

**Monocyclic Components for Evaluating Disease Resistance to *Cercospora arachidicola* and
Cercosporidium personatum in Peanut**

by

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ABSTRACT

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop that is produced in the United States and throughout the world. However, there are two major fungal pathogens of cultivated peanuts, and they each contribute to substantial yield losses of 50% or greater. The pathogens of these diseases are *Cercospora arachidicola* which causes early leaf spot (ELS), and *Cercosporidium personatum* which causes late leaf spot (LLS). While fungicide treatments are fairly effective for leaf spot management, disease resistance is still the best strategy. Therefore, it is important to evaluate and compare different genotypes for their disease resistance levels. The overall goal of this study was to determine resistance levels of different peanut genotypes to ELS and LLS. The peanut genotypes (Chit P7, C1001, Exp27-1516, Flavor Runner 458, PI 268868, and GA-12Y) used in this study include two genetically modified lines (Chit P7 and C1001) that over-expresses a chitinase gene. This overall goal was addressed with three specific objectives: 1) determine suitable conditions for pathogen culture and spore production in vitro; 2) determine suitable conditions for establishing infection in the greenhouse; 3) compare ELS and LLS disease reactions of young plants to those of older plants. The suitable culture medium for *C. arachidicola* was found to be potato dextrose agar and peanut oatmeal broth for *C. personatum*. The suitable sporulating medium was found to be V8 agar for both *C. arachidicola* and *C. personatum*. Greenhouse trials indicated inoculation with 5.0×10^3 conidia/ml was the best option because it resulted in distinct single lesions. Six peanut genotypes

that included two genetically modified lines, a parent line, and commercial standard lines at both vegetative and reproductive growth stages (VGS and RGS, respectively) were inoculated with 5.0×10^3 conidia/ml; both and *C. personatum* were separately evaluated. The monocyclic components evaluated were incubation period, number and size of lesions, and proportion of defoliation. Peanut plants inoculated with *C. arachidicola* at vegetative growth stage have significantly longer incubation period, fewer lesions, less defoliated leaflets at both 30 and 42 DAI than those inoculated at RGS. Peanut plants inoculated with *C. personatum* at vegetative growth stage has significantly longer incubation period, fewer lesions at both 30 and 42 DAI, smaller lesions, and fewer defoliation than those inoculated at RGS. Among all six genotypes evaluated, GA-12Y had the worst disease reaction to both ELS and LLS; in contrast Flavor Runner 458 has the greatest disease resistance. Genetically modified lines did not show different disease resistance levels compared to their parental line (Exp27-1516). Methods developed in this study could be used in other studies. These evaluations of monocyclic components as plant phenotype data could be used as a baseline for any disease resistance study.

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CHAPTER I Literature Review

1.1 PEANUT

Peanuts belong to legumes and are members of the genus *Arachis*. Peanuts are more closely related to beans and peas than to tree nuts (Toomer *et al.*, 2014). There are up to 69 wild species of *Arachis* that have been documented over the years, and all of these wild species of peanut are found only in South America (Hermann, 1954; Krapovickas *et al.*, 2007; Ucko and Dimbleby, 1969). The genus *Arachis*, based on morphology, geographic distribution, and cross compatibilities, consists of nine sections, which are *Arachis*, *Caulorrhizae*, *Erectoides*, *Extranervosae*, *Heteranthae*, *Procumbentes*, *Rhizomatosae*, *Trirectoids* and *Triseminatae* (Krapovickas *et al.*, 2007). Among these nine sections, the cultivated peanut, *Arachis hypogaea* L., is the only species to be selected due to its significant economic value and distribution in tropical, sub-tropical, and warm temperate zones throughout the world (Hammons, 1982).

Peanuts are a self-pollinating annual crop, which prefer well drained loamy sand, sandy loam, or sandy clay loam soils and warm weather with adequate rainfall during the growing season. Peanuts are also called groundnuts, because of their special reproductive structures. After flower fertilization, pegs form and elongate to penetrate soil, while at the tip of the peg the pod forms. About a hundred days after planting, peanuts reach maturity, although this varies by botanical variety. When the majority of full size pods contain fully grown kernels, the crop is ready to be dug out of the ground (Henning *et al.*, 1982; Moss and Rao, 1995).

1.1.1 Origin

The exact origin of the peanut remains unknown, but it is believed that the peanut is native to South America where all wild species are found (Martin *et al.*, 2006). Some peanuts

were introduced from Peru to Mexico, and then traded to the Philippines through the Acapulco-Manila galleon line (Ucko and Dimbleby, 1969). Other peanuts were introduced by Spaniards to the West Pacific, Malayan Archipelago, China, Indonesia, and eventually to Madagascar (Ucko and Dimbleby, 1969; Janick *et al.*, 1981). Later, during transportation of slaves, peanuts were returned from Africa to Tropical America and the United States as a food source for slaves, along with the sweet potato (Martin *et al.*, 2006).

Peanut cultivation was not extensive in the United States until the late 19th and early 20th centuries, due to the heavy workload of digging and shelling, as well its association with slaves. In the late 19th and early 20th centuries, the U.S. government promoted agricultural research and production of peanuts in order to feed the growing population. They also improved peanut cultivation technology to increase peanut production. Meanwhile, the rapidly increasing demand for food after the Civil War stimulated the expansion of the peanut production area into the western United States (Hammons, 1982).

Currently, peanut production in the United States is found in three major areas: the Southeast, Southwest, and the Central Piedmont area. The Southeast peanut production area includes Alabama, Florida, Georgia, and Mississippi. The Southwest peanut production area includes New Mexico, Oklahoma, and Texas, and the Central Piedmont area includes Virginia, North Carolina, and South Carolina (USDA, 2013).

1.1.2 Economic value

The global five-year average production of cultivated peanuts from 2009 to 2013 was 41.37 million metric tons (Fig. 1.1) (FAO, 2014). The United States ranked No. 4 in the world for cultivated peanut production. The five-year (2009 - 2013) average production of cultivated

peanuts in the U.S. was 2.03 million metric tons (Fig. 1.2) (USDA, 2014). There were changes in the production from year to year. For example, in the year 2014, peanut production was 2.36 million metric tons, which was 25% more than the production in 2013 (USDA, 2014; USDA, 2015). These changes were dependent on the decrease or increase in plant acreage related to the price of peanuts during previous years.

In the U.S., Georgia produced almost 1.10 million metric tons of peanuts in the U.S., followed by Florida and Alabama, which produced ~ 0.30 and 0.25 million metric tons of peanuts respectively (USDA, 2015). Peanuts are one of the five most important oil seeds produced in the U.S. In 2014, peanut production was 2.36 million metric tons, ranked third behind soybeans (108.01 million metric tons) and cottonseed (4.82 million metric tons) (USDA, 2015).

1.1.3 Nutritional value

Cultivated peanuts are an excellent vegetarian source of oil, protein, fiber, vitamins and minerals. They consist of 45 to 60% lipids, 20 to 35% protein, and 5 to 10% fiber (Baernstein, 1938; Liao, 2014). The major components of peanut lipids are oleic and linoleic acids with a total content of 82 and 34%, respectively. While manufacturers prefer high-oleic peanuts because of their longer shelf-life, consumers prefer peanuts, in part, because of their greater nutritional benefits (Costa de Camargo and Canniatti-Brazaca, 2014; Wang *et al.*, 2014). Consumption of oleic peanuts also leads to improved health such as better serum lipid and apolipoprotein profiles, higher insulin production, lower glucose level, suppression of lung tumorigenesis, and lower risk of cardiovascular disease (Mozaffarian *et al.*, 2010; O'Byrne *et al.*, 1997; Vassiliou *et al.*, 2009; Yamaki *et al.*, 2002). Peanuts are also rich in antioxidants, such as polyphenols and resveratrol,

which provide the benefit of antimicrobial, anti-inflammatory, anticarcinogenic, and antioxidant properties, as well as preventing heart disease and cancer (Costa de Camargo and Canniatti-Brazaca, 2014; Gonzalez and Salas-Salvadó, 2006; Jones *et al.*, 2014; Sales and Resurreccion, 2014).

1.1.4 Commercial types in U.S.

There are four types of peanuts grown commercially in the U.S. These are Runner, Virginia, Spanish, and Valencia-types, which are named based on their kernel characteristics (Chukwumah, 2011). Runner-type peanuts dominate the U.S. market with an 80% share of the peanut market, followed by Virginia-types, at approximately 15% of the market. The rest is shared by Spanish and Valencia-types (USDA, 2013).

Runner peanuts have uniformly larger seeds, higher yield and higher oil content than Spanish and Valencia-types. They are the most popular commercial type and are mainly used for production of peanut butter. There are several Runner-type peanut cultivars that are commercially available, including Georgia-06G, Georgia-07W, Georgia-09B, Georgia-12Y, FloRun-107, Tifguard, and Flavor Runner 458 (Beasley, 2013). Virginia peanuts are large-seeded and have been cultivated in Virginia since 1844 (Smith, 2002). These peanuts have heavy reddish stems and large leaves, and are mostly used for gourmet snacks (Martin *et al.*, 2006). Spanish peanuts have small-pods and were introduced into the U.S. from Malaga, Spain, in 1871 (Hammons, 1982). These peanuts require a relatively short growing season and have a broad range of environmental adaptability (Hammons, 1982). Spanish-type peanuts also have a good nutty flavor, better roasting characteristics than other peanut types. Because of these characteristics, they are used for a variety of cuisines. Valencia-type peanuts have three or more

kernels per shell with sweet flavor, and are used mostly for all-natural peanut butter or roasted and sold in shells. Domestically in the U.S., peanuts are mostly consumed in the form of whole peanuts, peanut butter, and peanut oil (Martin *et al.*, 2006).

In the past several decades, Runner-type peanut cultivars share the major peanut market (Branch, 2014). As the No. 1 peanut production area in the U.S., the Southeast planted a high percentage (77%) of the cultivar Georgia-06G in 2012. Peanut growers in the Southeast prefer this cultivar because it is a Runner-type peanut with resistance to *Tomato spotted wilt virus* (TSWV) and has a high yielding capacity (Beasley, 2013; Branch, 2007). There are two other Runner-type peanut cultivars worth mentioning, Georgia-09B and Georgia-12Y. Georgia-09B, a cultivar with resistance to TSWV with a high yielding capacity, is also a high-oleic cultivar (Branch, 2010). Georgia-12Y is also a high yielding cultivar with resistance to TSWV and white mold or stem rot (Branch, 2012).

1.2 PEANUT DISEASES

Globally, there are three major peanut foliar diseases: early leaf spot (caused by *Cercospora arachidicola* Hori), late leaf spot (caused by *Cercosporidium personatum* [Berk. & Curtis]), and rust (caused by *Puccinia arachidis* Sperg.) (Dwivedi *et al.*, 2003; Liao, 2014).

1.2.1 Early leaf spot

Early leaf spot is caused by *Cercospora arachidicola* (C.a.) and is a major fungal disease of peanuts, which regularly threatens peanut yield. Early leaf spot symptoms include necrotic lesions on leaves, petioles, stems, and pegs (Fig 1.3). The lesions usually appear to have a dark-brown center encircled with an obvious yellow halo (Porter *et al.*, 1982). The lesions on the

leaves cause a reduction in photosynthesis and also cause infected peanut leaves to drop prematurely, increasing the impact of this photosynthesis reduction. Loss of photosynthesis affects pod fill. Lesions on pegs can cause breakage during harvest, which also contributes to yield loss. Yield losses due to early leaf spot alone ranges from 10% to 50%, depending on other elements, such as climate, disease management, and peanut genotype (Cantonwine *et al.*, 2006; Cantonwine *et al.*, 2008; Mehan *et al.*, 1994; Waliyar *et al.*, 1995). Early leaf spot is found in all peanut production areas (Fig. 1.3 A).

Lesions due to early leaf spot range in size from 1 to 10 mm in diameter and are dark-brown on the upper leaf surface and light brown on the lower leaf surface, commonly with the presence of a yellow halo (Shokes and Culbreath, 1997). On the upper surface of these leaf lesions, conidiophores develop, which range in size from 15 to 45 by 3 to 6 μm . Conidia are elongated, subhyaline, have up to 12 septa, and range in size from 35 to 110 by 3 to 6 μm (Gremillion, 2007) (Fig. 1.3 B).

