non-inoculated control (Table 3). Fellers inoculated seedlings of all varieties had lower disease ratings that were not significantly different ($P > 0.05$) from the non-inoculated control. Averaging across *Rhizoctonia* isolates, seedlings of Jagger had significantly ($P \leq 0.05$) lower disease severity rating compared to 2174 (Table 4).

Shoot height was significantly ($P \leq 0.05$) affected by isolate but not affected by variety and there was no significant interaction ($P > 0.05$) between the two factors (Tables 3, 4 and 6). Across varieties, isolate G-24 inoculated seedlings had a significantly ($P \leq 0.05$) lower shoot height compared to Fellers and the non-inoculated control (Table 3). The mean shoot height for G-24 inoculated seedlings was 11.8 cm compared to 18.2 cm for Fellers inoculated seedlings and 18.1 cm for the non-inoculated control. Shoot height was not affected by the isolate Fellers and was not significantly different ($P > 0.05$) from the non-inoculated control (Table 3).

Shoot fresh weight was significantly (P≤0.05) affected by isolate but not affected by variety, and there was no significant interaction (P > 0.05) between the two factors (Tables 3, 4 and 7). G-24 -inoculated seedlings had a significantly (P≤0.05) lower shoot fresh weight compared to Fellers and non-inoculated control (Table 3). The mean shoot fresh weight for G-24-inoculated seedlings was 0.11 g compared to 0.14 g for Fellers and 0.15 g for non-inoculated control. Shoot fresh weight was not affected by the isolate Fellers and was not significantly different (P > 0.05) from the non-inoculated control (Table 3).

Root fresh weight was significantly (P≤0.05) affected by variety but not affected by isolate and there was no significant interaction (P > 0.05) between the two factors (Tables 3, 4 and 8). Averaging across the isolates to compare varieties, the mean root fresh weight of seedlings of Jagger was 0.053 g and was significantly (P≤0.05) higher compared to 2174 (Table 4). Root fresh weight of 2137 was not significantly different (P > 0.05) from either Jagger or 2174 (Table 4).

Wheat post-emergence test

Disease severity was significantly (P≤0.05) affected by variety and *Rhizoctonia* isolate, and there was a significant interaction between the two factors (Tables 9, 10 and 11). Non-inoculated seedlings of all varieties received the lowest disease severity rating (Table 9). *R. solani* isolate G-24-inoculated seedlings of all varieties received a significantly (P≤0.05) higher disease severity rating as compared to the *R. cerealis* isolate Fellers and the non-inoculated control (Table 9). Fellers inoculated seedlings of all varieties received a low disease

rating but were significantly (P≤0.05) higher than the non-inoculated control (Table 9). Averaging across isolates to compare varieties, seedlings of Jagger received a significantly (P≤0.05) lower disease severity rating compared to 2137 and 2174 (Table 10).

Shoot fresh weight was significantly (P≤0.05) affected by variety but not affected by isolate and there was no significant interaction (P > 0.05) between the two factors (Tables 9, 10 and 12). The mean shoot fresh weight of seedlings of Jagger was 0.53 g which was significantly (P≤0.05) lower than 2137 and 2174 (Table 10).

Root fresh weight was not significantly (P > 0.05) affected by variety or isolate and there was no significant interaction between the two factors (Tables 9, 10 and 13).

Peanut study

Disease severity was significantly (P≤0.05) affected by isolate but not affected by peanut genotype and there was no significant interaction (P > 0.05) between the two factors (Table 14, 15 and 16). Averaging across varieties to compare isolates, seedlings of all varieties inoculated with *R. solani* isolate G-24 received a significantly (P≤0.05) higher disease severity rating as compared to the seedlings inoculated with the *R. cerealis* isolate Fellers and the non-inoculated control (Table 14).

Shoot height was significantly (P≤0.05) affected by isolate and genotype and there was no significant interaction (P > 0.05) between the two factors

(Tables 14, 15 and 17). Averaging across varieties to compare isolates, G-24 inoculated seedlings had a significantly ($P \leq 0.05$) lower shoot height compared to Fellers and the non-inoculated control (Table 14). The mean shoot height for G-24 inoculated seedlings was 10.3 cm compared to 16.1 cm for Fellers inoculated seedlings and 16.1 cm for uninoculated control. Shoot height was not affected by the isolate Fellers and was not significantly different ($P > 0.05$) from the non-inoculated control (Table 14). Averaging across isolates to compare varieties, seedlings of Tamspan 90 recorded a significantly ($P \leq 0.05$) greater shoot height compared to other varieties (Table 15).

Shoot fresh weight was significantly ($P \leq 0.05$) affected by both isolate and genotype, and there was no significant interaction ($P > 0.05$) between the two factors (Tables 14, 15 and 18). G-24 inoculated seedlings had a significantly ($P \leq 0.05$) lower shoot fresh weight compared to seedlings inoculated with the isolate Fellers and non-inoculated control (Table 14). The mean shoot fresh weight for G-24 inoculated seedlings was 1.9 g compared to 2.9 g for Fellers and 2.9 g for the non-inoculated control. Shoot fresh weight was not affected by the isolate Fellers which was not significantly different ($P > 0.05$) from the non-inoculated control (Table 14). When averaged across isolates, Southwest Runner recorded a significantly ($P \leq 0.05$) higher shoot fresh weight followed by line C-209-6-60, Tamspan 90 and Okrun (Table 15).

Quantification of infection cushions formed on cellophane membrane by *Rhizoctonia* isolates

Peanut study

Formation of infection cushions per square (ICS) was significantly (P≤0.05) affected by isolate and cultivar and there was a significant interaction between the two factors (Tables 19, 20 and 21). Averaging across cultivars to compare isolates, *R. solani* isolate G-24 formed significantly (P≤0.05) more ICS compared to another *R. solani* isolate RSP, which formed significantly (P≤0.05) more number of ICS compared to the *R. cerealis* isolate Fellers (Table 19). G-24 formed 3.1 ICS compared to 1.4 by RSP and 0.3 for Fellers.

Averaging across isolates to compare genotypes, significant differences (P≤0.05) were observed among cultivars with respect to number of infection cushions per square (Table 20). The highest number of infection cushions per square were formed on Okrun whereas the lowest number were formed on Southwest Runner. No infection cushions were formed on the control (no host) confirming the fact that host stimulation is required for the formation of infection cushions. Tamspan 90 and C-209-6-60 had higher number of infection cushions per square but were not significantly different (P > 0.05) from Okrun and Southwest Runner (Table 20).

When the interaction between isolate and genotype was examined, G-24 significantly (P≤0.05) produced more infection cushions per square on Okrun, Tamspan 90 and Southwest Runner compared to RSP and Fellers whereas both

G-24 and RSP produced significantly (P≤0.05) more number of infection cushions per square in C-209-6-60 compared to Fellers (Table 21).

Wheat study

Formation of infection cushions per square (ICS) was significantly (P≤0.05) affected by cultivar (Table 23) but not by isolate (Table 22), and there was no significant interaction (P > 0.05) between the two factors (Table 24). Averaging across isolates to compare cultivars, Jagger and 2174 significantly had a higher number of infection cushions per square compared to 2137 and control. However, no significant difference (P > 0.05) in infection cushion number was observed between Jagger and 2174 (Table 23).

Averaging across cultivars, the numbers of infection cushions produced by *R. solani* isolate G-24 was not significantly different (P > 0.05) from *R. cerealis* isolate Fellers (Table 22).

DISCUSSION

R. solani isolates from peanut (G-24, JY-1, RS-00-07 and RSP) are clearly distinct in their characteristics compared to the *R. cerealis* isolates from wheat (Fellers, Marshall, Altus and Lahoma). Apart from the nuclear condition, isolates differed very much in their appearance, sclerotial development, growth rate and pathogenicity. *R. solani* isolates from peanut possessed dark colored mycelia, faster growth rates, multinucleate condition and copious sclerotial development. Since the peanut isolates were collected from other sources, the status of the

peanut fields where the isolates were originally collected is unknown. However, all the isolates were collected from pods possessing pod rot symptoms.

Cultural morphology of wheat isolates paralleled those reported previously for *R. cer*ealis (1, 9). All wheat isolates, irrespective of their origin in Oklahoma, possessed a yellow white to light tan colored mycelium, a significantly slower growth rate compared to the isolates from peanut and absence or very sparse sclerotial development. The wheat fields from where the isolates were collected did not show any major damage from the pathogen. The sharp eyespot lesions are not clearly visible in the field and in most cases, lesions were restricted to lower stem base. In some cases, sharp eyespot lesions are confused with the eyespot caused by the fungus *Psuedocercosporella herpotrichoides,* since both appear as eye shaped lesions. However, when observed closely, the margin of the sharp eyespot lesion is very distinct and sharp as opposed to wavy margins for the eyespot caused by *P. herpotrichoides*.

All *R.solani* isolates from peanut used in this study belonged to the AG 4 anastomosis group. The AG 4 group consists of isolates that are soilborne and cause damping off and root rot over a wide host range (2). Isolates belonging to AG4 are autotrophic for thiamine and are capable of hyphal fusion with members of AG 4 only (16). AG-4 is further subdivided based on the sclerotial form and DNA base sequence homology into AG 4 HG-I originally isolated from peanut and AG 4 HG-IIB that was isolated from sugar beet (16). Isolates of *R. cerealis* from wheat used in this study were all anastamosed with CAG-1 or AG-D

tester isolate which was originally isolated from wheat. CAG-1 group is distributed worldwide and includes pathogenic isolates causing sharp eyespot in cereals (2, 16).

Isolates of *R. solani* from peanut grew twice as fast as isolates of *R. cerealis* from wheat and these observations paralleled those previously reported (9). Among *R. solani* isolates from peanut, RSP grew significantly slower than other isolates. However, there is no significant difference in growth rates among *R. cerealis* isolates.

R. solani isolate G-24 isolated from peanut pods had caused significant damage to peanut and wheat seedlings during artificial inoculation in the greenhouse. Distinct necrotic lesions were seen on hypocotyls of inoculated peanut seedlings. Necrotic lesions resulted in stunted plants, decreased shoot fresh weight and in few cases complete death of the seedling.

In the wheat study, pre-emergence inoculation of the isolate G-24 resulted in non emergence of seedlings and decreased stand. Seedlings that emerged were stunted in appearance and produced distinct lesions on the base of the stem. Infection was restricted to the stem portion of the plant and roots were not affected. Hence, a significant reduction in shoot height and shoot fresh weight was observed but root fresh weight was not significantly affected. Lesions were not deep and were restricted to the outer leaf sheath. However, the G-24 isolate had a different effect when inoculated on 6-week old wheat plants, where the

severity of disease was very low with no significant effect on shoot and root fresh weights.

The *R. cerealis* isolate Fellers did not cause any damage to peanut and winter wheat in our greenhouse studies. In most cases, the effect of this isolate was not significantly different from the non-inoculated control. Among four isolates of *R. cerealis*, Fellers was chosen for this study since our preliminary data have shown that this isolate comparatively caused more disease than the other isolates. However, the pathogenicity of this isolate was negligible compared to G-24 and the other *R. solani* isolates from peanut.

Averaging across isolates to compare varieties, our pre-emergence wheat study showed that Jagger received a significantly lower disease severity rating compared to 2137 and 2174. However, no significant differences in shoot height, shoot fresh weight and root fresh weight were observed among the three varieties. Our post emergence study also showed significant differences in disease severity, with Jagger receiving a significantly lower disease severity rating compared to 2137 and 2174. However, the disease severity ratings were low, and the significance of this difference needs further substantiation.

Infection cushions formed by the *R. solani* isolate G-24 were more conspicuous and easy to read compared to the *R. cerealis* isolate Fellers. In the peanut study, signiciantly lower infection cushions were formed on Southwest Runner compared to the other peanut cultivars, and isolate G-24 produced a significantly higher number of infection cushions per cellophane square

compared to RSP and Fellers. It is important to note that the mean number of infection cushions produced by each isolate corresponded with the pathogenicity of the isolate that was previously tested in greenhouse studies. Results from the wheat study were somewhat surprising. There was no significant difference in the mean number of infection cushions produced by isolates G-24 and Fellers and since the disease ratings of *Rhizoctonia* isolates were very low on wheat seedlings, it is difficult to compare the number of infection cushions with the pathogenicity of the isolates. Overall, the results suggest that quantification of infection cushions can be a useful technique to evaluate peanut and wheat germplasms for resistance to *Rhizoctonia* diseases.

In conclusion, our greenhouse and lab studies on pathogenicity of *Rhizoctonia* spp. showed that *R. solani* isolates from peanut have the potential to cause significant damage to peanut and winter wheat, when the pathogen attacks at early stages of crop growth. Even though some resistance was observed in cultivars such as Jagger, field studies are needed to substantiate the results found under greenhouse conditions. On the other side, *R. cerealis* isolates, currently found in Oklahoma, are lowly virulent or non pathogenic and pose no risk to peanut and winter wheat productivity. Our results also indicate that planting peanuts in a wheat field with a history of sharp eyespot may not pose any risk to peanuts, but planting wheat in peanut field with a history of *Rhizoctonia* diseases may pose a significant risk to wheat productivity.

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Table 1. Source of *Rhizoctonia* isolates.

Isolate	Species	Host	Year	Geographic origin	Source
G-24	*R. solani*	Peanut	Unknown	Texas	T.A. Wheeler
JY-1	*R. solani*	Peanut	Unknown	Georgia	T. Brenneman
RS-00-07	*R. solani*	Peanut	Unknown	Georgia	T. Brenneman
RSP	*R. solani*	Peanut	1982	Oklahoma	H.A. Melouk
Fellers	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Marshall	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Altus	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Lahoma	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger

Table 2. Hyphal growth rates of isolates of *Rhizoctonia.*

Isolate	Species	Host	Anastamosis group	Growth rate (mm/24 hr)
G-24	*R. solani*	Peanut	AG 4	32.6 b*
JY-1	*R. solani*	Peanut	AG 4	36.1 a
RS-00-07	*R. solani*	Peanut	AG 4	28.6 c
RSP	*R. solani*	Peanut	AG 4	19.3 d
Fellers	*R. cerealis*	Winter wheat	CAG 1	15.3 e
Marshall	*R. cerealis*	Winter wheat	CAG 1	15.4 e
Altus	*R. cerealis*	Winter wheat	CAG 1	15.2 e
Lahoma	*R. cerealis*	Winter wheat	CAG 1	15.5 e

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 3. Disease severity, shoot height, shoot fresh weight and root fresh weight of winter wheat seedlings over genotypes as affected by pre-emergence inoculation with *Rhizoctonia* isolates.

Isolate	Disease severity[a]	Shoot height (cm)[b]	Shoot fresh weight (g)	Root fresh weight (g)
Non-inoculated	1.6 a[*]	18.1 a	0.15 a	0.05 a
G-24	3.4 b	11.8 b	0.11 b	0.04 b
Fellers	1.6 a	18.2 a	0.14 a	0.05 a

[a]Disease severity rating on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=no emergence).