1.2.2 Late leaf spot

Late leaf spot is caused by *Cercosporidium personatum*, which threatens peanut yield not only because of its effects on accelerating defoliation and reducing photosynthesis (Singh *et al.*, 2011a; Singh *et al.*, 2013), but also because the lesions on the pegs result in breakage and peanuts remain in the ground following inversion (Gremillion *et al.*, 2011). Late leaf spot is found in all peanut production areas (Fig. 1.3 C).

Lesions due to late leaf spot are light to dark brown, typically without the presence of a yellow halo compared to early leaf spot (Shokes and Culbreath, 1997). However, the presence of a yellow halo is also dependent on the genetics and nutritional status of the host, as well as

weather conditions. Thus, the presence of the halo is not a reliable distinction between early and late leaf spots (Porter and Smith, 1982). The major difference between early and late leaf spot is the side of leaf on which conidiophores form. For late leaf spot, conidiophores develop on the lower surface of the leaf and range in size from 10 to 100 by 3 to 6.5 μm . Conidia are cylindrical and slightly curved, have up to 9 septa, with a size range from 20 to 70 by 4 to 9 μm (Gremillion, 2007) (Fig. 1.3 D).

1.2.3 Rust

Peanut rust, caused by *Puccinia arachidis*, is one of the most destructive and economically important foliar diseases causing yield loss throughout the world. An early and intensive rust attack causes leaf necrosis. Leaves fail to attain normal size and fall to the ground prematurely, and the growth of the shoot is inhibited. Because of peanut rust, the life cycle of the peanut is shortened by 15 to 20 days, seeds are reduced in size and oil content (Bromfield, 1971), and pods are more readily detached at digging. Yield losses due to rust and leaf spots can range from 50% to 70% (Gibbons, 1980; Subrahmanyam *et al.*, 1980; Subrahmanyam *et al.*, 1984). Peanut rust was first recorded in the Soviet Union in 1910 and then by Mauritius in 1914 (Subrahmanyam *et al.*, 1984). Peanut rust was noted in China in 1937, but had not been a regular occurrence until the 1960s (Bromfield, 1971). Rust appeared to spread suddenly to many countries in Asia. It even spread to Australasia and Oceania during the early 1970s (Hammons, 1977) and eventually reached Africa (Subrahmanyam *et al.*, 1983; Subrahmanyam *et al.*, 1985; Savary *et al.*, 1988). Peanut rust has been noted in all peanut-producing areas since 1918 (Bromfield, 1971; Hammons, 1977; Hammons, 1987; Porter, 1982; Savary *et al.*, 1988). This

disease does not cause extensive losses in the Southeastern United States, but it does in other countries with warmer climates (Subrahmanyam *et al.*, 1984; Power *et al.*, 2012).

A peanut rust lesion, called uredial sorus, is found on the abaxial leaf surface. Uredial sori are orange or yellow in color, circular and ranging in size from 0.3 to 1.0 mm. Sori appear on all aerial plant structures except the flowers and pegs (Porter, 1982). Mature sori are called uredinia, and each uredinium ranges from 0.5 to 1.4 mm in diameter. Each uredinum may contain hundreds of asexual urediniospores (or uredospores), which can be ellipsoid or oval in shape with a thick brown wall, and are 16 to 11 by 23 to 29 μm (Arthur, 1934; Agrios, 2004; Subrahmanyam *et al.*, 1984). Urediniospores mature within uredial pustules and could become airborne and infect other host plants with appropriate temperature and moisture conditions (Bromfield, 1971).

1.2.4 Resistant cultivars

1.2.4.1 Early leaf spot

Singh *et al.* (1997) reported more than 30 germplasm lines that were identified as resistant to early leaf spot. Similarly, Bayo Grande (BG) and “CRSP” lines from the United States Agency for International Development’s Peanut Collaborative Research and Support Program (USAID Peanut CRSP) showed leaf spot resistance (Gremillion, 2007; Gremillion *et al.*, 2011). Li *et al.* (2012) also found that the cultivars Tifguard and Georganic showed resistance to leaf spots.

There are transgenic peanut plants to which a *chitinase* gene has been introduced, along with a promoter. This peanut plant, which is a Spanish transgenic peanut (*Arachis hypogaea* L. cv. TMV-2) plant with a tobacco *chitinase* gene, showed resistance to *C. arachidicola* (Rohini

and Rao, 2000). The genetically modified Valencia GOLDEN and BARI-2000 cultivars also showed higher resistance against *C. arachidicola* (Iqbal *et al.*, 2012). There is a need for studying transgenic Runner type of peanut plants since the Runner types are important to the peanut market (USDA, 2013).

1.2.4.2 Late leaf spot

There are commercially available late leaf spot resistant cultivars, such as those listed by McDonald *et al.* (1985) from ICRISAT. In addition to being resistant to early leaf spot, Gremillion (2007) reported BG and CRSP lines showed late leaf spot resistance. Chapin *et al.* (2010) found that the cultivars Bailey, N03088T, GA-03L, and N0390T showed significantly higher resistance to late leaf spot disease than the NC-V 11 standard (Wynne *et al.*, 1991). Singh *et al.* (2011b) discovered that York peanut cultivars have some resistance to *C. personatum*, which is the causal agent for late leaf spot. Vasavirama and Kirti (2012) found that the expression of SniOLP and Rs-AFP2 genes made the transgenic peanut plants less susceptible to late leaf spot disease. These lines were genetically modified from the Spanish JL-24 cultivar. Since Runner comprises more than 80% of the peanut market (USDA, 2013), more research needs to be done in order to develop transgenic Runner type peanuts that are more resistant to late leaf spot disease.

1.2.4.3 Rust

In immune genotypes, the fungus died shortly after entering the substomatal cavity (Subrahmanyam *et al.*, 1980). Different genotypes express their resistance to the rust fungus in different ways, such as having a longer incubation period, a decreased infection frequency, a

reduced pustule size, and spore production. These levels of resistance are associated with differences in rate and extent of mycelia development within the cavity and within leaf tissues (Subrahmanyam *et al.*, 1983).

High levels of resistance and immunity to rust have been found in some wild *Arachis* species (Subrahmanyam *et al.*, 1983), and these have been used in breeding. Mehan *et al.* (1994) studied more than 144 genotypes, 17 of which showed varied levels of resistance, especially ICG 10890, 10881, 10014, and 10940. ICG 10890 ranked No. 1 in rust resistance because this genotype had low infection frequency, with only 2.6 pustules per square centimeter of leaf area. Along with low infection frequency, ICG 10890 had a long incubation period of 18.7 days compared with other tested genotypes. It also had a small lesion diameter of 0.18 mm, a low level of damaged leaf area of 2.0 %, and a low sporulation index rating (2.0 on 1 to 5 scale, where 1 = no sporulation, and 5 = 76-100% sporulation). ICG 10881 was also rust resistant, showing a long incubation period of 17.0 days, a low damage leaf area of 3.1 %, and a low sporulation index rating of 2.3. Mehan *et al.* (1994) also found that, even though ICG 10014 did not have a longer incubation period, it still had a low infection frequency (3.5), a small lesion diameter (0.23 mm), and a low damaged leaf area (4.0%). ICG 10940 only showed rust resistance because it had a low infection frequency of 3.9.

In addition to the findings of Mehan *et al.* (1994), other researchers also found genotypes that were rust resistant. The study of Pande *et al.* (2001) showed ICG 8954 had high rust resistance with no leaf area damage. Power *et al.* (2012) reported the experimental lines GTC-20, Tarapoto, Ga-03L, 97x36-HO2-1-B2G3-1-2-2, Tifrust 13, and 99x33-1-B2G-13-1-1 have resistance to peanut rust. Kelly *et al.* (2012) found that cv. Sutherland also showed rust resistance of slower disease development as well as lower final disease ratings than cv. Menzies. Yeri *et al.*

(2014) studied heterogeneous inbred families (HIFs) derived near isogenic lines (NILs) and proved them rust resistant as well.

Although partially disease (early leaf spot, late leaf spot and rust) resistant commercial peanut cultivars exist, there is still a need for developing better resistant cultivars, and genetic engineering could contribute to this process (Rohini and Rao, 2000).

1.3 DISEASE EPIDEMIC

1.3.1 Monocycle

A monocycle is a single infection cycle and is the basic unit of a disease epidemic (Teng and Bowen, 1985). A group of monocycles together form infection chains, and the concatenation of infection chains can lead to an epidemic (Kranz, 1974). A monocycle includes the time between inoculation and the start of the production of spores by the resulting infection (Manners, 1993).

1.3.1.1 Monocyclic components

There are several important components included in one monocycle, and these are inoculum (infection), incubation period, onset, latent period, number and size of lesions, and sporulation (Fig. 1.4) (De Wolf and Isard, 2007). Inoculum is the biomass of a pathogen that is in contact with the plant host and available for infection. This infection represents the first sub stage of a monocycle-infection (Agrios, 2004; Termorshuizen and Jeger, 2014). The incubation period is the time between infection and the first disease symptom (onset) in the host. Latent period is the time between infection and the first reproduction of inoculum, which is sporulation (De Wolf and Isard, 2007). Incubation period, number and size of lesions, and latent period contribute to

disease severity, while sporulation, which indicates secondary infection, contributes to disease incidence (Carson, 1995).

1.3.2 Polycyclic disease

Polycyclic pathogens, also named multicyclic pathogens, have more than one generation in each growth season. They could cause many disease cycles each year, with multiplied inoculum for each following infection cycle. Polycyclic pathogens would lead to an explosive epidemic on most crops, including leaf spots and rusts (Agrios, 2004). Polycyclic components include primary infection and secondary infections (Fig. 1.5), which could repeat several times (Agrios, 2004).

1.3.3 Early leaf spot Disease cycle

One polycyclic disease is peanut early leaf spot. The primary inocula that cause peanut early leaf spot are mainly conidia, associated with local peanut debris in the soil; ascospores, chlamydospores and mycelial fragments are also potential sources (Hemingway, 1957, as cited by Porter *et al.*, 1982; Jenkins, 1938; Miller, 1953; Porter *et al.*, 1982; Shanta, 1960). Conidia of *C. arachidicola* germinate, and their germ tubes grow and penetrate the host either directly into plant tissue or through natural openings, such as stomata (Shokes and Culbreath, 1997). After infection, symptoms showed as dark-brown necrotic leaf lesions usually surrounded by a conspicuous yellow halo. These symptoms are followed by formation of stroma on the adaxial surface, then conidial sporulation, which become the secondary inocula (Jenkins, 1938).

1.3.3.1 Optimal conditions for disease development

1.3.3.1.1 Conditions for colonization

Both humidity and temperature are essential conditions for *C. arachidicola* infection. The optimum infection conditions are temperatures of 22 to 23 °C and 95% relative humidity for 48 to 84 hours (Olatinwo *et al.*, 2012; Wu *et al.*, 1999). When temperatures reach 19 to 31 °C with a moist environment caused by heavy dew or rainfall, conidia germinate and penetrate the host directly or indirectly, and starts the infection (Shokes and Culbreath, 1997). Temperatures for *C. arachidicola* to infect need to be higher than 19 °C and the humidity needs to be greater than 95%. After penetrating the host, *C. arachidicola* grows intercellularly and absorbs nutrients from host cells directly (Gremillion, 2007). The incubation period is from 11 to 17 days, after which lesions start to show (Nutter and Shokes, 1995; Shokes and Melouk, 1995). Later, asexual conidia are formed within the necrotic tissue of lesions (Gremillion, 2007).

1.3.3.1.2 Conditions for sporulation

The latent period (LP) is the number of days needed from inoculation to when lesions start to sporulate. The LP of early leaf spot ranges from 13 to 39 days, with temperatures ranging from 10 to 31 °C. The optimum daily mean temperature is around 25 °C (Wadia and Butler, 1994).

1.3.4 Late leaf spot Disease cycle

Similar to peanut early leaf spot, peanut late leaf spot is also a polycyclic disease. The primary inocula are also mainly conidia associated with local peanut debris in the soil. Ascospores, chlamydospores and mycelial fragments are also potential sources for peanut late leaf spot (Hemingway, 1957, as cited by Porter *et al.*, 1982; Jenkins, 1938; Miller, 1953; Porter

et al., 1982; Shanta, 1960). The differences are *C. personatum* does not kill host cells in advance of its proliferating intercellular hyphae (Jenkins, 1938; Woodroof, 1933). Furthermore, *C. personatum* forms its conidia on the abaxial surface, rather than adaxial surface. Conidial sporulation also becomes the secondary inocula (Porter *et al.*, 1982).

1.3.4.1 Optimal conditions for disease development

1.3.4.1.1 Conditions for colonization

Appropriate humidity and temperature are both essential conditions for *C. personatum* infection. Optimum infection conditions are 20 to 24 °C with a relative humidity greater than 93% for at least 12 hours per day over a six-day infection period (Olatinwo *et al.*, 2012; Shew *et al.*, 1988). Similar to *C. arachidicola*, when temperatures reach 25 to 31 °C, with a moist environment due to heavy dew or rainfall, conidia of *C. personatum* germinate and penetrate the host directly through cell walls, or indirectly through natural openings, such as stomata (Shokes and Culbreath, 1997). After penetrating the host, *C. personatum* grows intercellularly and forms haustoria to obtain nutrients. Lesions form 10 to 14 days after infection (Nutter and Shokes, 1995; Shokes and Melouk, 1995). Later, asexual conidia are formed within the necrotic tissue of lesions (Gremillion, 2007).

1.3.4.1.2 Conditions for sporulation

The latent period of late leaf spot ranges from 13 to 38 days, with temperatures ranging from 10 to 35 °C. The optimum daily mean temperature is around 25 °C (Wadia and Butler, 1994)

1.3.5 Rust Disease cycle

Peanut rust is also a polycyclic disease. The primary inoculum consists of urediniospores. Urediniospores of *P. arachidis* germinate on the leaf surface. Their germ tubes grow and form appressorium and penetrate the host through stomata with infection hypha, followed by the intercellular hyphae, and develop haustoria (Cook, 1980). After infection, uredial sori show on peanut leaves and stems, and produce urediniospores as secondary inocula (Agrios, 2004). Peanut rust does not over winter in the U.S. (Higgins, 1956, as cited by Hammons, 1987). However, urediniospores are introduced annually from other peanut producing countries through the wind (Harrison, 1972).

1.3.5.1 Optimal conditions for disease development

Peanut rust has been reported to attack only the genus *Arachis*, and all stages of peanuts are susceptible. The optimal conditions for urediniospore germination are high humidity, free water on the leaf surface (light rain showers rather than heavy showers), temperatures from 20 to 27 °C, and low light intensity. Low temperatures (< 15 °C) and high temperatures (> 30 °C) reduce urediniospore germination by at least 40%. High light intensity will also significantly reduce urediniospore germination, and once light intensity reaches 5000 lux, urediniospore germination could be totally inhibited (Subrahmanyam and McDonald, 1984; Savary *et al.*, 1988).

1.3.5.2 Conditions for colonization: rust develops rapidly during humid and wet weather

Peanut rust infection usually occurs late in the season, when plants are under severe drought stress (Hammons, 1987). Savary *et al.* (1988) reported the optimum infection

temperature for *P. arachidis* was 27 °C. Subrahmanyam *et al.* (1985) also found that the optimum temperatures range from 20 to 30 °C, combined with free water on the leaf surface and high relative humidity. However, the minimum wetness duration for infection by *P. arachidis* is not clear.

In the U.S., only the uredial stage of *P. arachidis* has been found (Higgins, 1956, as cited by Hammons, 1987). When temperatures reach 20 °C, urediniospores germinate on the host surface and penetrate indirectly through stomata, and the infection begins (Cook, 1980). Peanut rust uredia first appear on the lower surface of the leaf, and original pustules may be surrounded by secondary pustules (Bromfield, 1971). The optimal conditions for disease development are high relative humidity and leaf wetness with temperatures between 20 and 27 °C (Kelly *et al.*, 2012; Mallaiah and Rao, 1979; Savary *et al.*, 1988).

1.3.5.3 Conditions for sporulation

The latent period for peanut rust ranges from 12 to 49 days, with temperatures ranging from 12 to 40 °C. The optimum temperature for latent period is around 25 °C (Wadia and Butler, 1994). Urediniospores germinate, penetrate, and infect the new host within hours and mature within 10 days (Bromfield, 1971).

1.4 DISEASE MANGEMENT

The management of plant disease reduces economic and aesthetic damage caused by the different diseases. There are six principles to achieve this goal. They are avoidance, exclusion, eradication, protection, resistance, and therapy (Jacobsen, 2001). In order to manage peanut leaf spots and rust, chemical control and resistant cultivars are commonly used.

Chemical control methods, such as fungicides, are effectively used for management of both early and late leaf spots in the U.S. (Cook, 1981; Gibbons and Bailey, 1967; McDonald *et al.*, 1985). Commonly used fungicide programs are based upon seven sprays with a 14-day spray schedule, with the first spray at approximately 30 to 35 days after planting. There is another fungicide program called AU-Pnuts which focuses on leaf spot control and is based on the number of “rain events”. In this advisory, the first fungicide application is done after four rain events occur after cracking and when the five-day forecast calls for at least a 50% chance of rain; after five rain events following cracking with at least a 40% chance of rain over the next five days; or immediately after at least six rain events. The second spray should be scheduled ten or more days after the first spray. Additional fungicide applications are required based on rain events and the five-day average forecast: if rain events and the average chance of rain for the next five days is no less than 50%; if one rain event and the average chance of rain for the next five days is no less than 40%; or two rain events and the average chance of rain for the next five days is no less than 20%; or immediately after three rain events (Jacobi *et al.*, 1995; Jacobi and Backman, 1995).

Peanut rust can be controlled by fungicides, but the cost is too high for growers in most developing countries (Rao, 1987). Some ways to biologically control peanut rust have been studied. For example, the fungi, *Verticillium lecani* (Zimmerm), *Penicillium islandicum* Sopp., *Eudarluca caricis* (Fr.) O. Ericks, and *Acremonium persicinum* (Nicot). W. Gams have been found growing on *P. arachidis* (Subrahmanyam *et al.*, 1984), which contribute to rust control. However, intensive screening for rust resistance and high-yielding cultivars has become a major research topic (Rao, 1987).

Disease resistant cultivars are the best strategy for reducing crop yield losses from diseases (Porter and Smith, 1982). In addition to fungicide cost, large portions of grower's time for scheduling fungicides and the risk of development of resistance to fungicides (Gremillion *et al.*, 2011) make chemicals less desirable than host resistance. In addition, environmental hazards caused by different fungicides, such as potential negative effects of fungicides on water quality and non-target organisms, are also reasons why disease resistant cultivars should be prioritized over fungicides (Castillo *et al.*, 2000; Moser *et al.*, 2001; Yeri *et al.*, 2014).

1.4.1 Transgenic peanut plants with enhanced chitinase protection

1.4.1.1 Chitinase gene

Chitinases are hydrolytic digestive enzymes that break down glycosidic bonds in chitin, which is a component of the cell walls of fungi and exoskeletal elements of some animals (Sámi *et al.*, 2001). Chitinases are commonly found in organisms that either need to reshape their own chitin or to digest the chitin of fungi or animals. Some plants express a large number of genes encoding diverse proteins, such as chitinases, as pathogenesis related proteins that are induced as part of systemic acquired resistance to fungal and insect attack (Collinge *et al.*, 1993; Salzer *et al.*, 2000). Various chitinases have been found to accumulate around fungal hyphae and lyse hyphal tips *in vitro* (Broekaert *et al.*, 1988; Brogue *et al.*, 1991; Mauch *et al.*, 1988; Schlumbaum *et al.*, 1986). For example, a well-known potent inhibitor of numerous fungal chitinases, pseudotrisaccharide allosamidin, was found to have the ability of inhibiting the outgrowth of hyphae fragments in aging *P. chrysogenum* cultures (Sámi *et al.*, 2000). Therefore, the potential of chitinase to reduce the damage caused by pathogens has become of great interest (Brogue *et al.*, 1991).

Chitinase has been used as a biological control for fungi. For example, chitin-supplemented application of the chitinolytic bacteria, *Bacillus cereus*, has been proven effective in reducing early leaf spot of peanuts (Kokalis-Burelle *et al.*, 1992). Similarly, the chitinolytic bacteria, *B. circulans* GRS 243 and *S. marcescens* GPS 5, have been proven effective in reducing late leaf spot of peanuts (Kishore *et al.*, 2005). Additionally, the chitinolytic bacteria, *Stenotrophomonas maltophilia* C3, has improved the control of bean rust (Yuen *et al.*, 2001) and leaf spot of *Festuca arundinacea* (Zhang and Yuen, 1999).

1.4.1.2 Transgenic peanut plants with chitinase genes

Although the expression of chitinase in peanut is too weak to induce full protection against pathogens, there is the possibility of engineering constitutive expression of a defense protein that can enhance the ability to express chitinase and be used to develop transgenic plants. Overexpression of chitinase in transgenic plants would have the ability to enhance the resistance to fungal pathogens (Wu *et al.*, 2009). Recently, several studies found that transgenic peanut plants with enhanced chitinase expression showed stronger disease resistance ability. Rohini and Rao (2001) studied transgenic peanut plants with a tobacco chitinase gene and found better resistance to *C. arachidicola* attack as demonstrated by longer incubation periods. Another group found that transgenic peanut plants to which a rice chitinase-3 gene had been introduced with an enhanced version of the CaMV 35S promoter showed a higher resistance against *C. arachidicola* (Iqbal *et al.* 2012). Prasad *et al.* (2013) reported transgenic peanut plants with an overexpressed rice (*Rchit*) chitinase gene had longer incubation and latent periods for late leaf spot, lower damaged leaf area, fewer lesions per leaf, and lower infection frequency against *C. personatum*. Transgenic peanut plants also showed longer incubation and latent periods as well as lower

damaged leaf area, fewer pustule numbers per leaf, and lower infection frequency against *P. arachidis*.

1.4.1.3 Chitinase *BjCHII*

The *Brassica juncea* chitinase gene *BjCHII* is unique not only because it is the only chitinase to our knowledge with two chitin-binding domains, but also because it has both chitinase and agglutinin activities (Chye *et al.*, 2005; Tang *et al.*, 2004; Zhao and Chye, 1999). The expression of *BjCHII* is also reported to be highly induced by pathogenic fungal infection, as well as other means, such as wounding and caterpillar feeding (Wu *et al.*, 2009), suggesting it to be a pathogenesis related gene. Fung *et al.* (2002) reported transgenic tobacco plants which expressed *BjCHII* showed antifungal activity of reducing fungal hyphal diameter as well as hyphal branching and conidia size. In addition, Gao *et. al.* (2014) found transgenic *Arabidopsis* with enhanced *BjCHII* had higher resistance to *Botrytis cinerea* infection. Both transgenic plants with expression of *BjCHII* had enhanced antifungal activity, which provided the foundation of the possibility that transgenic peanuts with the *B. juncea* chitinase gene *BjCHII* would also have the ability of enhanced antifungal abilities.

1.5 SUMMARY

Cultivated peanut is an economically important crop that is produced worldwide. The United States ranks fourth in the world for cultivated peanut production averaging 2.03 million metric tons annually over a five year period (2009-2013) (USDA, 2014). However, there are two major fungal pathogens of cultivated peanuts, and they each contribute to substantial yield losses of 50% or greater. The pathogens *Cercospora arachidicola*, which causes early leaf spot, and

Cercosporidium personatum, which causes late leaf spot. While fungicide treatments are fairly effective for leaf spot management, disease resistance is still the best strategy for both environmentally benign and economic reasons. Therefore, it is important to evaluate and compare different genotypes for their disease resistance levels. The overall goal of this study was to determine resistance levels of different peanut genotypes against ELS and LLS. The peanut genotypes (Chit P7, C1001, Exp27-1516, Flavor Runner 458, PI 268868, and GA-12Y) include two genetically modified lines (Chit P7 and C1001) that over-expresses a chitinase gene. This overall goal was addressed with three specific objectives: 1) determine suitable conditions for pathogen culture and spore production in vitro; 2) determine suitable conditions for establishing infection in the greenhouse; 3) compare ELS and LLS disease reactions of young plants to those of older plants.

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Figure 1.1. World cultivated peanut production from FAO, 2014. The global five-year average peanut production from 2009 to 2013 was 41.37 million metric tons.