[b] Measured from the base of the stem to the tip of the second leaf.

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 4. Disease severity, shoot height, shoot fresh weight and root fresh weight of winter wheat seedlings over *Rhizoctonia* isolates as affected by cultivar in a pre-emergence test.

Cultivar	Disease severity[a]	Shoot height (cm)[b]	Shoot fresh weight (g)	Root fresh weight (g)
Jagger	2.0 b[*]	16.0 a	0.13 a	0.05 a
2137	2.3 ab	15.3 a	0.13 a	0.05 a
2174	2.4 a	16.8 a	0.14 a	0.04 a

[a]Disease severity rating on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=no emergence).

[b] Measured from the base of the stem to the tip of the second leaf.

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 5. *Rhizoctonia* disease severity of wheat seedlings as affected by isolate and cultivar in a pre-emergence pathogenicity test.

Isolate	Disease Severity[a]		
	Jagger	**2137**	**2174**
Non-inoculated	1.2 a[*]	1.6 a	2.1 a
G-24	3.3 b	3.4 b	3.5 b
Fellers	1.6 a	1.8 a	1.5 a

[a]Disease severity rating on a scale of 1-6 at 14 days after inoculation (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=no emergence).

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 6. Shoot height of wheat seedlings as affected by isolate and cultivar in a pre-emergence pathogenicity test.

Isolate	Shoot height (cm)[b]		
	Jagger	**2137**	**2174**
Non-inoculated	19.1 a[*]	16.9 a	18.3 a
G-24	11.5 b	10.4 b	13.7 a
Fellers	17.3 a	18.7 a	19.6 a

[b]Measured from the base of the stem to the tip of the second leaf.

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 7. Shoot fresh weight of wheat seedlings as affected by isolate and cultivar in a pre-emergence pathogenicity test.

Isolate	Shoot Fresh Weight (g)		
	Jagger	**2137**	**2174**
Non-inoculated	0.16 a[*]	0.14 a	0.14 a
G-24	0.10 ab	0.09 b	0.13 a
Fellers	0.13 a	0.15 a	0.14 a

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 8. Root fresh weight of wheat seedlings as affected by isolate and cultivar in a pre-emergence pathogenicity test.

Isolate	Root Fresh Weight (g)		
	Jagger	2137	2174
Non-inoculated	0.06 a*	0.04 a	0.04 a
G-24	0.05 a	0.04 a	0.03 a
Fellers	0.05 a	0.05 a	0.05 a

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 9. Disease severity, shoot fresh weight and root fresh weight of winter wheat plants as affected by post-emergence inoculation of *Rhizoctonia* isolates.

Isolate	Disease severity[a]	Shoot fresh weight (g)	Root fresh weight (g)
Non-inoculated	1.0 a*	0.61 a	0.49 a
G-24	1.4 c	0.59 a	0.44 a
Fellers	1.1 b	0.62 a	0.47 a

[a]Disease severity rating at 14 days after inoculation on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=complete death).
*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 10. Disease severity, shoot fresh weight and root fresh weight of winter wheat plants as affected by cultivar in post-emergence test.

Cultivar	Disease severity[a]	Shoot fresh weight (g)	Root fresh weight (g)
Jagger	1.1 a[*]	0.53 b	0.45 a
2137	1.2 ab	0.66 a	0.49 a
2174	1.3 b	0.63 a	0.47 a

[a]Disease severity rating at 14 days after inoculation on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6=complete death).
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 11. Disease severity of wheat plants as affected by isolate and cultivar in post-emergence pathogenicity test.

Isolate	Disease Severity[a]		
	Jagger	**2137**	**2174**
Non-inoculated	1.0 a[*]	1.0 a	1.0 a
G-24	1.3 b	1.3 b	1.6 b
Fellers	1.1 a	1.1 a	1.1 a

[a]Disease severity rating on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6= complete death).
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 12. Shoot fresh weight of wheat plants as affected by isolate and cultivar in post-emergence pathogenicity test.

Isolate	Shoot fresh weight (g)		
	Jagger	2137	2174
Non-inoculated	0.57 a*	0.62 a	0.62 a
G-24	0.49 a	0.65 a	0.60 a
Fellers	0.51 a	0.69 a	0.62 a

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 13. Root fresh weight of wheat plants as affected by isolate and cultivar in post-emergence pathogenicity test.

Isolate	Root fresh weight (g)		
	Jagger	2137	2174
Non-inoculated	0.50 a*	0.48 a	0.47 a
G-24	0.39 ab	0.43 a	0.48 a
Fellers	0.45 b	0.53 a	0.40 a

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 14. Disease severity, shoot height, shoot fresh weight of peanut seedlings as affected by inoculation of *Rhizoctonia* isolates.

Isolate	Disease severity[a]	Shoot height (cm)	Shoot fresh weight (g)
Non-inoculated	1.0 a*	16.1 a	2.9 a
G-24	4.1 b	10.3 b	1.9 b
Fellers	1.1 a	16.1 a	2.9 a

[a]Disease severity rating on a scale of 1-6 (1= no symptoms, 2= discoloration and/or small pinpoint lesions, 3 = small, distinct necrotic lesions, 4 = large necrotic lesions, 5 = girdling lesion, and 6 = dead seedling.

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 15. Disease severity, shoot height, shoot fresh weight of peanut seedlings as affected by genotype.

Cultivar	Disease severity[a]	Shoot height (cm)	Shoot fresh weight (g)
Okrun	2.1 a[*]	13.9 b	2.2 c
Tamspan 90	2.0 a	17.8 a	2.5 bc
Southwest Runner	2.0 a	15.1 b	2.9 a
C-209-660	2.1 a	10.1 c	2.5 b

[a]Disease severity rating on a scale of 1-6 (1= no symptoms, 2= discoloration and/or small pinpoint lesions, 3 = small, distinct necrotic lesions, 4 = large necrotic lesions, 5 = girdling lesion, and 6 = dead seedling.
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 16. Disease severity rating of peanut seedlings as affected by isolate and genotype.

Isolate	Disease severity rating[a]			
	Okrun	**Tamspan 90**	**Southwest Runner**	**C-209-660**
Non-inoculated	1.0 a[*]	1.0 a	1.0 a	1.0 a
G-24	4.1 b	3.7 b	4.0 b	4.4 b
Fellers	1.2 a	1.3 a	1.0 a	1.1 a

[a]Disease severity rating on a scale of 1-6 (1= no symptoms, 2= discoloration and/or small pinpoint lesions, 3 = small, distinct necrotic lesions, 4 = large necrotic lesions, 5 = girdling lesion, and 6 = dead seedling.
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 17. Shoot height of peanut seedlings as affected by isolate and genotype.

Isolate	Shoot height (cm)			
	Okrun	**Tamspan 90**	**Southwest Runner**	**C-209-660**
Non-inoculated	14.8 a[*]	20.4 a	17.4 a	11.5 a
G-24	11.2 b	12.5 b	10.1 b	7.2 b
Fellers	15.1 a	20.6 a	17.7 a	11.3 a

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 18. Shoot fresh weight of peanut seedlings as affected by isolate and genotype.

Isolate	Shoot fresh weight (g)			
	Okrun	Tamspan 90	Southwest Runner	C-209-660
Non-inoculated	2.4 a*	2.8 a	3.3 a	2.9 a
G-24	1.8 b	1.7 b	2.1 b	1.9 b
Fellers	2.4 a	2.9 a	3.7 a	2.7 a

*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 19. Formation of infection cushions by isolates of *Rhizoctonia* on cellophane membrane in response to stimulation by peanut root system.

Isolate	Infection cushions per square[a]
G-24	3.1 a*
RSP	1.4 b
Fellers	0.3 c

[a]Mean number of infection cushions per 2 cm^2 cellophane.

* Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 20. Formation of infection cushions on cellophane membrane by *Rhizoctonia* spp. in response to stimulation by peanut root system.

Cultivar	Infection cushions per square[a]
Okrun	2.4 a*
Tamspan 90	2.0 ab
Southwest Runner	1.6 b
C-209-660	1.9 ab
No host	0.0 c

[a]Mean number of infection cushions per 2 cm^2 cellophane.

* Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05

Table 21. Formation of infection cushions by isolates of *Rhizoctonia* on cellophane membrane in response to stimulation by peanut root system.

Isolate	Infection cushions per square[a]				
	Control	Okrun	Tamspan 90	Southwest Runner	C-209-6-60
G-24	0.0 a[*]	4.8 b	3.7 b	3.8 b	2.8 b
RSP	0.0 a	1.8 a	1.9 a	0.9 a	2.6 b
Fellers	0.0 a	0.6 a	0.5 a	0.7 a	0.4 a

[a]Mean number of infection cushions per 2 cm^2 cellophane.
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 22. Formation of infection cushions on cellophane membrane by isolates of *Rhizoctonia* in response to stimulation by wheat roots.

Isolate	Infection cushions per square[a]
G-24	2.2 a[*]
Fellers	2.7 a

[a]Mean number of infection cushions per 2 cm^2 cellophane.
[*] Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 23. Formation of infection cushions by *Rhizoctonia* spp. on cellophane membrane in response to stimulation by roots of different winter wheat cultivars.

Cultivar	Infection cushions per square[a]
Jagger	4.5 a[*]
2137	1.0 b
2174	3.7 a
No host	0.0 b

[a]Mean number of infection cushions per 2 cm^2 cellophane.
[*] Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05

Table 24. Formation of infection cushions on cellophane membrane by isolates of *Rhizoctonia* in response to stimulation by roots of winter wheat cultivars.

Isolate	Infection cushions per square[a]			
	Control	Jagger	2137	2174
G-24	0.3 a[*]	3.5 a	1.2 a	3.4 a
Fellers	0.0 a	5.5 a	1.0 a	3.9 a

[a]Mean number of infection cushions per 2 cm^2 cellophane.
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

CHAPTER IV

PATHOGENICITY OF

SCLEROTIUM ROLFSII ON PEANUT AND WINTER WHEAT

ABSTRACT

Sclerotium rolfsii Sacc. is an economically important pathogen on many crops in warmer regions of the world. The fungus can cause wide spread damage on peanut and to some extent on winter wheat, which are important crops in Oklahoma. The objective of this study was to determine the pathogenicity of isolates of *S. rolfsii* on peanut and winter wheat cultivars of Oklahoma. Pathogenicity of four isolates of *S. rolfsii* from peanut (Melouk, Ft. Cobb, Power St and Durant) and two from wheat (Wheat and ZP-3082) was determined on three peanut (Okrun, Southwest Runner and Tamspan 90) and three winter wheat cultivars (Jagger, 2137 and 2174) in greenhouse studies. Four plants of each cultivar were inoculated at the R-2 growth stage (peanut) or the two-leaf stage (winter wheat) by placing a 0.5 cm agar disk removed from a 3-day-old culture onto a 1 cm diameter filter paper that was then pressed to the base of the stem. Non-inoculated plants were used as the control. After inoculation, pots were covered with polythene sheets to maintain a high relative humidity, which were than maintained at 25 ± 2°C in the greenhouse. First assessment of disease

was made three days after inoculation (DAI) and every 2 days thereafter for a total of 6 assessments. An additional assessment was made on peanut at 24 DAI. A 1-6 scale was used for all disease assessments (1=no lesion; 6=complete death of plant). Additionally, lesion lengths were measured at each disease assessment on peanut and estimates of relative water content (RWC) of leaves were taken at the end of the experiment. On peanut, all isolates except Wheat and ZP-3082 induced blight symptoms. Wilting of plants occurred at nine DAI. On wheat, lesions were initially superficial, yellowish and water soaked. Lesions expanded and resulted in damping-off of seedlings. Disease severity was lowest at three and five DAI and highest at fifteen and twenty-four DAI. Peanut isolates (Melouk, Ft. Cobb, Power St and Durant) significantly ($P \leq 0.05$) produced a high disease severity on peanut and wheat cultivars compared to wheat isolates (Wheat and ZP-3082). In the peanut study, averaging across cultivars, isolate Durant significantly ($P \leq 0.05$) had a higher disease severity with longer lesions and lower RWC by the end of the experiment compared to the non-inoculated control and the wheat isolates. Averaging across isolates, Southwest Runner significantly ($P \leq 0.05$) had the lowest disease severity with shorter lesions and higher RWC. In the wheat study, averaging across cultivars, isolate Power St significantly ($P \leq 0.05$) had the highest disease severity by the end of the experiment compared to the control and the wheat isolates. Averaging across isolates, Jagger significantly ($P \leq 0.05$) had the lowest disease severity. Even though *S. rolfsii* is not expected to pose any significant risk to winter wheat

production, infection of wheat may enhance survival of *S. rolfsii* and facilitate infection and losses in a peanut crop following wheat. This is especially important in certain areas of Oklahoma where a wheat-peanut rotation is occasionally practiced.

INTRODUCTION

Sclerotium rolfsii Sacc. is a soilborne fungus that is widespread in tropical and warmer portions of the temperate zones. This fungus has a wide host range from mosses to composites and includes a large number of economically important crops (1). *S. rolfsii* causes stem and pod rot in peanut where losses are estimated at 7-10% annually (8). *S. rolfsii* is not known to significantly damage wheat even though sporadic occurrence of southern blight has been reported from some parts of wheat growing regions of the world (2, 4).

Currently, the pathogenicity of isolates collected from peanut growing regions of Oklahoma on Oklahoma peanut cultivars is unknown. Additionally, an isolate of *S. rolfsii* was collected in recent years from diseased winter wheat seedlings in OK, for which the pathogenicity is unknown. Host resistance can be used as an important component of an integrated management of *S. rolfsii* (10). Evidence of varietal resistance to *S. rolfsii* was demonstrated in runner peanuts (3, 6, 7, 9). Such resistance was attributed mainly to an impervious cuticle, thick-walled cortical cells and cork cambium activity (5). However, studies have not been found relating to resistance identification towards *S. rolfsii* in wheat

cultivars grown in United States. Currently, Okrun, a widely grown peanut cultivar in Oklahoma, is known to be susceptible towards southern blight (personal communication with Dr. Hassan Melouk). Hence, screening for resistance to identify tolerance in peanut and winter wheat cultivars to *S. rolfsii* infection is needed. Field experiments are not always reliable to confirm the reaction to the disease since distribution of sclerotial inoculum is non-uniform in the field (11). Hence, the objective of this research was to quantify the reaction of peanut and winter wheat to *S. rolfsii* under greenhouse conditions.

MATERIALS AND METHODS

Origin and maintenance of *S. rolfsii* isolates

The *S. rolfsii* isolates used in this study, along with their origin, host, and sources are given in Table 1. Isolates were maintained by placing surface disinfested (10% aqueous NaOCl for 2 min) sclerotia on SPDA [Potato Dextrose Agar amended with streptomycin sulfate (0.14 g/L)]. Mycelial plugs (0.5 cm) from three-day-old cultures were transferred onto fresh SPDA plates and incubated at 23 ± 2 C for three weeks to allow for sclerotial formation. Sclerotia were then collected and air-dried, placed into coin envelopes and stored at 23 ± 2 C in a desiccator containing anhydrous calcium sulfate.

Pathogenicity tests

Pathogenicity of six isolates of *S. rolfsii* (Table 1) obtained from peanut and wheat was determined on three peanut cultivars (Okrun, Tamspan 90 and

Southwest Runner) and three winter wheat cultivars (Jagger, 2137 and 2174) under greenhouse conditions. Reaction of peanut and wheat cultivars used in this study, to *S. rolfsii* infection is not known previously. Experiments were conducted separately on peanut and winter wheat. Except for Okrun, none of the other cultivars of peanut and winter wheat had been tested previously for their reaction to *S. rolfsii* under controlled conditions.

Peanut study

Peanut seeds were germinated in plastic containers lined with moist Whatman # 1 filter papers in an incubator at 30 C for 48 h. Four germinated seeds with good radical growth were selected and planted into 15 cm x 30 cm pots containing a mixture of sand, peat moss and topsoil (2:1:1;v/v/v).

Six weeks after planting, seedlings were inoculated using the mycelial disk technique (12). In this technique, a 0.5-cm mycelial disk was removed from a 3-day-old active *Sclerotium* culture and appressed to the base of the central stem of designated plants. A 1-cm diameter filter paper was placed underneath each mycelial disk to prevent contact with the soil. After inoculation, pots were wrapped with clear polyethylene bags to create a humid environment near the soil surface and plant crowns. Plants were watered as needed.

Disease assessment

First assessment of disease was made three days after inoculation (DAI) and every 2 days thereafter for a total of 6 assessments. An additional assessment was made 24 days after inoculation. A 1-6 scale was used for all disease

assessments, where, (1= no lesion on any of the three stems; 2= lesion on one stem per plant; 3= lesion on two stems per plant; 4= lesion on three stems per plant; 5= wilting or death of at least one stem;6= wilting or death of plant). Also, lesion lengths were taken at each disease assessment on each plant, and an average lesion length was calculated.

Relative Water Content

Relative water content (RWC) is the measure of plant water status in terms of the physiological consequence of cellular water deficit. RWC estimates the current water content of the sampled tissue. On the day of final disease assessment, three compound leaf samples were collected from each plant, totaling 4 samples from each pot. Using a cork borer, one cm diameter leaf disks were cut from each leaf, one per leaf. Leaf disks were weighed immediately to obtain a leaf sample fresh weight (W), after which the samples were hydrated to fully turgidity for 12 hrs. After 12 hrs, samples were taken out of water and dried of any surface moisture with filter paper and immediately weighed to obtain full turgid weight (TW). Samples were than oven dried at 70°C for 24 hrs and weighed to determine the oven dry weight (DW). RWC (%) was calculated as follows:

RWC (%) = [W-DW] / [TW-DW] x 100

Where,

W = sample fresh weight

TW= sample turgid weight

DW=sample dry weight.

Wheat study

Three hard red winter wheat cultivars, Jagger, 2137 and 2174 that are cultivated in Oklahoma, and which were never tested before for their reaction to *S. rolfsii* infection, were used in this study. Ten certified wheat seeds of each cultivar were planted in 12 cm dia. plastic pots at a depth of 2 cm containing a mixture of sand, soil and shredded peat moss (2:1:1; v/v/v). Four days after planting, seedlings were thinned to four seedlings per pot.

Inoculation with *S. rolfsii* was made at stage 1 on the Feekes' scale when the plants are at the two-leaf stage. A mycelial disk technique was used for inoculation (12). A 0.5-cm mycelial disk was removed from a 3-day-old active fungal culture and appressed to the base of the pseudostem of designated plants. A 1-cm dia. filter paper was than placed underneath each mycelial disk to prevent contact with the soil. After inoculation, pots were wrapped with clear polyethylene bags to create a humid environment near the soil surface and plant crowns. Plants were watered as needed.

Disease assessment

First assessment of disease was made three days after inoculation and every 2 days thereafter for a total of 6 assessments. A 1-6 scale was used for all disease assessments, where, 1= no infection on stem; 2= initial lesion on the stem;

3= expanded lesion on the stem; 4= expanded lesion with outer sheath lodging; 5= expanded lesion with whole plant lodging; 6= complete death of the plant.

Data analysis

The effect of isolate, time and cultivar on pathogenicity was determined using analysis of variance techniques with repeated measures. Data analyses were performed using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC). In the peanut study, the response variables considered were disease severity, lesion length and RWC (%) and independent variables were isolate, time and cultivar. In the wheat study, the effect of isolate, time and cultivar on disease severity was determined.

RESULTS

Peanut study

Isolates of *S. rolfsii* from peanut produced blight symptoms that included light brown lesions on main stems and branches, and wilting of leaves (Fig. 1b). Severity of disease varied significantly over the 24-day duration of the experiment depending on the isolate and peanut cultivar. Isolates from peanut showed a progressive increase in disease severity and lesion development with time whereas the wheat isolates were non-pathogenic during the entire duration of the experiment (Figs. 2 and 3).

Disease severity as affected by isolate x cultivar

Averaging across days when assessments were made, disease severity by isolates Durant and Ft. Cobb was significantly lower ($P \leq 0.05$) on Southwest Runner whereas Tamspan 90 exhibited the highest disease severity (Table 4). No significant differences ($P > 0.05$) in disease severity were observed among cultivars when inoculated with the other isolates and the non-inoculated control (Table 4).

Disease severity as affected by isolate

Disease severity was lowest at three and five days after inoculation and highest at 15 and 24 days after inoculation (Table 2). Severity of disease was relatively high on plants inoculated with peanut isolates (Durant, Ft. Cobb, Melouk and Power St) compared to wheat isolates (ZP-3082 and Wheat). Wheat isolates (Wheat, ZP-3082) did not differ significantly ($P > 0.05$) from the non-inoculated control during the entire duration of the experiment (Table 2). At 3 DAI, disease severity of isolates Power St and Durant were significantly different ($P \leq 0.05$) from the non-inoculated control and Wheat. No significant differences were observed among other isolates (Table 2). Starting from 5 DAI, peanut isolates Durant, Ft Cobb, Melouk and Power St had significantly ($P \leq 0.05$) higher disease severity ratings compared to Wheat, ZP-3082 and the non-inoculated control. Isolate Durant recorded significantly ($P \leq 0.05$) highest disease severity starting from 7 DAI, followed by isolate Power St (Table 2). Symptoms of wilting appeared on some of the infected plants starting at 11 DAI and became more

pronounced from 15 DAI. Disease severity on plants inoculated with isolate Durant and Power St was at its peak at 24 DAI and resulted in complete lodging and death of plants.

Disease severity as affected by cultivar

Significant differences in disease severity were observed among peanut cultivars Okrun, Southwest Runner and Tamspan 90 starting from 11 DAI (Table 3). Southwest Runner had a significantly lower disease severity from this period as compared to Okrun and Tamspan 90 (Table 3).

Lesion length as affected by isolate x cultivar

The length of lesions produced by isolates Durant and Ft Cobb was significantly higher ($P \leq 0.05$) on Tamspan 90 and low on Southwest Runner (Table 7). No significant differences ($P > 0.05$) in lesion lengths were observed among cultivars when inoculated with other isolates and non-inoculated control (Table 7).

Lesion length as affected by isolate

Early stage lesions were observed with peanut isolates at 3 DAI and no lesions were formed on plants inoculated with wheat isolates. No significant differences ($P > 0.05$) in lesions lengths were observed among isolates during the initial period (Table 5). However, length of lesion produced by peanut isolates was significantly higher starting from 7 DAI, as compared to the non-inoculated control and the Wheat isolates (ZP-3082 and Wheat). Lesion length produced by

wheat isolates (ZP-3082 and Wheat) was not significantly different from the non-inoculated control during the entire period of the experiment (Table 5). Length of lesion produced by the isolate Durant was significantly higher than other isolates starting from 13 DAI, followed by Power St (Table 5).

Lesion length as affected by cultivar

No significant differences ($P>0.05$) in lesion length were observed among cultivars Okrun, Southwest Runner and Tamspan 90 during the initial period (Table 6). However, significant differences ($P\leq0.05$) appeared starting from 9 DAI. From this period, Tamspan 90 significantly ($P\leq0.05$) had large lesions compared to other cultivars (Table 6). By the end of the experiment (24 DAI), Southwest Runner had significantly ($P\leq0.05$) smaller lesions followed by Okrun and Tamspan 90 (Table 6).

Relative water content

Significant differences ($P\leq0.05$) in RWC were observed among cultivars when inoculated with isolates Durant and Ft Cobb (Table 8). When inoculated with these isolates, Southwest Runner had the highest RWC whereas Tamspan 90 had lowest RWC (Table 8). No significant differences were observed among cultivars when inoculated with other isolates and non-inoculated control.

Wheat study

Isolates of *S. rolfsii* used in this study produced blight symptoms on young wheat seedlings (Fig. 1a). Lesions were initially yellowish and later turned light brown in color. Disease severity varied significantly over the 15-day duration of

the experiment depending on the isolate and winter wheat cultivar. All isolates showed a progressive increase in disease severity (Fig. 4). Disease severity was lowest at three and five days after inoculation and highest at fifteen days after inoculation (Table 9).

Disease severity as affected by isolate x cultivar

Averaging across days when disease assessment was made, disease severity of isolate Durant was significantly low ($P \leq 0.05$) on Jagger compared to 2137 (Table 11). No other significant differences ($P > 0.05$) in disease severity were observed among cultivars when inoculated with other isolates and the non-inoculated control (Table 11).

Disease severity as affected by isolate

Disease severity of peanut isolates (Durant, Ft. Cobb, Melouk and Power St) was significantly ($P \leq 0.05$) higher starting from 5 DAI, compared to the non-inoculated control and the wheat isolates, ZP-3082 and Wheat (Table 9). The wheat isolates (Wheat, ZP-3082) had a significantly ($P \leq 0.05$) higher disease severity compared to the non-inoculated control starting from 7 DAI (Table 9). Disease severity among peanut isolates did not differ significantly by the end of the experiment (15 DAI).

Disease severity as affected by cultivar

No significant differences in disease severity were observed among winter wheat cultivars (Table 10) at 3, 11 and 13 DAI. However, differences in disease severity appeared at 5, 7, 9 and 15 DAI (Table 10). At 5 and 9 DAI, 2137 had a

significantly (P≤0.05) higher disease severity compared to 2174 but was not significantly (P>0.05) different from Jagger (Table 10). At 7 DAI, 2137 had significantly (P≤0.05) high disease severity compared to Jagger and 2174 (Table 10). However, by the end of the experiment (15 DAI) 2174 has significantly higher disease compared to Jagger but was significantly different from 2137 (Table 10). Overall, the effect of cultivar on disease severity was not consistent over the duration of the experiment

DISCUSSION

Isolates of *Sclerotium rolfsii* used in this study came from two crops, peanut and wheat. *S. rolfsii* is known to cause major damage on peanut (1). However, pathogenicity on wheat is relatively unknown. Results from the experiments presented in this paper demonstrated that isolates of *S. rolfsii* from peanut were highly pathogenic on both peanut and winter wheat cultivars. Alternatively, *S. rolfsii* isolates from wheat were less virulent on winter wheat and non pathogenic on peanut cultivars.

Sclerotia of *S. rolfsii* of peanut isolates produced in culture were considerably larger than those of the wheat isolates. Isolate Wheat from Oklahoma had the smallest sclerotia of all the isolates used in this study followed by ZP-3082. Moreover, isolate Wheat had sparse mycelial formation both on plant tissue and on artificial media. Scleortial initials were formed as early as 4 days after incubation and this could be one reason that the isolate has the least

capability to proliferate and invade the host tissue (observation derived from *Sclerotinia minor* after personal communication with Dr. H.A. Melouk).

In the peanut study, isolate Durant was highly virulent on peanut cultivars compared to the other isolates. Both disease severity and length of lesions were significantly higher for this isolate. Wheat isolates, Wheat and ZP-3082 were non pathogenic on peanut cultivars and were not significantly different from the non-inoculated control. In the wheat study, both Power St and Ft. Cobb were highly pathogenic on winter wheat cultivars followed by the isolate Durant. The wheat isolates, Wheat and ZP-3082 showed limited virulence but were significantly higher than non-inoculated control.

To assess pathogenicity on peanut, disease severity (1-6 scale), lesion length and relative water content of the plant (RWC) were measured and the results from all three parameters agree with each other. Hence, any one of these parameters can be used to assess pathogenicity of the causal organism. However, under field conditions, disease severity is recommended, since measuring lesion lengths and RWC is impractical. *S. rolfsii* infection can initiate on any one or all of the branches in peanut plant. Rating the disease on just the main stem could bias the results if the infection occurs on side branches. Hence we devised our rating scale based on all three stems (main stem and two side branches). Lesion lengths were the average of measurements on all three stems. Our results clearly indicate that RWC in infected plants is a good indicator of disease severity.

Virulent isolates such as Durant that produced high disease severity resulted in low RWC plants and vice-versa with non-pathogenic isolates such as Wheat.

Among peanut cultivars, Southwest Runner had the lowest disease severity rating, smaller lesions and higher RWC compared to Okrun and Tamspan 90 suggesting that this runner market type has some resistance to *S. rolfsii* infection. Field studies in this aspect are needed. Tamspan 90 consistently had a higher disease severity rating and large lesions suggesting susceptibility to *S. rolfsii* infection. These observations agree with the general notion that plants with an upright growth habit such as Tamspan 90 are least resistant to *S. rolfsii* compared to cultivars with a spreading growth habit such as Okrun and Southwest Runner. However, reports on this concept were conflicting (3, 9).

Our data suggest that *S. rolfsii* from peanut has the potential to cause early damage on winter wheat seedlings in Oklahoma when warm temperatures prevail during the early part of the growing season. However, late infections on mature plants are not expected given the cold conditions that prevail during the later part of the crop-growing season. Greenhouse studies suggest that the isolate Wheat that was found in Oklahoma poses no risk to peanut or winter wheat production in Oklahoma. However, winter wheat planted in a peanut field with a history of Southern blight may have a risk of an early infection if conditions favor the pathogen. Alternatively, infection of wheat may enhance survival of *S. rolfsii* and facilitate infection and losses in a following peanut crop. This is

especially important in certain areas of Oklahoma where a wheat-peanut rotation is occasionally practiced.