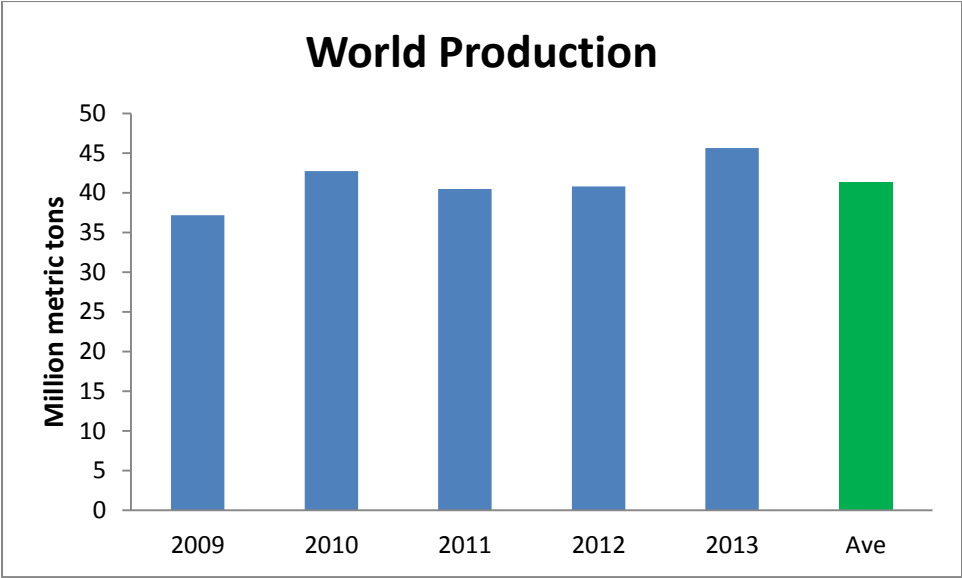


Figure 1.2. The U.S cultivated peanuts production from FAO, 2014. The U.S. five-year average peanut production from 2009 to 2013 was 2.03 million metric tons. The U.S. peanut production was 2.36 million metric tons in 2014.

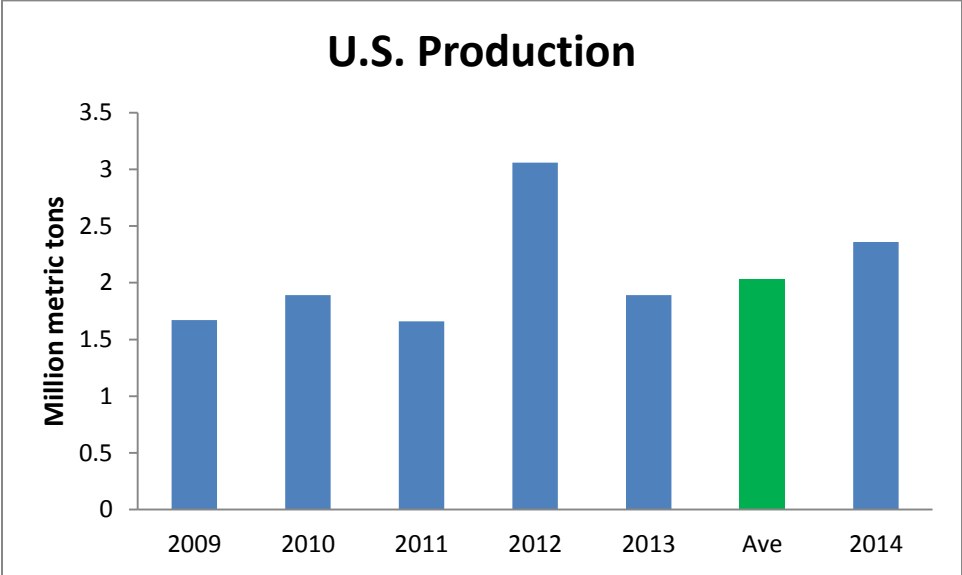


Figure 1.3. Symptoms and signs of peanut leaf spots: **A.** Peanut leaf with early leaf spot lesions. **B.** Conidia of early leaf spot causal agent (*Cercospora arachidicola*). **C.** Peanut leaf with late leaf spot lesions. **D.** Conidia of late leaf spot causal agent (*Cercosporidium personatum*).

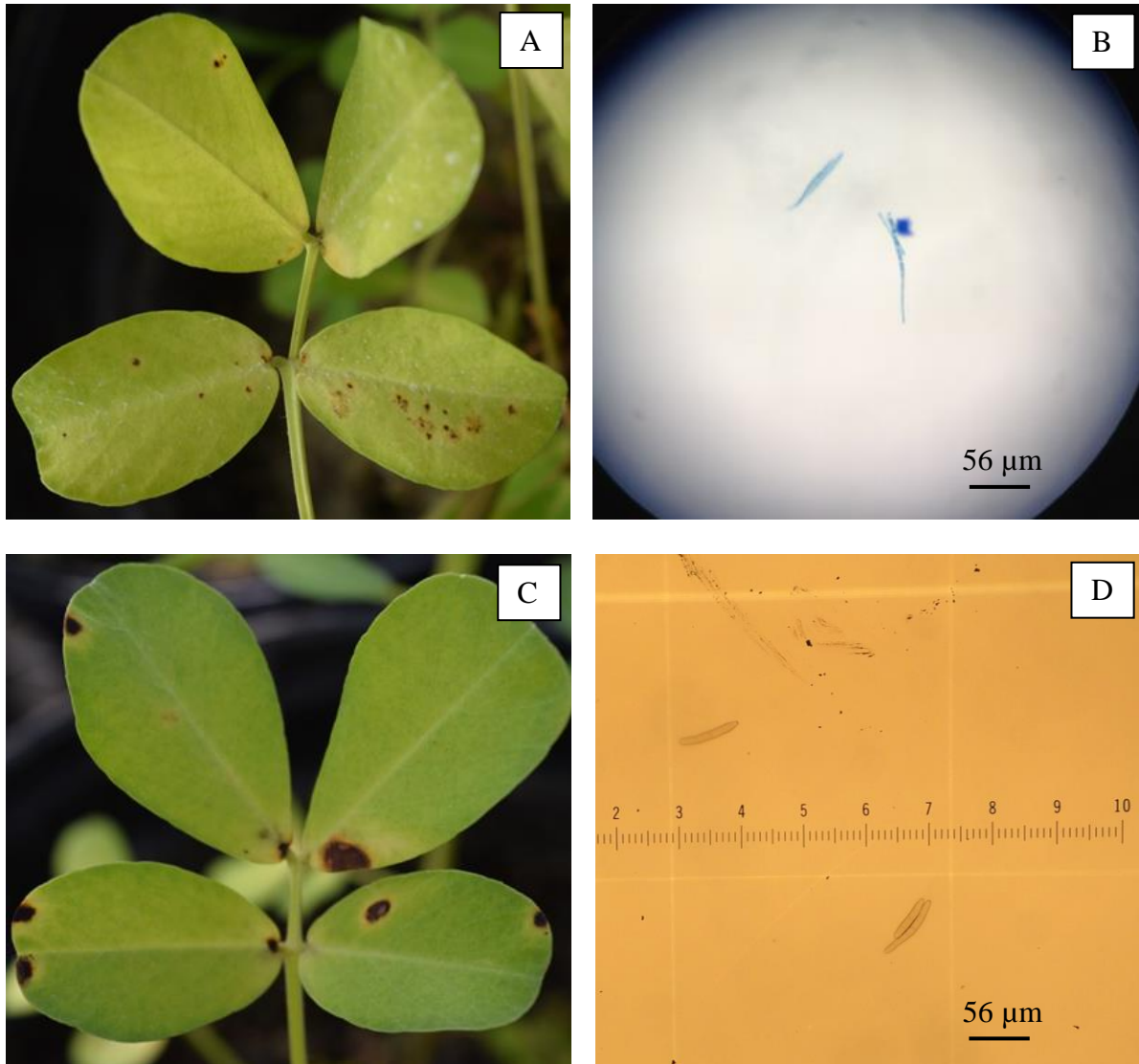


Figure 1.4. Monocyclic disease has a single infection cycle per season that includes several important components, which are inoculum, incubation period, onset, latent period, number and size of lesions, and sporulation.

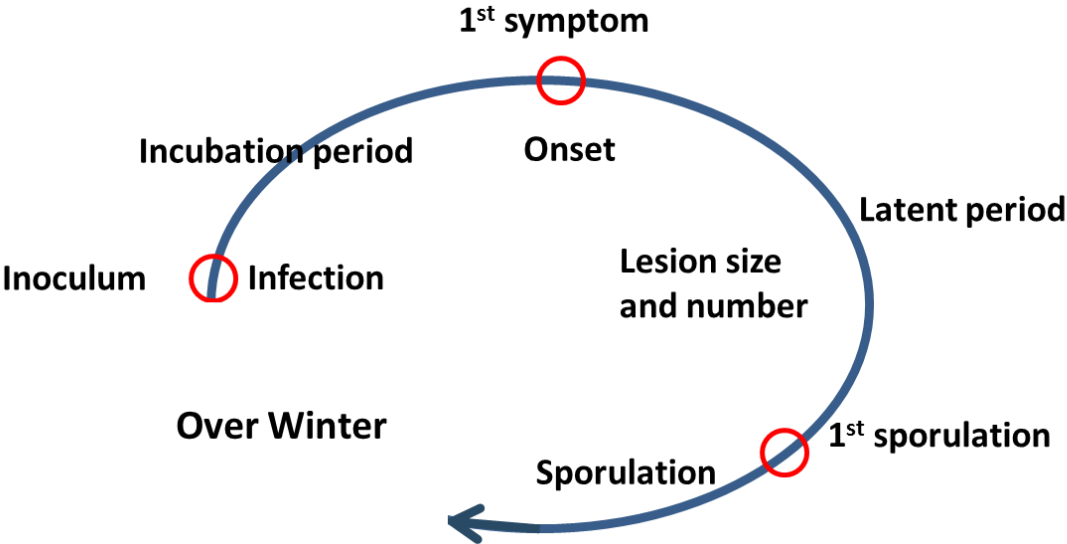
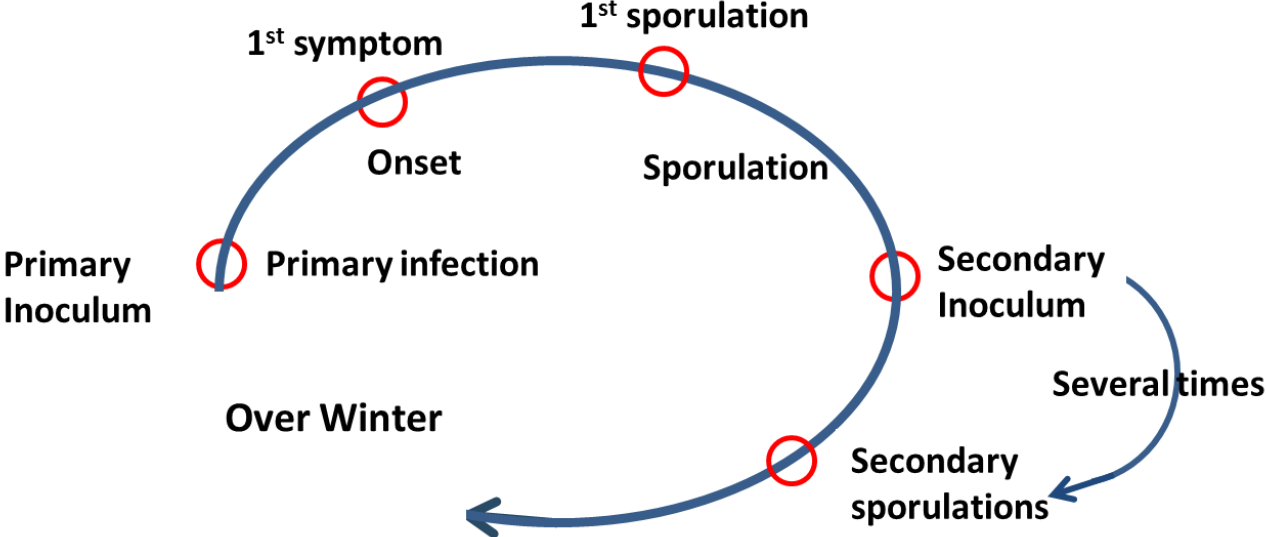


Figure 1.5. Polycyclic disease has multiple infection cycles in each growth season that includes primary infection and secondary infections, which could repeat several times.