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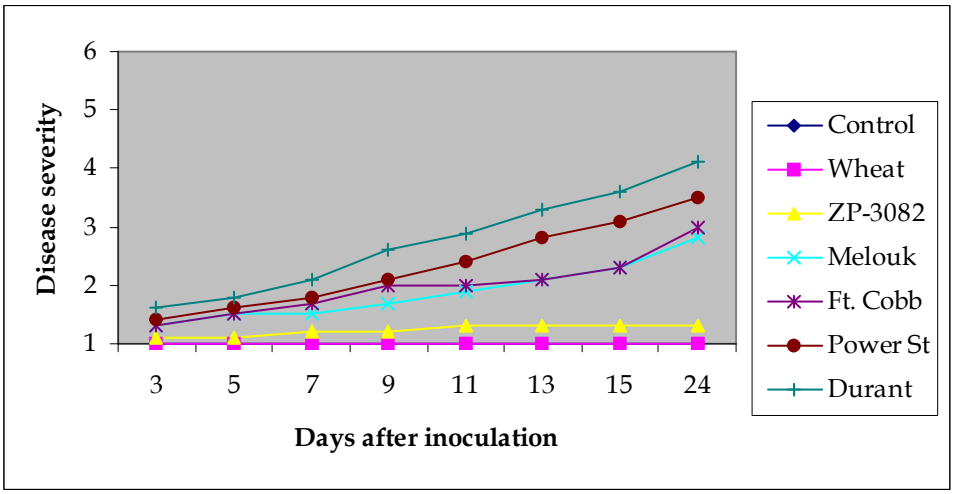
Fig. 1. (a) Southern blight on wheat (b) Sclerotium wilt on peanut.

Fig. 2. Disease severity of *S. rolfsii* isolates on peanut cultivars.



Disease severity rating on a scale of 1-6 (1= No lesion on any of the three stems; 2= Lesion on one stem per plant; 3= Lesion on two stems per plant; 4= Lesion on three stems per plant; 5= Wilting or death of at least one stem;6= Wilting or death of plant).

Fig. 3. Lesion development by *S. rolfsii* isolates on peanut cultivars.

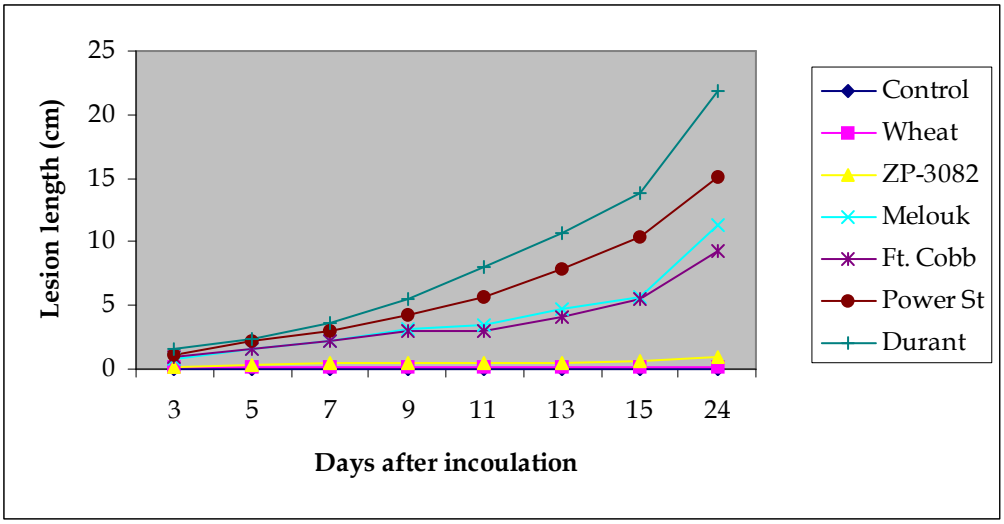
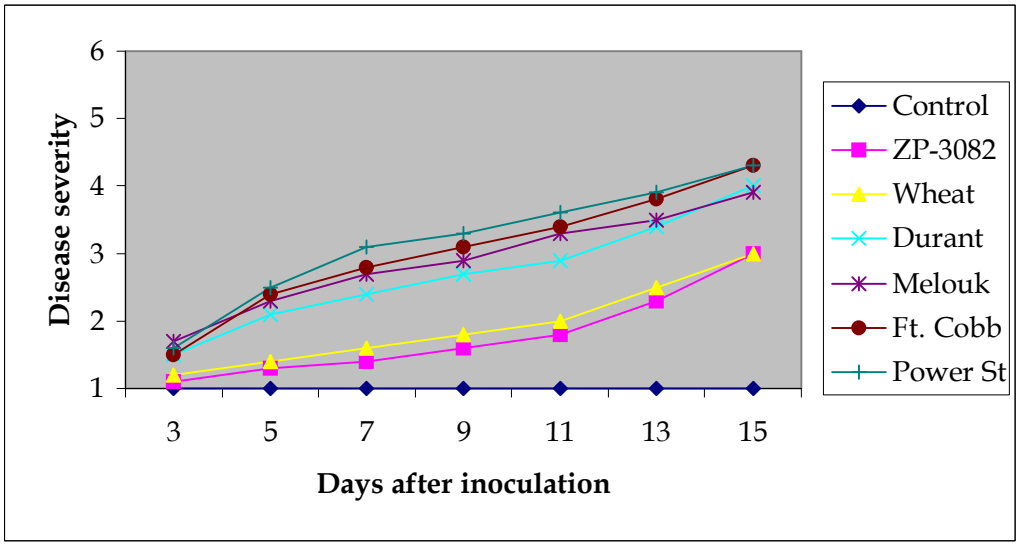


Fig. 4. Disease severity of *S. rolfsii* isolates on winter wheat cultivars.



Disease severity rating on a scale of 1-6 (1= No infection on stem; 2= Initial lesion on the stem; 3= Expanded lesion on the stem; 4= Expanded lesion with outer sheath lodging; 5= Expanded lesion with whole plant lodging;6=Complete death of the plant).

Table 1. Isolates of *Sclerotium rolfsii* used for pathogenicity studies on peanut and winter wheat.

Isolate	Host	Year	Location	Source
Melouk	Peanut	1992	Stillwater, OK	H.A. Melouk
Power St	Peanut	1997	Stillwater, OK	H.A. Melouk
Durant	Peanut	1992	Durant, OK	H.A. Melouk
Ft. Cobb	Peanut	1998	Ft. Cobb, OK	H.A. Melouk
Wheat	Wheat	1998	Stillwater, OK	R.M. Hunger
ZP-3082	Wheat	1993	Nepal	Z.K. Punja

Table 2. Disease severity of *Sclerotium rolfsii* on peanut cultivars as affected by isolate.

Isolate	Disease severity at (X) days after inoculation[a]							
	3	**5**	**7**	**9**	**11**	**13**	**15**	**24**
Non-inoculated	1.0 a[*]	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a
Wheat	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a
ZP-3082	1.1 a	1.1 a	1.2 a	1.2 a	1.3 a	1.3 a	1.3 a	1.3 a
Melouk	1.3 a	1.5 b	1.5 b	1.7 b	1.9 b	2.1 b	2.3 b	2.8 b
Ft. Cobb	1.3 a	1.5 b	1.7 bc	2.0 b	2.0 b	2.1 b	2.3 b	3.0 b
Power St	1.4 ab	1.6 b	1.8 bc	2.1 c	2.4 c	2.8 c	3.1 c	3.5 c
Durant	1.6 b	1.8 b	2.1 c	2.6 d	2.9 d	3.3 d	3.6 d	4.1 d

[a]Disease severity rating on a scale of 1-6 (1= No lesion on any of the three stems; 2= Lesion on one stem per plant; 3= Lesion on two stems per plant; 4= Lesion on three stems per plant; 5= Wilting or death of at least one stem;6= Wilting or death of plant).

[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 3. Disease severity of *Sclerotium rolfsii* on peanut as affected by cultivar.

Cultivar	Disease severity at (X) days after inoculation[a]							
	3	5	7	9	11	13	15	24
Okrun	1.3 a[*]	1.4 a	1.5 a	1.6 a	1.8 ab	2.0 b	2.2 b	2.5 b
Southwest Runner	1.2 a	1.3 a	1.4 a	1.6 a	1.6 a	1.7 a	1.8 a	2.1 a
Tamspan 90	1.2 a	1.4 a	1.5 a	1.7 a	1.9 b	2.1 b	2.2 b	2.6 b

[a]Disease severity rating on a scale of 1-6 (1= No lesion on any of the three stems; 2= Lesion on one stem per plant; 3= Lesion on two stems per plant; 4= Lesion on three stems per plant; 5= Wilting or death of at least one stem;6= Wilting or death of plant).
[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 4. Disease severity of isolates of *Sclerotium rolfsii* on peanut in response to cultivar.

Cultivar[b]	Disease severity[a]						
	Control	Durant	Ft. Cobb	Melouk	Power St	Wheat	ZP-3082
OK	1.0 a[*]	2.9 b	1.8 a	2.1 a	2.5 a	1.1 a	1.2 a
SW	1.0 a	2.3 a	1.7 a	1.8 a	2.2 a	1.0 a	1.1 a
TS	1.0 a	3.1 b	2.5 b	1.7 a	2.3 a	1.0 a	1.3 a

[a]Disease severity rating on a scale of 1-6 (1= No lesion on any of the three stems; 2= Lesion on one stem per plant; 3= Lesion on two stems per plant; 4= Lesion on three stems per plant; 5= Wilting or death of at least one stem;6= Wilting or death of plant).
[b]OK=Okrun; SW=Southwest Runner; TS=Tamspan 90.
[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 5. Length of lesions caused by *Sclerotium rolfsii* on peanut cultivars as affected by isolate.

Isolate	Length of lesion in cm at (X) days after inoculation							
	3	**5**	**7**	**9**	**11**	**13**	**15**	**24**
Non-inoculated	0.0 a*	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Wheat	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a
ZP-3082	0.2 a	0.3 a	0.4 a	0.5 a	0.5 a	0.5 a	0.6 a	1.0 a
Melouk	0.8 a	1.5 a	2.2 ab	3.1 b	3.5 b	4.7 b	5.7 b	11.3 b
Ft. Cobb	0.9 a	1.6 a	2.2 ab	3.0 b	3.0 b	4.1 b	5.5 b	9.3 b
Power St	1.1 a	2.2 a	3.0 b	4.2 b	5.7 bc	7.9 c	10.3 c	15.1 c
Durant	1.6 a	2.4 ab	3.6 b	5.5 bc	8.0 c	10.7 d	13.8 d	21.9 d

*Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 6. Length of lesion caused by *Sclerotium rolfsii* on peanut as affected by cultivar.

Cultivar	Length of lesion in cm at (X) days after inoculation[a]							
	3	5	7	9	11	13	15	24
Okrun	0.6 a*	1.0 a	1.3 a	1.9 ab	2.1 a	3.1 a	3.9 a	7.6 b
Southwest Runner	0.6 a	1.0 a	1.4 a	1.7 a	2.2 a	2.7 a	3.3 a	5.2 a
Tamspan 90	0.9 a	1.5 a	2.3 a	3.3 b	4.6 b	6.2 b	8.1 b	12.2 c

*Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 7. Lesion lengths[a] of isolates of *Sclerotium rolfsii* on peanut in response to cultivar.

Cultivar[b]	Isolate						
	Control	Durant	Ft. Cobb	Melouk	Power St	Wheat	ZP-3082
OK	0.0 a*	7.1 a	2.1 a	3.9 a	5.1 a	0.1 a	0.4 a
SW	0.0 a	4.4 a	2.4 a	3.0 a	5.9 a	0.0 a	0.2 a
TS	0.0 a	13.8 b	6.6 b	5.4 a	7.5 a	0.1 a	1.0 a

[a]Length of lesion measured in cm.
[b]OK=Okrun; SW=Southwest runner; TS=Tamspan 90.
*Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 8. Relative water content[a] in peanut cultivars as affected by inoculation of different isolates of *Sclerotium rolfsii*.

Cultivar[b]	Isolate						
	Control	Durant	Ft. Cobb	Melouk	Power St	Wheat	ZP-3082
OK	95.9 a[*]	78.6 b	95.2 b	84.8 a	81.0 a	93.2 a	92.0 a
SW	93.1 a	80.5 b	90.6 ab	91.1 a	82.9 a	94.4 a	92.6 a
TS	91.8 a	67.5 a	85.2 a	90.5 a	81.2 a	93.8 a	91.9 a

[a]Relative water content in % = [W-DW] / [TW-DW] x 100 Where, W =sample fresh weight, TW= sample turgid weight, DW=sample dry weight.
[b]OK=Okrun; SW=Southwest runner; TS=Tamspan 90.
[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 9. Disease severity in winter wheat as affected by *Sclerotium rolfsii* isolates.

Isolate	Disease severity at (X) days after inoculation[a]						
	3	5	7	9	11	13	15
Control[b]	1.0 a[*]	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a
ZP-3082	1.1 a	1.3 a	1.4 b	1.6 b	1.8 b	2.3 b	3.0 b
Wheat	1.2 a	1.4 a	1.6 b	1.8 b	2.0 b	2.5 b	3.0 b
Durant	1.5 ab	2.1 b	2.4 c	2.7 c	2.9 c	3.4 c	4.0 c
Melouk	1.7 b	2.3 b	2.7 c	2.9 c	3.3 c	3.5 c	3.9 c
Ft. Cobb	1.5 ab	2.4 b	2.8 c	3.1 c	3.4 cd	3.8 c	4.3 c
Power St	1.6 ab	2.5 b	3.1 cd	3.3 cd	3.6 cd	3.9 cd	4.3 c

[a]Disease severity rating on a scale of 1-6 (1= No infection on stem; 2= Initial lesion on the stem; 3= Expanded lesion on the stem; 4= Expanded lesion with outer sheath lodging; 5= Expanded lesion with whole plant lodging; 6= Complete death of the plant).
[b]Non-inoculated.
[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 10. Disease severity in winter wheat caused by isolates of *Sclerotium rolfsii.*

Cultivar	Disease severity at (X) days after inoculation[a]						
	3	5	7	9	11	13	15
Jagger	1.3 a[*]	1.8 ab	2.1 a	2.3 ab	2.4 a	2.7 a	3.2 a
2137	1.5 a	2.1 b	2.4 b	2.5 b	2.7 a	3.0 a	3.4 ab
2174	1.3 a	1.7 a	2.0 a	2.2 a	2.6 a	3.0 a	3.5 b

[a]Disease severity rating on a scale of 1-6 (1= No infection on stem; 2= Initial lesion on the stem; 3= Expanded lesion on the stem; 4= Expanded lesion with outer sheath lodging; 5= Expanded lesion with whole plant lodging; 6= Complete death of the plant).
[*]Means within a column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 11. Disease severity in winter wheat in response to cultivar caused by isolates of *Sclerotium rolfsii.*

Cultivar	Disease severity[a]						
	Control	Durant	Ft. Cobb	Melouk	Power St	Wheat	ZP-3082
Jagger	1.0 a[*]	2.3 a	3.1 a	2.7 a	3.2 a	1.9 a	1.6 a
2137	1.0 a	3.0 b	3.2 a	3.1 a	3.4 a	2.1 a	1.8 a
2174	1.0 a	2.8 ab	2.9 a	2.8 a	3.0 a	1.7 a	2.0 a

[a]Disease severity rating on a scale of 1-6 (1= No infection on stem; 2= Initial lesion on the stem; 3= Expanded lesion on the stem; 4= Expanded lesion with outer sheath lodging; 5= Expanded lesion with whole plant lodging; 6= Complete death of the plant).
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

CHAPTER V

PRODUCTION OF ENDO-POLYGALACTURONASE AND OXALIC ACID BY ISOLATES OF *SCLEROTIUM ROLFSII*

ABSTRACT

Sclerotium rolfsii produces endo-polygalacturonase (endo-PG) and oxalic acid to facilitate infection and degradation of host tissues. Our previous studies on pathogenicity showed that isolates of *S. rolfsii* from peanut are highly virulent on peanut and wheat, whereas isolates from wheat are less virulent on wheat and non-pathogenic on peanut. A study was initiated to determine the activity of endo-PG and oxalic acid by one isolate of *S. rolfsii* from peanut (Melouk) and one isolate from wheat (Wheat) and observe the relationship of this activity with the virulence. This study was conducted as three separate experiments. In the first experiment, production of endo-PG and oxalic acid were studied at different culture ages (3, 5 and 7 days) using sodium polypectate (Napp) as the source of carbon for fungal growth. In the second experiment, different carbon sources were used including glucose, Napp, fresh-, oven dried- and frozen-dried peanut and wheat stems. Activity of endo-PG and oxalic acid were then determined in 7-day old culture filtrates. In the third experiment, peanut (Okrun; 6 week-old) and winter wheat (Jagger; 11 day-old) plants were inoculated with *S. rolfsii* isolates

and activity of endo-PG and oxalic acid were determined in diseased tissues 7 and 14 days after inoculation in wheat and peanut, respectively. Endo-PG activity was determined using viscosity reduction and reducing sugar assays. Oxalic acid production was determined using a commercial oxalate test kit. Results from the first experiment indicated that culture filtrates from the peanut isolate Melouk had significantly higher endo-PG activity than the isolate Wheat. Oxalic acid concentration did not differ significantly between the two isolates. Results from the second experiment indicated that isolates produced negligible amounts of endo-PG in the presence of glucose. As determined by the reducing sugar assay, culture filtrates of the isolate Melouk had significantly higher activity of endo-PG in the presence of Napp, fresh peanut, or oven-dried wheat stems. Oxalic acid concentrations did not differ significantly between the two isolates except in the presence of fresh wheat stems, where the isolate Wheat had a significantly higher oxalic acid concentration than the isolate Melouk. Results from the third experiment indicated significantly higher endo-PG and oxalic acid activity in living tissues infected with the isolate Melouk as compared to the isolate Wheat, which suggested a positive correlation between isolate virulence and production of endo-PG and oxalic acid. However, the *in vitro* studies (experiments 1 and 2) indicate a correlation only with endo-PG and not with oxalic acid. In conclusion, results from these studies suggest that production of endo-PG and oxalic acid are influenced by the nature and type of carbon source. Use of host plant material as a carbon source for *in vitro* studies will support

abundant production of endo-PG and oxalic acid. However, use of host plant material as a carbon source does not necessarily simulate the actual conditions present in living host tissue. Further, our results suggest that *in vitro* studies by themselves are not always a good indicator of the actual nature of the pathogen in terms of production of endo-PG and oxalic acid.

INTRODUCTION

Production of endo-polygalacturonase (endo-PG) and oxalic acid are important for the infection process by *Sclerotium rolfsii* Sacc (7, 9, 11). Both of these compounds are reported to act synergistically and account for a rapid collapse and death of infected tissues (4). *S. rolfsii* is also known to produce other extracellular enzymes such as cutinase, phosphatidase and galactanase but their role in pathogenesis is not conclusively established (1, 13, 14). Previous reports have shown that virulence of *S. rolfsii* isolates is positively correlated with the production of endo-PG and oxalic acid and lowering the oxalic acid levels has been reported to result in less disease (9, 11).

Oxalic acid is proposed to remove calcium ions attached to pectin molecules and expose host cell walls to fungal enzymes such as endo-PG (4). Oxalic acid also reduces the pH of plant tissues close to the optimum for enzymes such as endo-PG (4). Oxalic acid was shown to alter guard cell osmoregulation and interferes with abscisic acid (ABA)-induced stomatal closure (12). Despite such reports, the role of oxalic acid in pathogenesis is not clearly

established and is subject to certain limitations. Virulence of an isolate is not affected until the oxalic acid levels are at the minimum concentration injurious to plant tissue. Hence final concentrations are needed when attempting to correlate differences in virulence with the observed differences in oxalic acid concentrations (9).

Past studies on production of endo-PG and oxalic acid were mostly conducted *in vitro* using different carbon sources that support abundant endo-PG and oxalic acid production (5, 10, 11). Sodium polypectate was normally used as a substrate during endo-PG assays. Sodium polypectate is a polymer of galacturonic acid molecules and endo-PG acts on the polymer by breaking it down into individual galacturonic acid molecules. In one study, bean seedlings inoculated with *S. rolfsii* exhibited a rapid reduction in galacturonic acid content due to the activity of endo-PG (3). Similarly, various carbon sources were defined for a culture medium that would support abundant oxalic acid production without affecting mycelial growth (10). However, studies have not been reported in which host plant material was used as the source of carbon. In this respect, studies were initiated with the following objectives:

1. Determine the activity of endo-PG and oxalic acid concentration in filtrates from different culture age using sodium polypectate as a carbon source.
2. Determine the activity of endo-PG and oxalic acid concentration in filtrates from cultures grown in media with different carbon sources.

3. Determine the activity of endo-PG and oxalic acid concentration in host tissue in response to infection by *S. rolfsii*.

MATERIALS AND METHODS

Origin and maintenance of *Sclerotium rolfsii* isolates

Sclerotium rolfsii isolates (Melouk & Wheat) were used throughout this investigation. Isolate Melouk was collected from a *S. rolfsii* infected peanut in Oklahoma in 1992 (Courtesy: Dr. H. A. Melouk). Isolate Wheat was originally collected from winter wheat seedlings showing symptoms of *Sclerotium* blight in Oklahoma in 1998 (Courtesy: Dr. R.M. Hunger). Pathogenic studies have shown that isolate Melouk was virulent on both peanut and winter wheat cultivars whereas the isolate Wheat was non-pathogenic on peanut and less virulent on wheat (Data presented previously).

Isolates were maintained by placing surface disinfested (10% aqueous NaOCl for 2 min) sclerotia on SPDA [Potato Dextrose Agar amended with streptomycin sulfate (0.14 g/L)]. Mycelial plugs (0.5 cm) from three-day-old cultures were transferred onto fresh SPDA in plates and incubated at 23 ± 2 C for three weeks for production of sclerotia. Sclerotia were then collected from the cultures, air-dried, placed into coin envelopes and stored at 23 ± 2 C in a desiccator containing anhydrous calcium sulfate.

In vitro activity of endo-PG and oxalic acid concentration in cultures of different ages

Culturing procedure and culture-filtrate collection

Isolates of *S. rolfsii* (Melouk and Wheat) were grown on a liquid basal medium containing KNO_3 10 g; KH_2PO_4, 5g; $MgSO_4.7H_2O$, 2.5g; $FeCl_3$, 0.02g that had been supplemented with 0.5% of sodium polypectate (NaPP). Fifty ml of the medium was placed in to 125 ml Erlenmeyer flasks and autoclaved for 20 min at 121 C. After autoclaving, flasks were inoculated with three mycelial plugs (0.5 cm) of 3-day-old fungus and incubated on a rotary shaker (110 rpm) at 25 C. Culture filtrates were collected 3, 5, and 7 days after incubation by filtration through glass microfibre filter paper (Whatman®). One hundred µl of 20% sodium azide was added to the filtrate which was stored at 4 C until used. The combination of two isolates, non-inoculated control, and three culture ages (3, 5 and 7 days) resulted in 9 treatments. Each treatment was replicated three times (i.e., three flasks), and the experiment was conducted twice.

Determination of endo-PG activity

Endo-PG activity of the culture filtrates was determined by both the viscosity reduction assay and the reducing sugar assay using Napp in a 0.05 *M* acetate buffer (pH 5.0) as the substrate. The viscosity reduction assay was conducted as follows: Equal volumes of culture filtrate and Napp solution were mixed for 10 seconds and transferred to a Cannon-Fenske viscometer tube No. 300, which was immersed in a water bath at 30 C. Initial viscosity was measured

one minute after adding culture filtrate to the substrate. Subsequent readings were taken at suitable intervals. Viscosity was expressed as time required in seconds for the solution to flow from one mark point to another in a Cannon-Fenske viscometer tube. Relative activity (RA) of the enzyme was expressed as the reciprocal value of incubation time (t) in minutes required to reduce the initial viscosity of mixture of culture filtrate and the 2% sodium polypectate solution at 30 C by 50%, multiplied by 100, i.e. 1/t x 100 (8).

The reducing sugar assay was based on the hydrolytic release of reducing groups from polygalacturonic acid (6). A reaction mixture (1.8 ml total volume) containing 1.35 ml of 0.1% Napp solution, 0.225 ml of 50 mM Na-acetate buffer (pH 5.0) and 0.225 ml of culture filtrate were incubated at 30 C in a water bath. In order to quantify released reducing sugars with 2-cyanoacetamide, reactions were terminated at four regular intervals (0, 2.5, 5.0 and 7.5 min) by mixing with 1.2 ml of cold 100 mM borate buffer (pH 9.0) containing 0.2% 2-cyanoacetamide (Sigma-Aldrich, St. Louis, USA) and immersion into a boiling water bath for 10 min. Samples were allowed to cool to room temperature and absorbance at 276 nm was determined using a Beckman Model 25 spectrophotometer. Enzyme activity (EA) was measured in units (i.e. μmole/min) per ml of culture filtrate and calculated as follows:

Units per ml of culture filtrate = (ΔA_{276}/min) • (6.29) • (1/ml of enzyme in 1.8 ml of reaction mixture).

Determination of oxalic acid concentration in culture filtrates

Oxalic acid concentration in culture filtrates was determined using the oxalate test kit (Trinity Biotech USA, St. Louis, MO). The enzymatic reactions involved in the assay procedure are as follows:

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate oxidase}} 2CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{Peroxidase}} \text{Indamine Dye} + H_2O.$$

Oxalate is oxidized to carbon dioxide and hydrogen peroxide by oxalate oxidase. The hydrogen peroxide reacts with 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) in the presence of peroxidase to yield an indamine dye which has an absorbance maximum at 590 nm. The absorbance of the color produced is linear with the concentration of oxalate in the sample. Thus, the oxalate concentration was calculated as:

Oxalate concentration (mM/L) = $((A_{sample} - A_{blank}) / (A_{standard} - A_{sample}))$

where,

A_{sample} = Absorbance of sample at 590 nm

A_{blank} = Absorbance of blank at 590 nm

$A_{standard}$ = Absorbance of standard at 590 nm

Data analysis

The effect of isolate and culture age on endo-PG activity and oxalic acid concentration were performed using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC). The response variables considered were the relative

activity (RA) of an enzyme obtained from the viscosity reduction assay, the enzyme activity (EA) obtained from the reducing sugar assay and the oxalic acid concentration obtained from the oxalic acid assay. Isolate and culture age were considered as independent variables.

In vitro activity of endo-PG and oxalic acid concentration in culture filtrates collected from media containing various carbon sources

Culturing procedure and culture-filtrate collection

Isolates of *S. rolfsii* (Melouk and Wheat) were grown in a liquid basal medium containing KNO_3 10 g; KH_2PO_4, 5g; $MgSO_4.7H_2O$, 2.5g; $FeCl_3$, 0.02g and supplemented with one of the following carbon sources listed in Table 1. Fifty ml of the medium was placed in each 125 ml Erlenmeyer flask and autoclaved for 20 min at 121 C. After autoclaving, medium in flasks were inoculated with three mycelial plugs (0.5 cm) excised from a 3-day-old fungus culture. These were incubated on a rotary shaker (110 rpm) at 25 C. Culture filtrates were collected 7 days after inoculation by filtration through glass microfibre filter paper (Whatman®, New Jersey, USA). One hundred µl of 20% sodium azide were added to the filtrate, which was stored at 4 C until used. Endo-PG activity and oxalic acid concentration were measured in the sample as previously described.

The combination of two isolates and nine carbon sources (including a no carbon source control) resulted in 18 treatments. Each treatment had three replicated flasks, and the experiment was conducted twice.

Data analysis

The effect of isolate and carbon source on endo-PG activity and oxalic acid concentration was determined using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC). The response variables considered were the relative activity of the enzyme obtained from the viscosity reduction assay, the enzyme activity obtained from the reducing sugar assay and the oxalic acid concentration obtained from the oxalic acid assay. Isolate and carbon source were used as independent variables.

Activity of endo-PG and oxalic acid concentration in host tissue subjected to inoculation with *S. rolfsii*

Peanut study

Peanut seeds of cultivar Okrun were germinated in plastic containers lined with moist Whatman # 1 filter papers in an incubator at 30 C for 48 h. Three germinated seeds with uniform radical growth were planted in 2:1:1 (v/v) mixture of sand, peat moss and topsoil contained in 15 cm x 30 cm pots. Six weeks after planting, seedlings were inoculated using a mycelial disk technique. In this technique, a 0.5-cm mycelial disk was removed from a 3-day-old active *Sclerotium* culture (Isolate Melouk or Wheat) and appressed to the base of the central stem. A 1-cm diameter filter paper was placed underneath each mycelial disk to prevent contact with the soil. After inoculation, pots were wrapped with clear polyethylene bags to maintain high humidity near the soil surface and plant

crowns. Pots were watered as required. Non-inoculated plants were used as the control.

Fourteen days after inoculation, a 2 cm length of main stem measured from the base was cut from each inoculated plant and macerated in 10 ml of deionized water using a Tekman Tissuemizer (1 min at 24,000 RPM). Samples were then centrifuged at 10,000 g for 10 min and the supernatant was collected. One hundred µl of 20% sodium azide were added to the supernatant and stored at 4 C. Endo-PG activity and oxalic acid concentration were measured in the sample as previously described.

The experiment tested the production of endo-PG activity and oxalic acid concentration *in vivo* for the two *Sclerotium* isolates. Hence, the combination of two isolates and the non-inoculated control resulted in three treatments. The treatments were arranged in a randomized complete block design with three replications, and the experiment was conducted twice.

Wheat study

Twelve certified seeds of the winter wheat cultivar Jagger were planted 2 cm deep in a mixture of sand, soil and shredded peat moss (2:1:1; v/v/v) contained in 12 cm dia. plastic pots. Four days after planting, seedlings were thinned to eight seedlings per pot. The seedlings were inoculated with the fungus at growth stage 1 (two leaf stage) on the Feekes' scale. A mycelial disk technique was used for inoculation as described previously for peanuts. After inoculation, pots were wrapped with clear polyethylene bags to maintain high

humidity near the soil surface and plant crowns, and plants were watered as needed.