CHAPTER II Evaluation of *in vitro* culture and sporulation media for *Cercospora arachidicola* and *Cercosporidium personatum*

2.1 ABSTRACT

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop worldwide. *Cercospora arachidicola* and *Cercosporidium personatum* are the causal agents of peanut early leaf spot and late leaf spot, respectively, two of the most important fungal diseases in all peanut producing areas. Due to slow growth and poor sporulation of these fungi *in vitro*, it is difficult to obtain uniform conidia as inoculum for research. Few studies have been done on *C. arachidicola* and *C. personatum* culture and sporulation. There is a need to improve *in vitro* culture and spore production conditions for both *C. arachidicola* and *C. personatum*. This study used different culture and sporulation media and determined that a suitable culture medium for *C. arachidicola* and *C. personatum* culture was potato dextrose agar (PDA) and peanut oatmeal broth (POB), respectively. This study also determined that the suitable sporulation medium was V8A for both the fungi.

Keywords: *in vitro* culture, sporulation

2.2 INTRODUCTION

Cercospora arachidicola and *Cercosporidium personatum* (Porter *et al.*, 1982) are the causal agents of early leaf spot (ELS) and late leaf spot (LLS), respectively, the most important fungal diseases of peanut worldwide. Together these pathogens can threaten peanut yields with 50% losses or more. Due to their slow growth and poor sporulation capacity *in vitro*, researchers in need of inoculum collect peanut leaves with spots from the field and rinse conidia from these leaves (Cantonwine *et al.*, 2008). However, this method does not ensure uniform conidia from

the same isolate. To improve the uniformity of inoculum, optimized *in vitro* fungal culture and sporulation protocols are needed for controlled peanut studies (Abdou and Cooper, 1974; Reddy and Subbayya, 1987).

Previous studies indicate that potato dextrose agar (PDA, Thermo Fisher Scientific, Pittsburgh, PA) and peanut leaf extract agar (PLA, Abdou and Cooper, 1974) could be used as growth media for *C. arachidicola*; PLA, peanut oatmeal agar (POA, Smith, 1971), as well as V-8 agar (Dhingra and Sinclair, 1985) could be used as sporulation media for *C. arachidicola* (Abdou and Cooper, 1974; El-Gholl *et al.*, 1981; Landers, 1964). Based on Kleb's law (Dahlbery, 1982), nutrient deficiency contributes to the induction of fungal sporulation; there is a need to compare whether *C. arachidicola* sporulates better when cultured in low-nutrient media.

Few studies have been done previously regarding *C. personatum* culture and sporulation. Abdou and Cooper (1974) reported that growth on PLA and POA resulted in good yields of conidia. Reddy and Subbayya (1987) reported that Czapek's yeast extract agar provided good growth of *C. personatum* but not sporulation. However, both of these studies provided a conidia yield per microscopic field, but did not provide a total number of harvestable conidia. It is difficult to evaluate the efficiency of their sporulation protocol. There is a need to determine the suitable conditions for *C. personatum* culture and spore production *in vitro*.

The overall goal of this study was to optimize the conditions for *C. arachidicola* and *C. personatum* sporulation *in vitro* for further experimental study. The three specific objectives of this work were: 1) to isolate and identify *C. arachidicola* and *C. personatum*; 2) to evaluate the suitable culture media for growth of these two pathogens; and 3) to evaluate the suitable sporulation media for each of these two pathogens.

2.3 MATERIALS AND METHODS

2.3.1 Isolation

Peanut leaves with ELS and LLS were collected from a peanut field at the E. V. Smith Research Center in Tallahassee, AL in the Fall of 2013. Leaves with sporulating conidia were surface disinfected with 70% ethanol for 5 s and rinsed twice with sterile deionized water (diH₂O) for 1 min. One leaf spot with conidia was pressed onto the surface of water agar, and each single conidium was hand-picked with a dissection needle and transferred to another water agar plate.

2.3.2 Culture

All culture plates were maintained in a dark incubator at 24 °C. Five germinated *C. arachidicola* conidia were transferred to each culture plate (60×15 mm); both PDA and PLA were used as media with three replicates. Five germinated *C. personatum* conidia were transferred to each culture plate; PDA, PLA, and POA were used as media with three replicates, and peanut oatmeal broth (POB, Smith, 1971) was tested in three replicates. When cultured in POB, some conidia attached to the inner surface of the glass tube and grew. A thin, soft, and transparent ruler was used to measure the diameters of three individual colonies among these attached colonies weekly.

2.3.3 Identification

Confirmation of the identity of *C. arachidicola* and *C. personatum* from the single spore cultures was done through sequence analysis of the internal transcribed spacer (ITS).

2.3.3.1. DNA Extraction

A small piece of mycelium from a single culture was placed in a clean 1.5 ml Eppendorf tube; 90µl of 0.5 M NaOH was added to the tube with the mycelium. The fungus was ground with a clean konte pestle (Kimble Chase, Rockwood, TN) until the sample was liquefied; a 1µl aliquot of the solution was transferred to a new tube containing 99µl 100 mM Tris-HCl buffer, pH 8.0, and mixed thoroughly (Wang *et al.*, 1993).

2.3.3.2. PCR for Fungal ITS Region

Extracted DNA (8 µl) was placed in a 100 µl tube with 15 µl double-distilled water (ddH₂O) and 1 µl 10 µM Primer ITS1 and Primer ITS4. The tube was then put into a thermal cycler, and the ITS program was used with 30 s annealing at 55 °C (White *et al.*, 1990).

2.3.3.3. Gel Electrophoresis of PCR amplicons

PCR product (4 µl) was mixed with 1 µl of 5X loading dye, and analyzed by agarose gel electrophoresis using GelRED (Biotium., Hayward, CA). Four µl of DNA ladder was loaded into a well, and then the power of the electrophoresis box was turned on to run for 30 minutes at 100 volts. Image was captured on a Alpha Innotech gel imaging system (Proteinsimple, San Jose, CA) for imaging.

2.3.3.4. QIAquick PCR Purification (Qiagen, Hamburg, Germany)

Twenty µl of PCR product was mixed with 100 µl Buffer PB in a 2 ml collection tube, and then centrifuged for 1 min at 13,000 rpm. Flow-through was discarded, the QIAquick column (Qiagen, Hilden, Germany) was placed back into the same tube, 0.75 ml Buffer PE was placed on the QIAquick column, and the tube was centrifuged for 1 min at 13,000 rpm to wash

the PCR product. Flow-through was discarded, and the QIAquick column was placed back into the same tube and centrifuged for 1 additional min to completely remove residual ethanol. The QIA column was placed in a clean 1.5 ml microcentrifuge tube, 30 µl Buffer EB was added to the center of the QIAquick membrane, and the column stood for 1 min before being centrifuged for 1 min; the liquid in the bottom of the clean 1.5 ml microcentrifuge tube was the purified PCR product.

2.3.3.5. Sequencing and Analyzing sequencing data

Purified PCR products were then quantified by the NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific, Waltham, MA). The samples were submitted to the Auburn University Genomics & Sequencing Lab for sequencing. The sequences were modified by the BioEdit software (Ibis Biosciences, Carlsbad, CA), and then used to compare with known sequences by basic local alignment search tool (BLAST, National Center for Biotechnology Information).

2.3.4 Sporulation

When colonies reached 4 mm diameter, each colony was ground and evenly spread on sporulation medium. POA, PDA, V8A, 1/10 PDA, and 1/10 PDA with sterile filter paper covering its surface were used for both *C. arachidicola* and *C. personatum* sporulation evaluations with three replicates. All plates were maintained at room temperature (around 22 °C) with 12 h day light.

2.3.5 Conidia harvest

A daily microscopic examination was performed to observe sporulation on each plate. When conidia were observed, 2 ml of sterile diH₂O with 0.005% Tween 20 was used to rinse conidia off for collection in 15 ml sterile tubes. This harvest procedure was repeated until few conidia were observed floating on the surface of diH₂O. The conidia suspension was brought to 15 ml with diH₂O containing 0.005% Tween 20. The harvested conidia were counted with a hemocytometer; three samples from the conidial suspension were counted. The same plate was air-dried and sealed for microscopic examination and conidia harvest the next day.

2.3.6 Data analysis

For single colony identification, BioEdit software was used to modify the DNA sequence, and BLAST was used to compare the modified sequence. For sporulation experiments, effects of medium, conidial harvest time point, and the two-way interaction on number of harvested conidia were determined with mixed model analysis (Proc Glimmix, SAS 9.4). A *P*-value less than or equal to 0.10 was considered significantly different. Means for treatments were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.1$.

2.4 RESULTS

2.4.1 Culture

C. arachidicola conidia grew well and reached 4 mm diameter within 3 months on both PDA and PLA; because PDA has a clear background, it was chosen for further study. *C. personatum* conidia started to sporulate on PLA plates shortly after transfer instead of growing; *C. personatum* conidia grew slowly on PDA and POA, and quickly developed white mycelium

with declining sporulation ability on PDA. Therefore, PDA, PLA, and POA were all excluded as culture media for *C. personatum*. *C. personatum* grew relatively better on POB; even though conidia on the surface of POB developed white mycelium, there were still many that grew well below the broth surface and reached 4 mm diameter size in 3 months. POB was chosen for further study.

2.4.2 Isolation and Identification

A bright band showed clearly in the gel (Fig 2.1, arrow), indicated the PCR product.

BioEdit software was used to modify the DNA sequence from both ends to eliminate background noise for the best sequence data.

The modified sequence for *C. arachidicola* ITS 1 is:

```
CGAGCCCGACCTCCAACCCTTTGTGCACCAACTCTGTTGCTTCGGGGGCGACCCCGC
CGTCTGGGCGACGGCGCCCCCGGAGGTCGTCAAACACTGCATCTCTGCGTCGGAG
TCGTCAAGTAAATTGAAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGACCGCACATTGCGCCCCGTGGTATTCCGCGGGGCATGCCTGTTTCGAG
CGTCATTTACCACTCAAGCCTAGCTTGGTATTGGGCGTCGCGGTTCCGCGCGCCTT
AAAGTCTCCGGCTGAGCAGTCCGTCTCTAAGCGTTGTGGCACATATTTTCGCTGCAGA
GTCCGGGCGGCTTTTCGGCCG---
```

The modified sequence for *C. personatum* ITS 1 is:

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AGCCCGACCTCCGACCCTTTGGTGAACCAACCCTGNTTGCTTCGGGGGCGACCCCGC
CGTCCC GGCGACGGCGCCCCCGGAGGTCATCAAACACTCTGCATCCGTGCGTCGGAGT
GGTCAAGTAAATTCCAACAAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCCGCGGCATTCCGCGGGGCATGCCTGTTTCGAGC
GTCATTTACCGCTCAAGCCTAGCTTGGTATTGGGCGTCGCGGTTCCGCGCGCCTTG
AAGTCCCCGGCTGAGCAGTCCGTCTCTAAGCGGCGTGGCATATATTTTCGCTGAAGAG
TTCGGGCGGCTTTTGGCCGTTAAATCTTTCTCAAGGTTGACCTCGGATCAGGTAGGG
ATACCCGCTGAACTTAAGCATATCAATA---
```

The BLAST results showed that there were several known sequences similar to these sample sequences: *Mycosphaerella arachidis* and *Mycosphaerella berkeleyi* (teleomorph names for *C. arachidicola* and *C. personatum*, respectively), and they were all in the same strain, but in different groups. From the Taxonomy report for BLAST, the sample belongs to Genus *Mycosphaerella*, Family *Mycosphaerellaceae*, Order *Capnodiales*, Division *Ascomycota*, Kingdom *Fungi*. From the Distance Tree of BLAST, the sample is more closely related to group AP4M2 in strain USGA05 (Fig 2.2). The alignment result of BLAST shows that the sample sequence is identical with the known sequences, compared to group AP4M3; only 1 nucleic acid is different, which in the sample was C, and in the know group AP4M3 sequence, that location was A instead (Fig 2.3).

2.4.3 Sporulation

For *C. arachidicola* sporulation, POA was excluded because the conidial suspension had a substantial amount of mycelial debris even after filtering through 4 layers of cheesecloth. Based on daily microscopic examination, conidia were optimally harvested from the 5th day of sporulation until the 8th day. V8A yielded the highest number of conidia compared to the remaining three sporulation media (Table 2.1). One-tenth PDA had higher conidial yields compared to PDA for all harvest durations; 1/10 PDA with filter paper yielded a higher number of conidia on the 6th day compared to 1/10 PDA, and it also yielded a higher number of conidia compared to PDA through all harvest durations. Low nutrient content of media, as with 1/10 PDA, does contribute to the induction of *C. arachidicola* sporulation. Furthermore, continuous *C. arachidicola* conidia formation *in vitro* was observed over the duration of this study.

For *C. personatum* sporulation, PDA was excluded because few if any conidia appeared to form on the fungus on this media. POA was excluded as *C. arachidicola* sporulation media for the same reason. V8A allowed continuously good yields each day from the 8th until 11th day with at least 17,500 conidia/ml for 15 ml (Table 2.2).

2.5 DISCUSSION

Both *C. arachidicola* and *C. personatum* cultures were confirmed through sequencing. This study confirmed that a suitable culture medium for *C. arachidicola* is PDA, which agrees with previous findings by El-Gholl *et al.* (1981). This study also found that a suitable culture medium for *C. personatum* is POB, which is comparable with previous findings by Abdou and Cooper (1974) about the POA as a sporulation medium for *C. personatum*. However, we modified the medium from agar to broth, which drastically improved fungal growth. This study also confirmed that V8A is a suitable sporulation medium for *C. arachidicola*, which agrees with previous findings from El-Gholl *et al.* (1981) about V8A as a sporulation medium. El-Gholl *et al.* (1981) reported a mean of 3,400 spores counted from ten 0.5-1.0 mm diameter cultures, and approximately 133,000 spores per plate with an average of 100 colonies. We were able to obtain approximately 138,525 spores per plate per day for at least four days, which is a higher yield than noted in previous studies. This study determined that V8A is a suitable sporulation medium for *C. personatum*, which is different from the findings from Abdou and Cooper (1974) that indicated PLA and POA provided good yields of conidia for *C. personatum*. Because Abdou and Cooper (1974) only provided the number of conidia per microscopic field rather than the conidia harvest from the whole plate, it is difficult to make a direct comparison to our findings. The current study also confirmed that nutritional deficiency contributes to the induction of *C.*

arachidicola sporulation on PDA plates. Further, it was observed that both *C. arachidicola* and *C. personatum* have the ability to sporulate continuously *in vitro* which is the first report of this aspect. For fungi, *C. arachidicola* and *C. personatum* grow very slowly and produce few conidia (Abdou and Cooper, 1974), thus the observation of continuous *in vitro* sporulating ability is vital. Researchers who need inoculum will not need to spend several months repetitively culturing these two fungi, nor do they need to collect peanut leaves with spots from field in order to rinse off conidia from these leaves.

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Table 2.1. *Cercospora arachidicola* conidia harvested from different sporulation media at different time points.

Source of variation	<i>P</i> -value
Time point ^a	0.017
Medium	0.083
Two way interaction	0.303
Concentration of harvested conidia ^b	
Time point ^a	
5	7375 ^b B ^c
6	7225 B
7	8250 A
8	7150 B
Medium	
V8A	9235 A
PDA	5050 C
1/10 PDA	7650 B
1/10 PDA with filter paper	7925 B

^aTime point is the number of days after colony was ground and spread on the sporulation medium.

^bThe unit for all concentrations of harvest conidia should be conidia/ml, there are a total 15 ml of harvest conidial suspension.

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Table 2.2. *Cercospora personatum* conidia harvested from different sporulation media at different time points.

Source of variation	<i>P</i> -value
Time point ^a	0.976
Medium	0.090
Two way interaction	0.993
Concentration of harvested conidia ^b	
Time point ^a	
8	7771 ^b A ^c
9	7821 A
10	7802 A
11	7859 A
Medium	
V8A	9371 A
PDA	7295 A
1/10 PDA	7295 A
1/10 PDA with filter paper	7295 A

^aTime point is the number of days after colony was ground up and spread on the sporulation medium.

^bThe unit for all concentrations of harvest conidia should be conidia/ml, there are a total 15 ml of harvest conidial suspension.

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Figure 2.1. The fragments amplified by ITS 1 and 4. The sequence (5'-3') of ITS 1 is TCCGTAGGTGAACCTGCGG; the sequence (5'-3') for ITS 4 is TCCTCCGCTTATTGATATGC. PCR product from mycelium sample is indicated by the arrow.

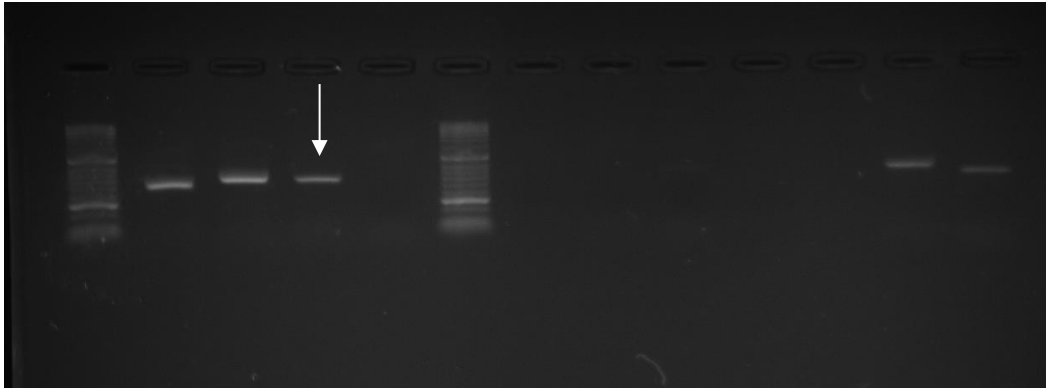


Figure 2.2. Distance tree suggests the query sequence aligns to the database sequences of *Mycosphaerella arachidis* (teleomorph name for *C. arachidicola*). Query sequence of mycelium sample is indicated by the arrow.

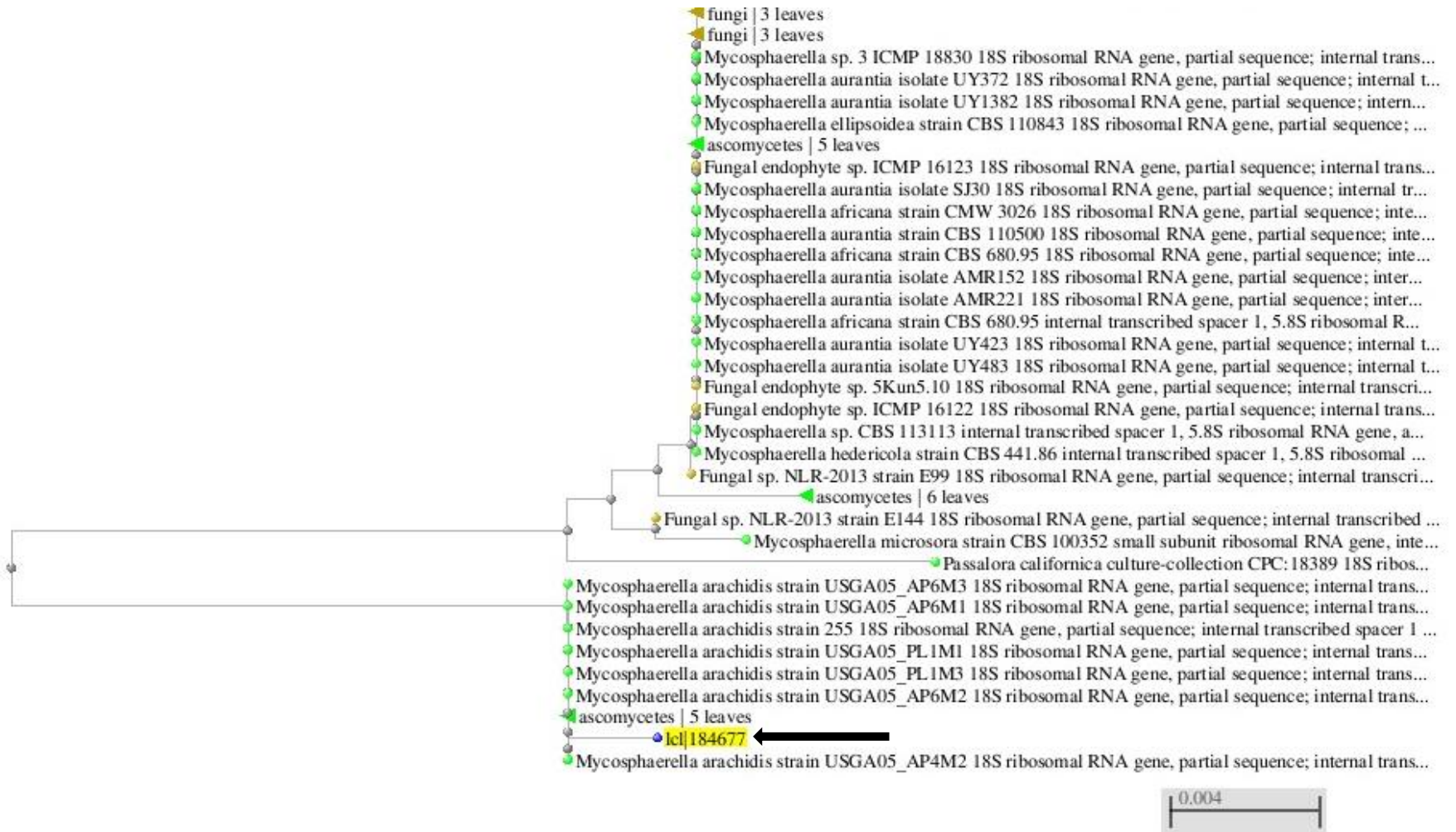


Figure 2.3. Alignment of the query sequence with database sequences.

Alignments

Download ▾ GenBank Graphics ▼ Next ▲ Previous ▲ Descriptions

Mycosphaerella arachidis strain USGA05_AP4M3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
 Sequence ID: [gb|EF157739.1](#) Length: 617 Number of Matches: 1

Range 1: 171 to 587 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Related Information

Score	Expect	Identities	Gaps	Strand
765 bits(414)	0.0	416/417(99%)	0/417(0%)	Plus/Plus

```

Query 1  CGAGCCCGACCTCCACCCCTTTGTGCACCAACTCTGTTGCTTCGGGGCGACCCCGCCGT 60
Sbjct 171 .....
Query 61  CTGGGCGACGGCGCCCCCGGAGGTCGTCAAACACTGCATCTCTGCGTCGGAGTCGTCAA 120
Sbjct 231 .....
Query 121  GTAAATGAAACAAAACITTCACCAACGGATCTCTTGGTTCGGCATCGATGAAGAACGC 180
Sbjct 291 .....
Query 181  AGCGAAATGCGATAAGTAATGTGAATTCAGAAATTCAGTGAATCATCGAATCTTTGACCG 240
Sbjct 351 .....A..
Query 241  CACATTGCGCCCCGTGGTATCCGCGGGGCATGCCCTGTCGAGCGTCATTTCACTCA 300
Sbjct 411 .....
Query 301  AGCCTAGCTTGGTATGGGCGTCGCGGTCCGCGCCCTTAAAGTCTCCGCTGAGCAGT 360
Sbjct 471 .....
Query 361  CCGTCTCTAAGCGTTGTGGCACAATTTGCTGCGAGTCCGGGCGGCTTTCGGCCG 417
Sbjct 531 .....
  
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CHAPTER III An evaluation of *Cercospora arachidicola* monocyclic components of three newly released peanut cultivars in greenhouse studies

3.1 ABSTRACT

Early leaf spot, caused by *Cercospora arachidicola*, is one of the most economical important fungal diseases of cultivated peanuts (*Arachis hypogaea* L.). To evaluate and compare peanut early leaf spot monocyclic components of three recently released peanut cultivars (Georgia-06G, Georgia-09B, and Georgia-12Y), an intermittent mist system simulated dew for optimized infection conditions. The monocyclic components include incubation period, number and size of lesions, and defoliation for early leaf spot, which were examined daily on whole peanut plants arranged in a randomized complete block. Based on a significantly shorter incubation period, higher lesion counts, larger lesion size, and higher defoliation than other cultivars, the most susceptible cultivar was Georgia-09B of the three cultivars evaluated. This study used whole peanut plants rather than detached peanut leaves, with an intermittent mist system in a greenhouse, which not only mimicked the natural environment for peanut plant growth, but also used a better setting that extended the ability to examine whole plants.