Seven days after inoculation, a 2 cm length of the main stem measured from the base was cut from each inoculated plant and macerated in 10 ml of deionized water using a Tekman Tissuemizer (1 min at 24,000 RPM). Samples were then centrifuged at 10,000 g for 10 min and the supernatant was collected. A 100 micro liter of 20% sodium azide was added to the supernatant, which was stored at 4 C. Endo-PG activity and oxalic acid concentration were measured in the sample as previously described for the *in vitro* experiment.

The experiment tested the *in vivo* endo-PG activity and oxalic acid concentration for the two *Sclerotium* isolates. Hence, the combination of two isolates and the non-incoulated control resulted in three treatments. The treatments were arranged in a randomized complete block design with three replications and the experiment was conducted twice.

Data analysis

The effect of isolate on endo-PG activity and oxalic acid concentration was determined using PROC MIXED (SAS Version 9.1, SAS Institute Inc., Cary, NC). The response variables considered were as described previously for the peanut study.

RESULTS

In vitro activity of endo-PG and oxalic acid concentration in cultures of different ages

Endo-PG activity and oxalic acid concentration were significantly ($P \leq 0.05$) affected by isolate and age of culture (Tables 2, 3 and 4). Endo-PG activity found in 3-, 5-, and 7-day-old cultures of *S. rolfsii* and as determined by viscosity reduction assay and reducing sugar assay are shown in Tables 2 and 3. Highest enzyme activity was detected in filtrates from cultures 7 days old. Both assays revealed a significantly higher ($P \leq 0.05$) endo-PG activity in filtrates of the *S. rolfsii* isolate Melouk from peanut as compared to the isolate Wheat which in turn, showed a greater activity than the non-inoculated control (Tables 2 and 3).

Concentration of oxalic acid was highest in filtrates from 5- and 7- day-old cultures (Table 4). When compared across 3-day-old cultures, oxalic acid concentration was significantly higher in filtrates of the isolate Melouk compared to the isolate Wheat which, in turn, was significantly higher than the control ($P \leq 0.05$). However, oxalic acid concentration increased dramatically by day 5 in filtrates of isolate Wheat and was not significantly different from isolate Melouk ($P > 0.05$). Data also show that oxalic acid concentration stopped increasing from 5- to 7-day-old cultures.

***In vitro* activity of endo-PG and oxalic acid concentration in culture filtrates collected from media containing various carbon sources**

Endo-PG activity and oxalic acid concentration were significantly ($P \leq 0.05$) affected by the type of carbon source, but were not affected by isolate (Tables 5, 6 and 7). When comparing between the two isolates (Melouk and Wheat), endo-PG activity as determined by a viscosity reduction assay was not significantly different ($P > 0.05$) between the two isolates except when sodium polypectate was used as a carbon source (Table 5). For sodium polypectate, the activity of endo-PG in culture filtrates of the isolate Melouk was significantly higher than for the isolate Wheat (Table 5). When compared across different carbon sources, culture filtrates with sodium polypectate as a carbon source tend to have higher activity of endo-PG (Table 5). This particular trend was observed with isolate Melouk but not with isolate Wheat (Table 5).

In the reducing sugar assay, endo-PG activity was not significantly different ($P \leq 0.05$) between the two isolates except when sodium polypectate, fresh peanut stems, or oven dried wheat stems were used as a carbon source (Table 6). In these cases, the culture filtrates of isolate Melouk had a significantly higher endo-PG activity compared to the isolate Wheat (Table 6). When compared across different carbon sources, culture filtrates of isolate Melouk or isolate Wheat suspended with either plant material (fresh, oven-dried and frozen dried peanut and wheat stems) or sodium polypectate had a significantly ($P \leq 0.05$) higher endo-PG activity compared to the control and glucose (Table 6).

In particular, culture filtrates of isolate Melouk suspended with sodium polypectate, fresh peanut stems, fresh wheat pseudo stems or frozen dried wheat stems had significantly ($P \leq 0.05$) higher endo-PG compared to the other carbon sources. Similarly, culture filtrates of isolate Wheat suspended with fresh wheat pseudo stems had a significantly ($P \leq 0.05$) higher endo-PG activity compared to the other carbon sources (Table 6).

No significant difference ($P > 0.05$) in oxalic acid concentrations was observed between the two isolates except when fresh wheat pseudo stems were used as carbon source, where the culture filtrate of the isolate Wheat had a significantly ($P \leq 0.05$) higher oxalic acid concentration compared to isolate Melouk (Table 7). Isolates grown on media containing different carbon sources had a significantly ($P \leq 0.05$) higher amounts of oxalic acid compared to control (Table 7). However, oxalic acid concentrations did not vary significantly among different carbon sources except with culture filtrates of isolate Melouk suspended with fresh wheat pseudostems, where a significantly ($P \leq 0.05$) lower oxalic acid concentrations compared to the other carbon sources was observed (Table 7).

Activity of endo-PG and oxalic acid concentration in host tissue subjected to inoculation with *S. rolfsii*

Peanut study

Apparent endo-PG activity in peanut stems as determined by a viscosity reduction assay and a reducing sugar assay was not significantly ($P > 0.05$) affected by isolate (Table 8). However, concentration of oxalic acid was significantly ($P \leq 0.05$) affected by the isolate Melouk, where a significantly higher concentration of oxalic acid was observed as compared to the isolate Wheat and the non-inoculated control (Table 8).

Wheat study

Activity of endo-PG in wheat seedlings as determined by the viscosity reduction assay and the reducing sugar assay was significantly ($P \leq 0.05$) affected by isolate (Table 9). Endo-PG activity as determined by viscosity reduction assay was significantly ($P \leq 0.05$) higher in stems of the winter wheat variety Jagger inoculated with the isolate Melouk as compared to the isolate Wheat which in turn was significantly ($P \leq 0.05$) higher than the control (Table 9). As determined by reducing sugar assay, endo-PG activity was significantly ($P \leq 0.05$) higher in stems of the winter wheat variety Jagger inoculated with the isolate Melouk as compared to the isolate Wheat and the non-inoculated control and isolate Wheat did not differ significantly ($P > 0.05$) from the non-inoculated control (Table 9).

The concentration of oxalic acid also was significantly ($P \leq 0.05$) affected by isolate (Table 9). Wheat seedlings inoculated with the isolate Melouk had a significantly ($P \leq 0.05$) higher concentration of oxalic acid compared to the isolate Wheat and the non-inoculated control (Table 9).

DISCUSSION

Production of endo-polygalacturonase (endo-PG) and oxalic acid by *Sclerotium rolfsii* was reported to play a vital role in the pathogenesis of the fungus (7, 9, 11) and our studies did observe the production of these constituents during the infection process. Presence of endo-PG and oxalic acid in young cultures (3 days old) where mycelial growth was limited indicates that secretion of these components does not necessarily require high mycelial growth.

Results from the first experiment have shown that the isolate Melouk from peanut produced significantly higher levels of endo-PG as compared to the isolate Wheat from wheat. However, oxalic acid levels did not differ between the two isolates and we did not observe a rapid mycelial growth rate as previously reported for some virulent strains of *S. rolfsii* (11).

Production of endo-PG and oxalic acid is induced in presence of carbon source. However, the activity of these substances varies based on the type of carbon source (10). In our second experiment, we tested the effect of different carbon sources on production of endo-PG and oxalic acid in culture. Production

of endo-PG in the presence of glucose as a carbon source was negligible. However, production of oxalic acid was not affected. These observations suggest that production in the presence of a readily available carbon source such as glucose, enzyme production is not required by the fungus to obtain carbon. Plant material (peanut or wheat) had an effect similar to that of sodium polypectate in the production of endo-PG, as determined by the viscosity reduction assay. However, the reducing sugar assay revealed differences among type of plant materials (fresh, oven dried or freeze dried) as a carbon source. Plant material induced similar amounts of oxalic acid from two isolates as sodium polypectate and no differences were observed among fresh, oven dried or freeze dried material.

In the *in vivo* study, the isolate Melouk produced distinct lesions on peanut and wheat stems whereas isolate Wheat had limited pathogenicity. Water related activity of endo-PG and concentration of oxalic acid were relatively high in plant tissues infected with isolate Melouk whereas plant tissues inoculated with isolate Wheat had these constituents at very low levels. These results indicate that isolate virulence is correlated with the production of endo-PG and oxalic acid. Our results also support previous work by Bateman and Beer (1965), which showed the necessity of simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii* (4).

Water related activity of endo-PG and concentration of oxalic acid measured in the *in vivo* study are of fungal origin since control plants did not

show presence of these constituents, eliminating the possibility of host origin. This is important since higher plant tissues are known to produce oxalic acid. However, it is to be noted that endo-polygalacturonase has not been found as a normal constituent of higher plant tissues (2).

A previous study with *Rhizoctonia solani* demonstrated that endo-PG from diseased tissue converted sodium polypectate to three lower molecular weight polymers, whereas culture produced endo-PG converted this substrate to galacturonic acid (2). These results indicate that enzyme systems differ from culture and disease tissue. We currently do not know if this is true with *S. rolfsii* and further studies in this aspect are needed.

Activity of endo-PG and concentration of oxalic acid produced by two isolates in host tissues are no way close to what was found in culture filtrates grown on synthetic media. Even though isolate virulence appears to be correlated with activity of endo-PG and oxalic acid concentration from the *in vivo* study, it did not appear to be true from the results of the *in vitro* study. Various factors could have been attributed to this difference which may include but not limited to the nature and availability of carbon source. The production of endo-PG and oxalic acid *in vitro* may have been impacted by soluble nature of carbon source in the basal medium.

In conclusion, results from these studies suggested that production of endo-PG and oxalic acid are influenced by the nature and type of carbon source. Use of host plant material as a carbon source for in vitro studies will support

abundant production of endo-PG and oxalic acid. However, use of host plant material as a carbon source does not necessarily simulate the actual conditions present in living host tissue. Further, our results suggest that in vitro studies by themselves are not always a good indicator of actual nature of pathogen in terms of production of endo-PG and oxalic acid.

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Table 1. Carbon sources used to measure *in vitro* endo-PG activity and oxalic acid concentration produced by two isolates of *Sclerotium rolfsii*.

Carbon source	Concentration (% w/v)
Control	0.0
Glucose	0.5
Sodium polypectate	0.5
Fresh peanut stems	2.0
Fresh wheat pseudostems	2.0
Oven dried, ground peanut stems	2.0
Oven dried, ground wheat pseudostems	2.0
Frozen dried, ground peanut stems	2.0
Frozen dried, ground wheat pseudostems	2.0

Table 2. Activity of endo-PG produced *in vitro* by *Sclerotium rolfsii* isolates as determined by a viscosity reduction assay.

Isolate	Endo-PG activity at (X) days of culture age*		
	3	5	7
Control	0.0 a A**	0.0 a A	0.0 a A
Wheat	5.2 a A	21.3 b B	32.2 b C
Melouk	13.8 b A	52.0 c B	52.0 c B

*Activity expressed as reciprocal value of incubation time (t) in minutes required to reduce the initial viscosity of mixture of culture filtrate and 2% sodium polypectate solution at 30 C by 50%, multiplied by 100 i.e. (1/t x 100).

**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 3. Activity of endo-PG produced *in vitro* by *Sclerotium rolfsii* isolates as determined by a reducing sugar assay.

Isolate	Endo-PG activity at (X) days of culture age*		
	3	5	7
Control	0.04 a A**	0.12 a A	0.04 a A
Wheat	0.09 ab A	0.49 b B	1.32 b C
Melouk	0.29 b A	1.09 c B	1.88 c C

*Activity expressed as units per ml of culture filtrate.
**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 4. Concentration of oxalic acid produced *in vitro* by *Sclerotium rolfsii* isolates as determined by an oxalate assay.

Isolate	Oxalate concentration at (X) days of culture age*		
	3	5	7
Control	0.00 a A**	0.03 a A	0.09 a A
Wheat	1.78 b A	3.70 b B	3.70 b B
Melouk	2.90 c A	3.70 b B	3.70 b B

* Concentration expressed as mmol/L.
**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 5. Activity of endo-PG produced *in vitro* by *Sclerotium rolfsii* isolates in media containing different carbon sources.

Carbon source	Endo-PG activity*	
	Isolate Melouk	Isolate Wheat
Control	0.2 a A**	0.0 a A
Glucose	5.1 a A	3.6 a A
Sodium polypectate	142.7 b A	33.8 a B
Fresh peanut stems	30.8 a A	27.2 a A
Fresh wheat pseudostems	23.3 a A	30.9 a A
Oven dried, grounded peanut stems	14.5 a A	17.6 a A
Oven dried, grounded wheat pseudostems	19.8 a A	17.3 a A
Frozen dried, grounded peanut stems	16.7 a A	21.9 a A
Frozen dried, grounded wheat pseudostems	35.1 a A	32.7 a A

*Enzyme activity was determined by viscosity reduction assay, where activity expressed as reciprocal value of incubation time (t) in minutes required to reduce the initial viscosity of mixture of culture filtrate and 2% sodium polypectate solution at 30 C by 50%, multiplied by 100 i.e. 1/t x 100.
**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 6. Activity of endo-PG produced *in vitro* by *Sclerotium rolfsii* isolates in media containing different carbon sources.

Carbon source	Endo-PG activity*	
	Isolate Melouk	Isolate Wheat
Control	0.1 a A**	0.0 a A
Glucose	0.1 a A	0.0 a A
Sodium polypectate	2.3 d A	1.2 b B
Fresh peanut stems	2.2 d A	1.5 d B
Fresh wheat pseudostems	2.5 d A	3.1 f A
Oven dried, grounded peanut stems	1.4 b A	1.5 d A
Oven dried, grounded wheat pseudostems	1.7 c A	1.0 b B
Frozen dried, grounded peanut stems	1.6 c A	1.4 c A
Frozen dried, grounded wheat pseudostems	2.3 d A	2.2 e A

* Enzyme activity was determined by reducing sugar assay, where activity was expressed as units per ml of culture filtrate.
**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 7. Concentration of oxalic acid produced by *Sclerotium rolfsii* isolates in media containing different carbon sources.

Carbon source	Oxalate concentration*	
	Isolate Melouk	Isolate Wheat
Control	0.5 a A**	0.5 a A
Glucose	1.8 c A	1.9 b A
Sodium polypectate	1.9 c A	2.3 b A
Fresh peanut stems	1.9 c A	2.0 b A
Fresh wheat pseudostems	1.3 b A	1.9 b B
Oven dried, grounded peanut stems	1.8 c A	1.8 b A
Oven dried, grounded wheat pseudostems	1.8 c A	1.9 b A
Frozen dried, grounded peanut stems	1.9 c A	2.0 b A
Frozen dried, grounded wheat pseudostems	2.0 c A	2.0 b A

* Concentration expressed as mmol/L.
**Means in the same column followed by the same lower case letter, and means in the same row followed by the same upper case letter are not significantly different according to LSMEANS at P≤0.05.

Table 8. *In vivo* activity of endo-PG and oxalic acid concentration produced by *Sclerotium rolfsii* isolates in the peanut cv "Okrun".