Keywords: inoculation level, monocyclic components, disease resistance, early leaf spot

3.2 INTRODUCTION

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop not only in the United States, but worldwide. The United States ranks fourth in the world for cultivated peanut production with 2.03 million metric tons as a five-year (2009-2013) average production (USDA, 2014). Globally, one of the most important fungal diseases of peanut is early leaf spot (ELS), caused by *Cercospora arachidicola* S. Hori. This disease causes necrotic lesions on

leaves, petioles, stems, and pegs, and eventual defoliation (Porter *et al.*, 1982). ELS causes yield losses of 10 to 50 % annually, depending on other elements such as climate and peanut cultivar (Cantonwine *et al.*, 2006; Cantonwine *et al.*, 2008; Waliyar *et al.*, 1995). While fungicide treatments are fairly effective for ELS management, disease resistance is still the best strategy for both environmental and economic reasons. In addition, cultivar resistance does not contribute to the development of resistance to fungicides (Castillo *et al.*, 2000; Gremillion *et al.*, 2011; Porter *et al.*, 1982). Therefore, it is important to evaluate and compare newly released peanut cultivars for their disease resistance levels.

Discovering how to evaluate newly released peanut cultivars accurately, efficiently, and affordably is critical. Peanut early leaf spot is a polycyclic disease, which means it has more than one generation in each growing season (Agrios, 2004); this makes resistance evaluation difficult. However, the polycycle is comprised of many single cycles or monocycles; monocyclic components include incubation period, number and size of lesions, defoliation and sporulation. Incubation period, the time between infection and the first disease symptom in the host (De Wolf and Isard, 2007), and number and size of lesions contribute to disease severity; defoliation is another important disease index, while sporulation drives secondary infection (Carson, 1995). By evaluating and comparing peanut ELS monocyclic components, resistance levels of newly released peanut cultivars can be estimated.

The amount of material used for experiments may be another critical aspect for evaluating how a disease epidemic is affected by varying values of monocyclic components. Small closed containers have been previously used in research trials to maintain the high humidity needed for *C. arachidicola* infection. Due to the space limitation of closed containers, researchers either used detached peanut leaves or whole peanut plants in small containers. For

example, Cantonwine *et al.* (2008) used detached peanut leaves in a transparent enclosure in a growth chamber, while Wu *et al.* (1999) had whole peanut plants in 12-cm-diameter plastic cups inside dew chambers for resistance evaluations. Unrestricted whole peanut plants are more likely to reflect natural conditions since defense mechanisms in leaves may be altered with detachment. Disease symptom development in detached leaves have been proven to be uncoupled from plant processes in several ways, such as failure to develop basal penetration resistance, induced systemic resistance, and expression of defense-responsive genes (De Vleeschauwer *et al.*, 2009; Liu *et al.*, 2007). Furthermore, small containers limit plant size; therefore, larger pots, which can support healthier peanut root systems, are more desirable for resistance evaluation studies.

Besides the amount of plant material, inoculating whole peanut plants under a more natural environment, such as natural day/night cycle and dew simulation, could be important. A greenhouse provided a natural day/night cycle. Beckman and Payne (1983) observed lesion development on corn plants using a mist system after *Cercospora zea-maydis* inoculation. This mist system was adapted for use in the current study with modifications to provide a more suitable environment for *C. arachidicola* infection of peanut.

The objective of this study was to evaluate and compare ELS monocyclic components of three recently released peanut cultivars (Georgia-06G, Georgia-09B, and Georgia-12Y), using whole peanut plants in both a growth chamber and in a greenhouse under intermittent mist. The three specific objectives of this experiment were: 1) to optimize *C. arachidicola* inoculation level; 2) to establish greenhouse experimental condition; 3) to evaluate and compare three recently released peanut cultivars ELS resistant levels.

3.3 MATERIALS AND METHODS

3.3.1 Inoculum production

Peanut leaves with ELS were sampled from a peanut field at the E.V. Smith Research Center, Tallassee, AL; *C. arachidicola* conidia from a single lesion on these leaves were collected and single spores were cultured on PDA medium for three months. Identity of cultures was confirmed, using internal transcribed spacer (ITS) sequence analysis, as *C. arachidicola*. One clone was ground using a 15 ml VWR (Radnor, PA) conical tissue grinder and evenly spread on a V8 agar plate (Dhingra and Sinclair, 1985), allowed to dry in a hood for 30 min, and then the plate was parafilmmed and placed under a grow light with a 12 h photoperiod for one week. A daily microscopy check revealed peak sporulation between three and seven days. A solution of 0.005% Tween 20 was used to rinse conidia off the medium using gentle pipetting for higher yield. Eight layers of cheese cloth were used to filter any mycelia residue. Conidia were counted with a hemacytometer then adjusted to three concentration levels, 2.5×10^3 /ml, 5×10^3 /ml, and 7.5×10^3 /ml.

3.3.2 Peanut plants

3.3.2.1 Growth chamber

Three recently released peanut cultivars (Georgia-06G, Georgia-09B, and Georgia-12Y) (Branch, 2007; 2010; 2013) were evaluated. Twelve seeds of each cultivar were individually planted on September 3, 2014 in Magenta GA-7 plant culture boxes ($7.62 \times 7.62 \times 10.16$ cm³) containing potting mix (Sunshine mix # 8, Sun Gro Horticulture, Vancouver, Canada) and sand in a 1:1 (vol/vol) mixture, with 100 ml mixture in the bottom of the box, seed in the middle, and 50 ml mixture on the top. Thirty ml of water was applied onto the media in each culture box.

Three weeks after germination, peanut plants had reached the V4-V5 growth stages (Boote 1982); nine out of the twelve plants were chosen for use in studies based on their uniform size.

A randomized complete block design (RCBD) was used with three cultivars and three *C. arachidicola* conidial suspensions for a 3×3 factorial set of treatments. Three replications of all treatments were included. The growth chamber was set at 26 °C with light from 7 a.m. until 8:30 p.m., and 21 °C without light from 8:31 p.m. until 6:59 a.m. Plants were watered as needed and fertilized once a week with 20-10-20 Peat-Lite (J.R. Peters Inc., Scotts, Marysville, OH) at a rate of 250 ppm N. The three *C. arachidicola* conidia concentrations were used to spray-inoculate plants 30 days after planting (DAP). A total volume of 0.5 ml conidial suspension was applied to each plant. After inoculation, each culture box was covered with another transparent culture box to maintain high humidity.

3.3.2.2 Greenhouse

Two experiments were carried out in the greenhouse. The first (GH-1) experiment was similar to the growth chamber experiment with the same three cultivars and three *C. arachidicola* conidial suspensions for a 3×3 factorial in an RCB. Two replications were used. Twelve seeds of each cultivar were individually planted in seedling starter trays on September 3, 2014. Three weeks after germination, 6 of the 12 plants were chosen based on their uniformity and transferred to one gallon pots containing potting mix and sand in a 1:1 (v/v) mixture. The second (GH-2) experiment used the same three cultivars, with *C. arachidicola* conidial suspensions of 5×10^3 /ml. Thirty-six seeds of each cultivar were individually planted in seedling starter trays on February 28, 2015. One week after germination, 13 uniform seedlings were selected for each cultivar and were transferred to one gallon pots containing potting mix and

sand in a 1:1 (v/v) mixture. A RCBD was used with 13 replications of each treatment. For both greenhouse experiments, all pots were arranged under Coolnet Pro 4-way Fogger nozzles, each attached to the 61 cm micronet hanging assembly (Netafim, Fresno, CA), and separated by one meter. Intermittent mist was scheduled for 6 s duration every 15 min from 10 p.m. to 10 a.m. daily with a Drip Irrigation Greenhouse 6 (DIG Corp., Vista, CA) station controller. The greenhouse temperature was set at 26 °C from 7 a.m. until 8:30 p.m. and 21 °C from 8:31 p.m. until 6:59 a.m. Plants were watered as needed and fertilized weekly with 20-10-20 Peat-Lite (J.R. Peters Inc., Scotts, Marysville, OH) at the rate of 250 ppm N. In both greenhouse experiments, conidia suspensions were used to spray-inoculate plants 30 days after seeds were planted (DAP). A total volume of 0.5 ml conidial suspension was sprayed on each peanut plant in the evening and peanut plants reached V6-V8 vegetative growth stages (Boote, 1982).

3.3.3 Disease evaluation

Five leaves of each plant were examined daily after *C. arachidicola* inoculation for evaluating the monocyclic components (incubation period, number and size of lesions, defoliation, and sporulation) of ELS. Incubation period was defined as the number of days from inoculation to the first day on which lesions were observed. Size of lesions was determined as the average diameter of the three largest lesions on the five evaluated leaves of each plant. Defoliation was determined by the number of defoliated leaflets from these same five leaves of each plant. Sporulation was the number of sporulating lesions from these five leaves of each plant. Area under disease progress curves (AUDPCs) were calculated based on number of lesions on the five leaves (Shaner and Finney, 1977).

3.3.4 Data analysis

For both growth chamber and greenhouse studies, effects of cultivar, inoculation rate, and the two-way interaction on monocyclic components was determined with mixed model analysis (Proc Glimmix, SAS 9.4). A p-value less than or equal to 0.10 was considered significantly different. Means are significantly different according to Fisher's protected least significant difference (LSD) test ($P \leq 0.1$).

3.4 RESULTS

3.4.1 Growth chamber

In the growth chamber experiment, cultivar had a significant effect ($P < 0.05$) on incubation period, number of lesions at 8 DAI, largest lesion size, and number of sporulating lesions (Table 3.1). Inoculation concentration had a significant effect ($P < 0.005$) on both the largest lesion size and number of sporulating lesions (Table 3.1). The two-way interactions were not significant for any parameter.

Georgia-09B had an incubation period less than half that of Georgia-06G or Georgia-12Y (Table 3.1). Georgia-09B also had a greater number of lesions than Georgia-06G or Georgia-12Y at 8 DAI and this trend continued up to 25 DAI (Table 3.1, Fig. 3.1A). AUDPCs for Georgia-06G, Georgia-09B, and Georgia-12Y were 438.5, 450.8, and 506.9, respectively, and were not significantly different. Georgia-09B had a numerically greater number of defoliated leaflets than the other two cultivars until 38 DAI; at 42 DAI, defoliation of Georgia-06G was similar to that of Georgia-09B, while Georgia-12Y had more defoliated leaflets than Georgia-09B (Fig. 3.1B). At 42 DAI, Georgia-09B had larger lesions than Georgia-12Y. Georgia-09B also had more sporulating lesions than Georgia-06G or Georgia-12Y at 42 DAI (Table 3.1).

There was no significant difference between inoculation concentrations for incubation period or number of lesions. Based on number of lesions, AUDPCs for inoculation concentrations 2.5×10^3 /ml, 5×10^3 /ml, and 7.5×10^3 /ml were 361.5, 527.1, and 526.2, respectively, and were not statistically different. However, there were significant differences for both the largest lesion size and number of sporulating lesions (Table 3.1, Fig. 3.2A). The highest inoculation rate (7.5×10^3 /ml) yielded the largest lesion size (diameter) at 42 DAI than the lower conidial suspensions (Table 3.1). The lowest inoculation rate (2.5×10^3 /ml) also resulted in the fewest number of sporulating lesions at 42 DAI, compared with higher inoculation concentrations (Table 3.1). The inoculation rate of 5×10^3 /ml had a greater number of defoliated leaflets than the other two levels through 38 DAI; at 42 DAI, the lowest inoculation rate had the highest number of defoliated leaflets (Fig. 3.2. B).

3.4.2 Greenhouse

In the GH-1 experiment, both cultivar and inoculation rate had a significant effect ($P < 0.05$) on number of lesions (Table 3.2). The two-way interactions were also significant for number of lesions at 35 DAI ($P < 0.05$) as well as largest lesion size ($P < 0.10$) (Table 3.2). Georgia-12Y had about 40% fewer lesions than Georgia-06G or Georgia-09B at 35 DAI. Georgia-12Y had half the number of lesions than Georgia-09B at 38 DAI (Table 3.2). Even though there was no significant difference, Georgia-12Y had a numerically lower AUDPC (912.8) than that of Georgia-06G or Georgia-09B (989.8 or 1170.4, respectively). Georgia-06G tended to have a longer incubation period than Georgia-09B and Georgia-12Y (Table 3.2). Inoculation rate also had a significant effect on number of lesions from 22 DAI through 38 DAI. The highest inoculation rate (7.5×10^3 conidia/ml) led to a greater number of lesions, but

sometimes not significantly greater than 5×10^3 conidia/ml (Table 3.2). The two-way interactions had a significant effect on number of lesions; the highest inoculation rate (7.5×10^3 conidia/ml) on Georgia-06G or Georgia-09B had highest number of lesions at 35 DAI and 38 DAI (Table 3.2). At 42 DAI, the lowest inoculation rate (2.5×10^3 conidia/ml) on Georgia-06G or Georgia-12Y had smallest lesion size (Table 3.2).