Isolate	Peanut		
	VRA[a]	RSA[b]	OA[c]
Control	0.0 a*	0.00 a	0.03 a
Wheat	1.7 a	0.13 a	0.04 a
Melouk	3.9 a	0.21 a	0.14 b

[a]Activity of endo-PG in units of reciprocal time, as determined by viscosity reduction assay.
[b]Activity of endo-PG in units per ml of culture filtrate, as determined by reducing sugar assay.
[c]Concentration of oxalic acid in mmol/L as determined by oxalate assay.
*Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 9. ***In vivo*** **activity of endo-PG and oxalic acid concentration produced by** ***Sclerotium rolfsii*** **isolates in the winter wheat cv "Jagger".**

Isolate	Winter wheat		
	VRA[a]	RSA[b]	OA[c]
Control	0.0 a[*]	0.09 a	0.01 a
Wheat	3.9 b	0.15 a	0.04 a
Melouk	5.0 c	0.44 b	0.10 b

[a]Activity of endo-PG in units of reciprocal time, as determined by viscosity reduction assay.
[b]Activity of endo-PG in units per ml of culture filtrate, as determined by reducing sugar assay.
[c]Concentration of oxalic acid in mmol/L as determined by oxalate assay.
[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

CHAPTER VI

GENETIC VARIABILITY OF *RHIZOCTONIA* SPP. ISOLATED FROM PEANUT AND WINTER WHEAT

ABSTRACT

Rhizoctonia spp. is a soilborne fungus responsible for important diseases in peanut and wheat. *Rhizoctonia* isolates that appear to be closely related may differ genetically but such differences are difficult to identify using conventional techniques. In such cases, molecular techniques such as the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique are helpful in identifying the genetic variability among closely related isolates. In this study, RAPD-PCR was used to identify genetic variability among isolates of *Rhizoctonia solani* (RSP, JY-1, G-24 and RS-00-07) and *Rhizoctonia cerealis* (Altus, Lahoma, Marshall and Fellers) obtained from peanut and wheat, respectively. DNA polymorphisms were detected among the isolates using 15 oligonucleotide primers. Fragment profiles were analyzed using the RAPDistance program version 1.04 and a dendrogram was generated using the Neighbor-joining method. Isolates were clearly separable into two distinct groups based on the species. Among the isolates of *R. solani*, JY-1, G-24 and RS-00-07 appeared to be more closely related to each other, than to RSP. This differentiation supports the

variation in virulence of the isolates, since RSP was least virulent compared to the other three isolates. Geographical origin may also have impacted isolate placement within the dendrogram since isolates JY-1 and RS-00-07, which were obtained from Georgia, were clustered more closely than RSP, that was obtained from Oklahoma. No major genetic divergence was observed among *R. cerealis* isolates. However, Fellers, Lahoma and Marsall were clustered relatively closely than Altus. The results indicate that RAPD-PCR is a valuable technique for differentiating isolates of *Rhizoctonia* spp.

INTRODUCTION

Rhizoctonia spp. is an important soilborne pathogen associated with diseases of many crops including peanut and wheat (2, 23). In peanut, *Rhizoctonia solani* causes seedling disease on young plants, pods and pegs and limb rot on mature plants (2). In wheat, *R. solani* is associated with damping off and root rot (5, 6, 13). Additionally, a binucleate species of *Rhizoctonia* called *Rhizoctonia cerealis* (teleomorph: *Ceratobasidium gramineum*), causes sharp eye spot in wheat and other cereals (3, 10). This pathogen, however, was not reported to cause any damage on peanut.

Isolates of *R. solani* and *R. cerealis* are classified based on anastomosis groups (AGs) (17). Isolates within a particular anastomosis group (AG) are reported to be closely related in terms of pathogenicity and hosts they infect. However, evidence from some studies suggest that variation in pathogenicity exists among strains belonging to the same AG (16, 21). In such cases, a further attempt to classify isolates into subgroups is a difficult task using conventional techniques.

Molecular tools such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) are used to detect variation among closely related strains of fungi and in population analysis (12, 19, 22, 24). The technique was used previously to determine variation among several AGs of *Rhizoctonia solani*

(5, 18). This technique has also been used successfully in place of conventional diagnosis to detect the presence of fungi in plants (15, 20).

The objective of this study was to determine the genetic relatedness among the isolates of *R. solani* and *R. cerealis* obtained from peanut and wheat respectively, using the RAPD-PCR technique.

MATERIALS AND METHODS

Collection and maintenance of *Rhizoctonia* isolates

Isolates of *Rhizoctonia* were obtained from different sources during 2004 (Table 1). Peanut isolates, which were obtained from cooperators were maintained on SPDA [Potato Dextrose Agar amended with streptomycin sulfate (0.14 g/L)]. Isolates from wheat were obtained from wheat collected from northern and southwestern Okalahoma that exhibited characteristic sharp eye spot symptoms. Small sections of the infected tissue having the sharp eye lesion were cut and surface sterilized for 3 minutes with 2% sodium hypochlorite, rinsed in sterile distilled water, plated on 2% water agar and incubated at room temperature (~24° C). After 48 h, fungal colonies with mycelia characteristics of *Rhizoctonia* spp. were transferred and maintained on SPDA. The isolates were reinoculated to wheat to fulfill Koch's postulates.

DNA extraction

Isolates were grown in potato dextrose broth for seven days on a rotary shaker at 24°C and the mycelial mat harvested by vacuum filtration and blotted dry. Dried mycelial mats were stored at -80°C until used. A *Sclerotium rolfsii* isolate (Melouk) was included as an out group.

Genomic DNA was extracted according to the protocol of Lee & Taylor (9). Fifty mg of dry mycelia was finely ground in a mortar and pestle, suspended in 400 µL of lysis buffer (50 mM Tris-HCl at 7.2 pH, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol) and incubated at 65°C for 1 hr. The supernatant obtained after centrifuging at 10,000 g for 15 min at 25°C, was extracted with an equal volume of chloroform : TE- saturated phenol (1:1, v:v) and precipitated with 0.54 volumes of isopropanol. Samples were then centrifuged at 10,000 g for 2 min at 25°C and the genomic DNA pellet was washed with 70% ethanol and air dried for 30 min at 25°C. The dried DNA pellet was resuspended in 100 µL of sterile Tris-EDTA (TE) buffer and stored at -20°C until used. DNA was quantified using a spectrophotometer (Beckman DU-7000).

RAPD fingerprinting

A 50 µL volume of PCR reaction mixture was prepared for each sample containing 36.5 µL distilled water, 5 µL 10X PCR buffer (Qiagen Inc, Valencia, CA), 4 µL of dNTPs (Promega, Madison, WI) 1 µL of primer (50 picomoles/µL), 0.5 µL Taq polymerase (5U) (Qiagen Inc, Valencia, CA), 2 µL $MgCl_2$ and 1 µL of template DNA. Template DNA was amplified using 15 different primers (Table

2). PCR reactions were carried out in a thermal cycler (PTC-100, MJ Research, Watertown, MA) programmed to 40 cycles of 94°C for 1 min (2 min at first cycle), 35°C for 1 min and 72°C for 3 min (10 min at final cycle).

The amplified products were loaded into 1.0% agarose/Tris-Acetate EDTA (TAE) buffer gel followed by electrophoresis (75 V for 3 hrs) and staining with ethidium bromide. Each reaction was performed in triplicate to verify the reproducibility of banding patterns.

Band scoring and analysis

Bands obtained for each primer were visualized using BioRad gel documentation system (Bio-Rad laboratories, Hercules, CA). Amplified bands were scored as 1 when the band was present and 0 when the band was absent. The data were analyzed using the RAPDistance program version 1.04 (1). A matrix of genetic distance values was calculated and a dendrogram was generated using the Neighbor-joining method (NJTREE, version 2.0 and TDRAW version 1.14).

RESULTS AND DISCUSSION

All isolates used in this study were initially characterized using conventional methods such as determination of the nuclear condition and anastamosis grouping. Isolates from peanut were found to be multinucleate whereas the isolates from winter wheat were binucleate. Hence isolates from peanut were categorized as *Rhizoctonia solani* and those from winter wheat as

Rhizoctonia cerealis. When isolates were anastamosed with tester isolates, all peanut isolates anastamosed with the AG-4 tester isolate whereas the isolates from winter wheat all anastamosed with the CAG-1 tester isolate. Hence, a clear distinction was identified between the isolates of peanut and winter wheat in terms of nuclear condition and anastomosis grouping. However, it is difficult to identify variation within isolates that belong to the same anastomosis group using conventional methods. So, RAPD-PCR was employed to identify variations among closely related isolates.

PCR reactions with the fifteen different primers gave amplification products that generated consistently reproducible polymorphisms. Using the band data for all isolates from 15 primers (Table 1), pair-wise distances between the samples were calculated using 15 different algorithms in the form of a triangular matrices. This triangular matrix was used to show the degree of relatedness between the isolates in the form of a dendrogram (Fig. 1). Dendrograms generated by 15 different algorithms were similar and the one generated by algorithm 5 (14) was chosen for further analysis.

Some bands were common among isolates RSP, JY-1, G-24 and RS-00-07 that were recovered from peanut, as can be seen in the pattern generated by primer 324 and 327 (Fig. 2 and 3). However, there also were bands that were unique to a particular isolate (Fig. 2 and 3; depicted with yellow circles). There were fewer commonalities in band pattern between the isolates of Altus, Lahoma, Marshall and Fellers from winter wheat.

Results from the RAPD-PCR test did not suggest a major genetic divergence within the isolates obtained from the same host and that belonged to the same AG. However, as seen from the dendrogram (Fig. 1), isolates were clearly demarcated into two groups based on the species. Group 1 (G-1) consisted of *R. solani* isolates RSP, JY-1, RS-00-07 and G-24 that were recovered from peanut from different geographical locations in Oklahoma, Georgia and Texas. Within this group, isolates JY-1, RS-00-07 and G-24 clustered more closely than isolate RSP (Fig.1). Group 2 (G-2) consisted of *R. cerealis* isolates Altus, Lahoma, Marshall and Fellers that were recovered from winter wheat in Oklahoma. Isolate Melouk belonging to *Sclerotium rolfsii* which was used as an out group, separated clearly from other groups as expected. It is also to be noted that even though *S. rolfsii* separated out clearly, group 2 (G-2) representing *R. cerealis* isolates appear to be relatively close to the out group than *R. solani* isolates. This may suggest the proximity of *R. cerealis* to *S. rolfsii* in evolutionary stand point.

Among the *R. solani* isolates from peanut, isolate RSP branched out differently from rest of the peanut isolates. This could indicate differences in virulence, effect of geographical location and other factors. Previous investigations have confirmed the significant inter-isolate variability within subgroups of *R. solani* and variations seem to be correlated with geographical origin and virulence (5, 11, 18, 21). Genetic relatedness of isolates of *R. solani* is influenced greatly by the geographic origin (7, 18). Isolates of *R. solani* belonging

to AG1-IA that was recovered from the same area but from different hosts were clustered more closely than isolates from a different location (18). However, in our study, the relationship between geographical location, i.e. where isolates were collected and grouping is not clear. For example, isolates collected from the same location in Georgia (JY-1 and RS-00-07) were clustered closely with isolate obtained from Texas (G-24). These results indicate a high level of genetic relatedness which could be explained by cross introduction into the two locations. Among *R. cerealis* isolates collected from winter wheat in Oklahoma, Marshall, Fellers and Lahoma were clustered more closely than Altus. It is to be noted that Marshall, Fellers and Lahoma were collected from Northern Oklahoma whereas Altus was collected from the Southwestern Oklahoma, which explains partly, the possible relatedness of the three isolates (Marshall, Fellers and Lahoma) as a result of geographical proximity.

Variation in terms of virulence is possible either due to the heterokaryotic nature of the fungus or by the teleomorph (4, 8). RAPD analyses of *R. solani* isolates belonging to AG1-IA have shown that highly virulent isolates were clustered more closely as one group and least virulent as another group (18). Our preliminary testing of virulence of peanut isolates on peanut cultivar Okrun has indicated that RSP was least virulent whereas G-24, JY-1 and RS-00-07 were highly virulent (Table 3). Results from the dendrogram are also in line with the variation observed in virulence test. The highly virulent isolates G-24, JY-1 and RS-00-07 were clustered together whereas RSP has branched out separately

(Fig.1). Virulence of *R. cerealis* isolates was very less and the variation was not significant (Table 4). Even though non-significant, Altus was relatively less virulent compared to other *R. cerealis* isolates. The branching pattern among *R. cerealis* isolates as seen from the dendrogram is partially in line with the slight variation that was observed in the virulence test (Fig.1). Altus which was slightly less virulent has branched out separately whereas the other three isolates (Fellers, Marshall and Lahoma) were clustered more closely (Fig.1).

Our study may have found more variation among isolates of *R. solani* and *R. cerealis* if additional isolates obtained from outside the U.S.A or from different hosts would have been included. Previous studies have demonstrated such differences among fungal isolates obtained from different geographical locations (18, 19). Even though, occurrence of *R. cerealis* on peanut is not known, presence of *R. solani* on wheat roots is well known and results would have been more interesting if we would have obtained *R. solani* isolates from wheat root rots. However, no *R. solani* isolates from wheat were ever found in the sampled fields during this study.

In summary, this study demonstrated that RAPD-PCR is a reliable tool for detecting genetic variation within isolates of *Rhizoctonia* spp. and is sensitive and useful for detecting genetic differences among closely related strains.

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Figure 1. Dendrogram generated using fifteen primers in RAPD analysis of 8 *Rhizoctonia* isolates. Isolates of group G-1 belong to *Rhizoctonia solani* and were obtained from peanut. Isolates of group G-2 belong to *Rhizoctonia cerealis* and were obtained from winter wheat.

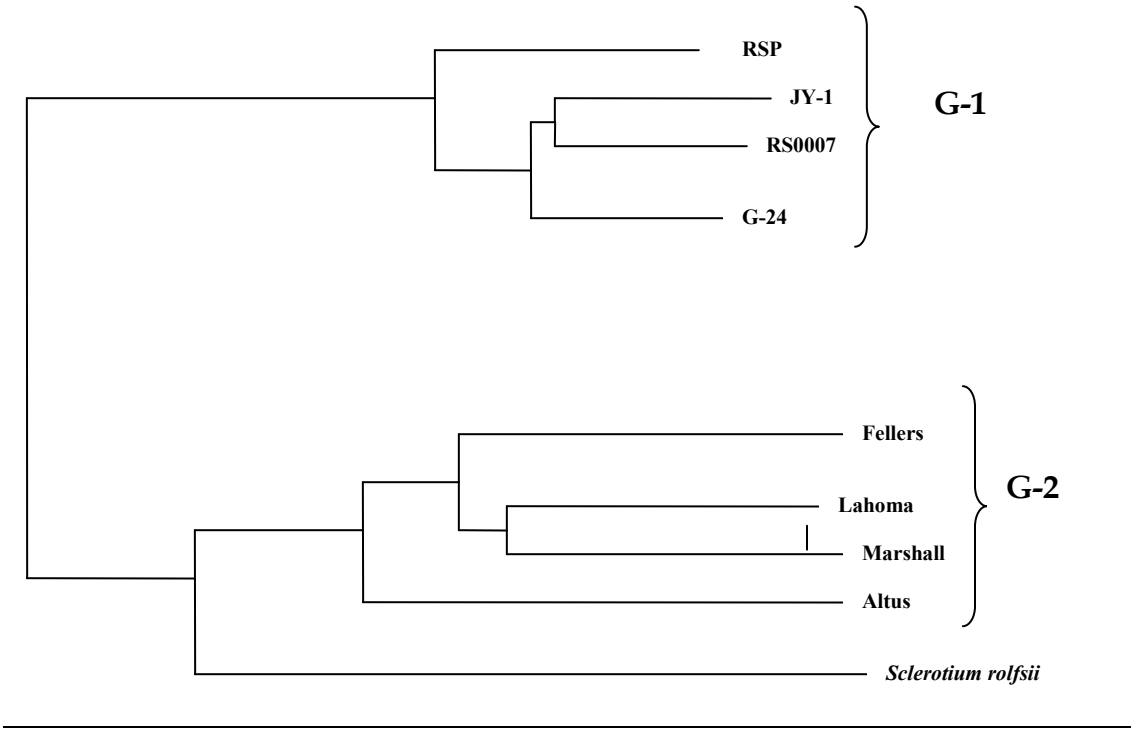

Figure 2. Random amplified polymorphic DNA banding pattern of *Rhizoctonia* isolates collected from peanut and winter wheat generated with primer 324. Lanes, M, marker (100 bp ladder); 1-8 (Isolates RSP, JY-1, G-24, RS-0007, Altus, Lahoma, Marshall and Fellers respectively); OG (out group; *Sclerotium rolfsii*); C (Control; no template). Yellow circles indicate bands that are unique to a particular isolate within the same species.

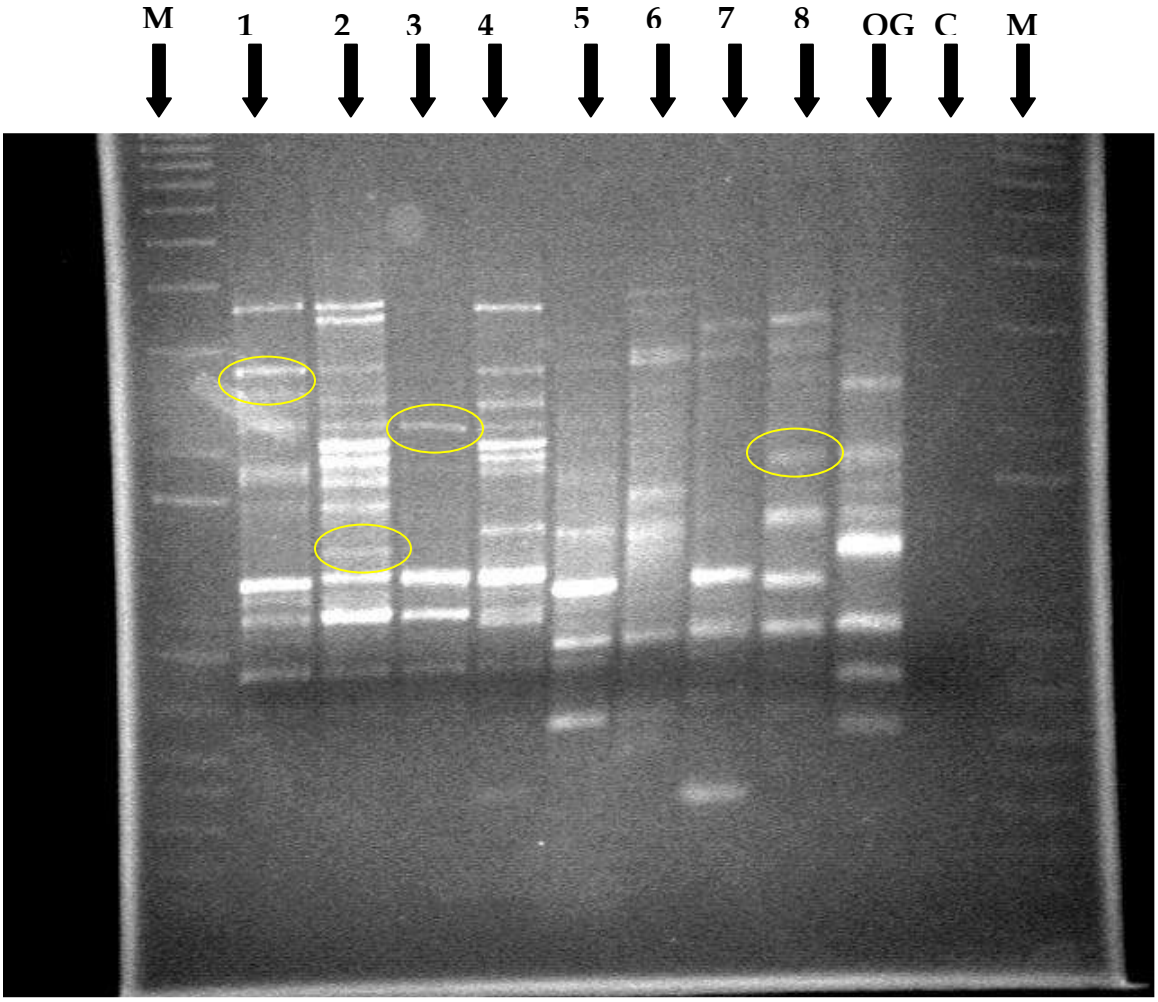

Figure 3. Random amplified polymorphic DNA banding pattern of *Rhizoctonia* isolates collected from peanut and winter wheat generated with primer 327. Lanes, M, marker (100 bp ladder); 1-8 (Isolates RSP, JY-1, G-24, RS-0007, Altus, Lahoma, Marshall and Fellers respectively); OG (out group; *Sclerotium rolfsii*); C (Control; no template). Yellow circles indicate bands that are unique to a particular isolate within the same species.

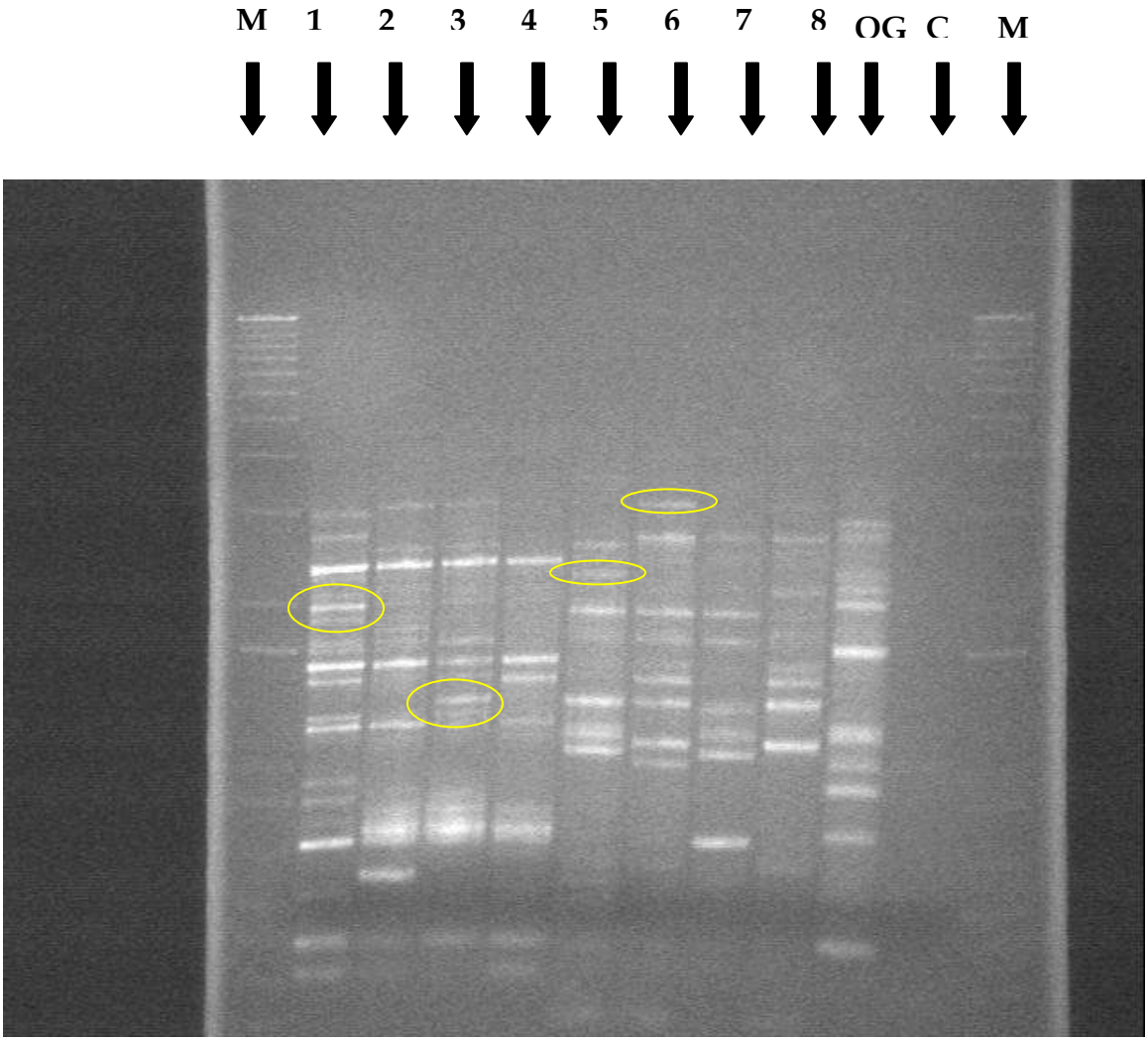

Table 1. Isolates of *Rhizoctonia* used to determine the genetic variability

Isolate	Species	Host	Year	Geographic Origin	Source
G-24	*R. solani*	Peanut	Unknown	Texas	T.A. Wheeler
JY-1	*R. solani*	Peanut	Unknown	Georgia	T. Brenneman
RS-00-07	*R. solani*	Peanut	Unknown	Georgia	T. Brenneman
RSP	*R. solani*	Peanut	1982	Oklahoma	H.A. Melouk
Fellers	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Marshall	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Altus	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger
Lahoma	*R. cerealis*	Winter wheat	2004	Oklahoma	R.M. Hunger

Table 2. Sequence of oligonucleotide primers used for RAPD analysis along with the number of amplified and polymorphic bands generated.

Primer	Sequence 5′-3′	Total amplified bands	Polymorphic bands
P14	CCACAGCACG	38	16
R28	GATAACGCAC	41	15
RC09	ATGGATCCGC	32	12
308	AGCGGCTAGG	23	14
318	CGGAGAGCGA	26	8
320	CCGGCATAGA	24	9
324	ACAGGGAACG	39	14
327	ATACGGCGTC	62	19
329	GCGAACCTCC	44	15
335	TGGACCACCC	45	11
337	TCCCGAACCG	47	15
340	GAGAGGCACC	44	7
350	TGACGCGCTC	45	10
353	TGGGCTCGCT	52	9
361	GCGAGGTGCT	55	6
Total		**617**	**180**

Table 3. Disease severity of peanut seedlings of cv. Okrun as affected by inoculation of *Rhizoctonia solani* isolates from peanut.

Isolate	Disease severity[a]
Control	**1.0 a[*]**
G-24	**5.0 c**
JY-1	**4.6 c**
RS-00-07	**4.8 c**
RSP	**2.6 b**

[a]Disease severity rating on a scale of 1-6 (1= no symptoms, 2= discoloration and/or small pinpoint lesions, 3 = small, distinct necrotic lesions, 4 = large necrotic lesions, 5 = girdling lesion, and 6 = dead seedling.

[*]Means in the same column followed by the same letter are not significantly different according to LSMEANS at P≤0.05.

Table 4. Disease severity of winter wheat seedlings of cv. Jagger as affected by pre-emergence inoculation of *Rhizoctonia cerealis* isolates from wheat.

Isolate	Disease severity[a]
Control	**1.0 a[*]**
Fellers	**1.6 a**
Marshall	**1.5 a**
Altus	**1.3 a**
Lahoma	**1.6 a**

[a]Disease severity rating on a scale of 1-6 (1= healthy; 2= slight discoloration of leaf sheath or inner stem; 3= distinct lesion on the leaf sheath or inner stem; 4= rotting at the base of the stem; 5=damping off or yellowing; 6= complete death).

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: PATHOGENICITY OF *RHIZOCTONIA* SPP. AND *SCLEROTIUM ROLFSII* ON WHEAT AND PEANUT AND GENETIC VARIATION AMONG *RHIZOCTONIA* ISOLATES

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: PATHOGENICITY OF *RHIZOCTONIA* SPP. AND *SCLEROTIUM ROLFSII* ON WHEAT AND PEANUT AND GENETIC VARIATION AMONG *RHIZOCTONIA* ISOLATES

Pages in Study: 126 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Pathology

Scope and Method of Study: Studies were conducted to determine the pathogenicity of two soilborne fungi, *Rhizoctonia* spp. and *Sclerotium rolfsii* which cause important diseases on peanut and wheat. In *Rhizoctonia* study, pathogenicity of isolates G-24 (*Rhizoctonia solani*) and Fellers (*Rhizoctonia cerealis*) were determined on three winter wheat cultivars (Jagger, 2137 and 2174) and four peanut genotypes (Okrun, Tamspan 90, Southwest Runner and C 209 6-60). Disease severity was measured on a 1-6 scale and also by quantifying the number of infection cushions. In *Sclerotium rolfsii* study, pathogenicity of four isolates of *S. rolfsii* from peanut (Melouk, Ft. Cobb, Power St and Durant) and two from wheat (Wheat and ZP-3082) was determined on cultivars previously described for *Rhizoctonia*. In addition, *in vitro* and *in vivo* assays were conducted to determine the activity of endo-polygalacturonase (endo-PG) and oxalic acid produced by two isolates of *S. rolfsii* (Melouk and Wheat).

Findings and Conclusions: *Rhizoctonia* isolate G-24 from peanut was the most virulent on all peanut and winter cultivars with significantly more number of infection cushions as compared to the *R. cerealis* isolate Fellers. Results from *Rhizoctonia* study suggest that quantification of infection cushions can be a useful technique for screening cultivars for resistance to infections caused by *Rhizoctonia*. In *S. rolfsii* study, all isolates from peanut caused significant damage on peanut and wheat cultivars used in the study. Among peanut cultivars, Southwest Runner showed significant resistance to *S. rolfsii* infection. *In vitro* Activity of endo-PG and oxalic acid produced by *S. rolfsii* isolates differed based on the type of carbon source used. *In vivo* study suggested a definite correlation between the activity of endo-PG and oxalic acid and the virulence of the *S. rolfsii* isolate.

ADVISER'S APPROVAL: Hassan A. Melouk