In the GH-2 experiment, cultivar had a significant effect ($P < 0.05$) on incubation period, number of lesions, largest lesion size at 30 DAI, and number of defoliated leaflets (Table 3.3). Georgia-09B had a shorter incubation period than Georgia-12Y or Georgia-06G (Table 3.3). Georgia-09B had more lesions than either Georgia-06G or Georgia-12Y, which had similar numbers of lesions (Table 3.3). For Georgia-06G, Georgia-09B, and Georgia-12Y, AUDPCs were 481.1, 4256.2, and 431.1, respectively (Fig. 3.3A). Georgia-09B had larger lesions than Georgia-06G or Georgia-12Y at 30 DAI; at 42 DAI, there was no difference in lesion size between these three cultivars (Table 3.3).

There was no significant difference in defoliation among these three cultivars until 34 DAI, after which Georgia-12Y had fewer defoliated leaflets than Georgia-06G or Georgia-09B; while Georgia-06G had less defoliation than Georgia-09B (Table 3.3, Fig. 3.3B).

3.5 DISCUSSION

In the growth chamber experiment, higher conidial concentrations for inoculation resulted in larger lesions and a greater number of sporulating lesions. The highest inoculation rate also resulted in largest lesion size, mostly because lesions coalesced to form one larger lesion. The middle inoculation rate (5×10^3 conidia/ml) actually resulted in distinct single lesions. Even though there was a numerical difference on the number of sporulating lesions between

inoculation concentrations of 5×10^3 conidia/ml and 7×10^3 conidia/ml, there was no significant difference. In the GH-1 experiment, the higher inoculation rate led to the greater number of lesions. These results are comparable with the findings of Ricker *et al.* (1985) in which higher inoculation concentrations (5×10^3 conidia/ml and 7×10^3 conidia/ml) led to a statistically similar numbers of lesions in the first 31 DAI. In the growth chamber study, the higher inoculation rate resulted in a numerically shorter incubation period, and this result is also similar to the findings of Ricker *et al.* (1985). Therefore, the inoculation rate of 5×10^3 conidia/ml was considered the most suitable inoculation density for further studies. This inoculation density is almost half of that used by Ricker *et al.* (1985), which was 9×10^3 conidia/ml. It should be noted that the plant material Ricker *et al.* (1985) used were rerooted shoot cuttings and detached leaves maintained at broader temperature ranges (19-39°C/ 14-28°C) than this study in which whole peanut plants were maintained in a greenhouse with temperature settings at 21-26°C. The optimal temperature for elongation of germ tubes of *C. arachidicola* is 22°C (Alderman and Beute, 1986), and the optimal temperature for highest lesion density was 22.8°C (Wu *et al.*, 1999). Therefore, our experiments had consistently closer temperature settings to optimal temperatures; under these more conducive environmental conditions, our inoculation density was expected to be lower compared to that used by Ricker *et al.* (1985).

All experiments indicated that Georgia-09B was the most susceptible cultivar of the three cultivars evaluated based on incubation period, greater numbers of lesions, larger lesion sizes, higher defoliation, and greater number of sporulating lesions. Georgia-09B showed consistently higher lesion and defoliation counts than the other two cultivars in both growth chamber and greenhouse experiments. This result is comparable with both “2015 Peanut Update” (Prostko *et al.*, 2015) and observations made by H. L. Campbell in 2014 (personal communication). Prostko

et al. (2015) gave leaf spot points for Georgia-06G, Georgia-09B, and Georgia-12Y as 20, 25, and 20, respectively, indicating that Georgia-09B was the most susceptible cultivar based on its higher peanut leaf spot points. Campbell observed that Georgia-09B scored 4.0 compared to scores of 3.5 and 3.2 for Georgia-06G and Georgia-12Y, respectively, using the Chiteka *et al.* (1988) scale (Florida 1 to 10 leaf spot rating scale where 3 = few lesions noticeable in lower and upper leaf canopy and 4 = some lesions noticeable with slight defoliation (< 10%)). In the growth chamber experiment, Georgia-09B had greater defoliation during the first 30 DAI; however, after 31 DAI, Georgia-12Y had a rapid increase in number of lesions as well as defoliated leaflets. This observation is comparable with unpublished field data (H. L. Campbell personal communication) in which Georgia-12Y had very good performance until some point late in the season, when many leaves defoliated. This led to a higher score of 6.1 for Georgia-12Y by the end of the season compared with 4.8 and 5.4 on Georgia-06G and Georgia-09B, respectively, based on the Florida 1 to 10 leaf spot rating scale in an irrigated field experiment (Hagan *et al.*, 2014a). In another study, Georgia-12Y had a score of 6.0 by the end of the season compared with 4.5 and 5.7 from Georgia-06G and Georgia-09B, respectively, based on the Florida leaf spot rating scale in a rain fed field experiment (Hagan *et al.*, 2014b). Another factor that should be considered is that Hagan *et al.* (2014 a; b) rated disease at 137, 150 and 158 DAP, much later than done herein (through 42 DAI, about 70 DAP); they also rated early and late leaf spots together. In the current growth chamber and greenhouse experiments, only ELS was evaluated since only *C. arachidicola* was inoculated on peanut plants.

Georgia-09B did not have a significantly higher AUDPC than the other two cultivars in the growth chamber, but this parameter was significantly higher for Georgia-09B in the greenhouse. Also, Georgia-09B had a significantly greater number of sporulating lesions than the

other cultivars in the growth chamber experiment; however, sporulating lesions were not observed in the greenhouse experiments. The differences in forming sporulating lesions between the growth chamber and the greenhouse experiments could be because the smaller container used in the growth chamber led to a less vigorous condition for peanut plants, causing plant stress and accelerated progress through growth stages, which may have contributed to the sudden increase in number of lesions and defoliated leaflets of Georgia-12Y. This was observed in other whole season field experiments (Hagan *et al.*, 2014a; b), but not in our greenhouse experiment due to a shorter experiment duration. This could also lead to the lack of sporulation in greenhouse since plants maintained in the greenhouse appeared healthier and more vigorous. It is possible that without the hosts reaching a certain growth stage, pathogens (e.g., *C. arachidicola*) might not reach their reproductive stage. Evaluation of pathogen response to host growth stages is needed to determine the actual mechanisms of the secondary infection.

Overall, both the growth chamber experiment and the greenhouse experiments led to a similar result, which is that the most susceptible cultivar was Georgia-09B of the three evaluated cultivars. Field screening is time-consuming and presents challenges due to presence of non-target pathogens; this adapted platform of an intermittent mist system in a greenhouse not only mimicked the natural environment for peanut plant growth and *C. arachidicola* infection, but also that extended the ability to examine larger plants in an open area rather than in limited growth chamber spaces.

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Table 3.1. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on whole peanut plants in growth chamber.

Source of variation	Incubation period	Number of lesions			Largest lesions diameter 42 DAI	Number of sporulating lesions 42 DAI
		8 DAI	31 DAI	42 DAI		
				<i>P</i> -value		
Cultivar	0.012	0.024	0.384	0.112	0.023	0.024
Inoculation level	0.258	0.213	0.465	0.741	0.001	0.004
Two way interaction	0.805	0.221	0.339	0.158	0.596	0.177
	days ^a	number			mm ^b	number
Cultivar						
GA ^c -06G	20.5 A ^d	0.8 B	9.3 A	50.1 A	3.4 AB	0.9 B
GA-09B	9.1 B	7.4 A	7.9 A	40.8 A	5.0 A	2.2 A
GA-12Y	26.0 A	0.1 B	3.2 A	103.1 A	2.5 B	0.7 B
Inoculation level						
2.5×10 ³ /ml	23.1 A	0.2 A	3.7 A	77.0 A	1.7 B	0.1 B
5×10 ³ /ml	17.9 A	4.6 A	8.9 A	64.2 A	3.3 B	1.3 A
7.5×10 ³ /ml	14.6 A	3.6 A	7.9 A	52.8 A	6.0 A	2.3 A

^aLeast square means from Proc Glimmix of days after inoculation until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves.

^cGA stands for Georgia.

^dMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Table 3.2. Factor significance and factor means in monocyclic components of *Cercospora arachidicola* on whole peanut plants in first greenhouse trial.

Source of variation	Incubation period	Number of lesions ^a				Largest lesions diameter 42 DAI	
		22 DAI	31 DAI	35 DAI	38 DAI		
- - - - - <i>P</i> -value - - - - -							
Inoculation level	0.212	0.089	0.075	0.000	0.004	0.228	
Cultivar	0.222	.	.	0.009	0.030	0.195	
Two way interaction	0.667	.	.	0.011	0.062	0.094	
Cultivar	days ^b	- - - - - number - - - - -				mm ^c	
GA ^d -06G	7.5 A ^e	.	.	40.2 A	69.2 AB	5.9 A	
GA-09B	6.7 A	.	.	44.7 A	78.8 A	7.2 A	
GA-12Y	5.5 A	.	.	26.7 B	40.0 B	3.7 A	
Inoculation level							
2.5×10 ³ /ml	5.5 A	11.5 B	15.8 B	19.2 C	32.5 B	3.8 A	
5×10 ³ /ml	7.0 A	18.0 A	23.8 AB	28.7 B	54.8 B	6.8 A	
7.5×10 ³ /ml	7.2 A	21.9 A	35.3 A	63.7 A	100.7 A	6.2 A	
Two way interaction							
2.5×10 ³ /ml	GA-06G	6.5 A	.	.	12.5 C	17.5 B	1.8 C
	GA-09B	5.0 A	.	.	28.0 BC	53.0 B	6.5 ABC
	GA-12Y	5.0 A	.	.	17.0 BC	27.0 B	3.0 C
5×10 ³ /ml	GA-06G	7.0 A	.	.	29.0 BC	63.0 B	13.0 A
	GA-09B	7.5 A	.	.	27.0 BC	50.5 B	5.0 ABC
	GA-12Y	6.5 A	.	.	30.0 B	51.0 B	2.5 C
7.5×10 ³ /ml	GA-06G	9.0 A	.	.	79.0 A	127.0 A	3.0 BC
	GA-09B	7.5 A	.	.	79.0 A	133.0 A	10.0 AB
	GA-12Y	5.0 A	.	.	33.0 B	42.0 B	5.5 ABC

^aCertain data points are not available due to entry restrictions for the greenhouse (due to chemical application). Time points are chosen based on their *P*-value as well as experimental time line.

^bLeast square means from Proc Glimmix of days after inoculation until first lesion observed.

^cLargest lesions diameter (mm, mini meter) is the mean diameter of three largest lesions from each plant's five evaluated leaves.

^dGA stands for Georgia.

^eMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Table 3.3. Factor significance and factor means in monocyclic components of *Cercospora arachidicola* on whole peanut plants in second greenhouse trial.

Cultivar	Incubation period (days) ^a	Number of lesions				Largest lesions diameter (mm) ^b		Number of Defoliation	
		7 DAI	22 DAI	30 DAI	42 DAI	30 DAI	42 DAI	34 DAI	42 DAI
GA ^c -06G	17.5 A ^d	0.1 B	9.7 B	19.2 B	33.0 B	1.1 B	1.1 A	3.1 A	5.8 A
GA-09B	7.5 B	9.5 A	136.9 A	139.6 A	118.5 A	2.0 A	1.2 A	3.4 A	9.2 A
GA-12Y	14.8 A	0.0 B	8.2 B	15.1 B	27.1 B	1.3 B	1.1 A	0.2 B	1.3 B
<i>P</i> -value	<0.001	0.046	<0.001	<0.001	0.012	0.040	0.650	0.017	0.002

^aLeast square means from Proc Glimmix of days after inoculation until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves. Due to defoliation, largest lesions diameter at 42 DAI are generally smaller than largest lesions diameter at 30 DAI.

^cGA stands for Georgia.

^dMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Figure 3.1. Disease progress curves by cultivar in a growth chamber: **A.** Disease progress curves based on number of lesions by cultivars on whole peanut plants. **B.** Number of defoliated leaflets per plant on each cultivar from whole peanut plants maintained.

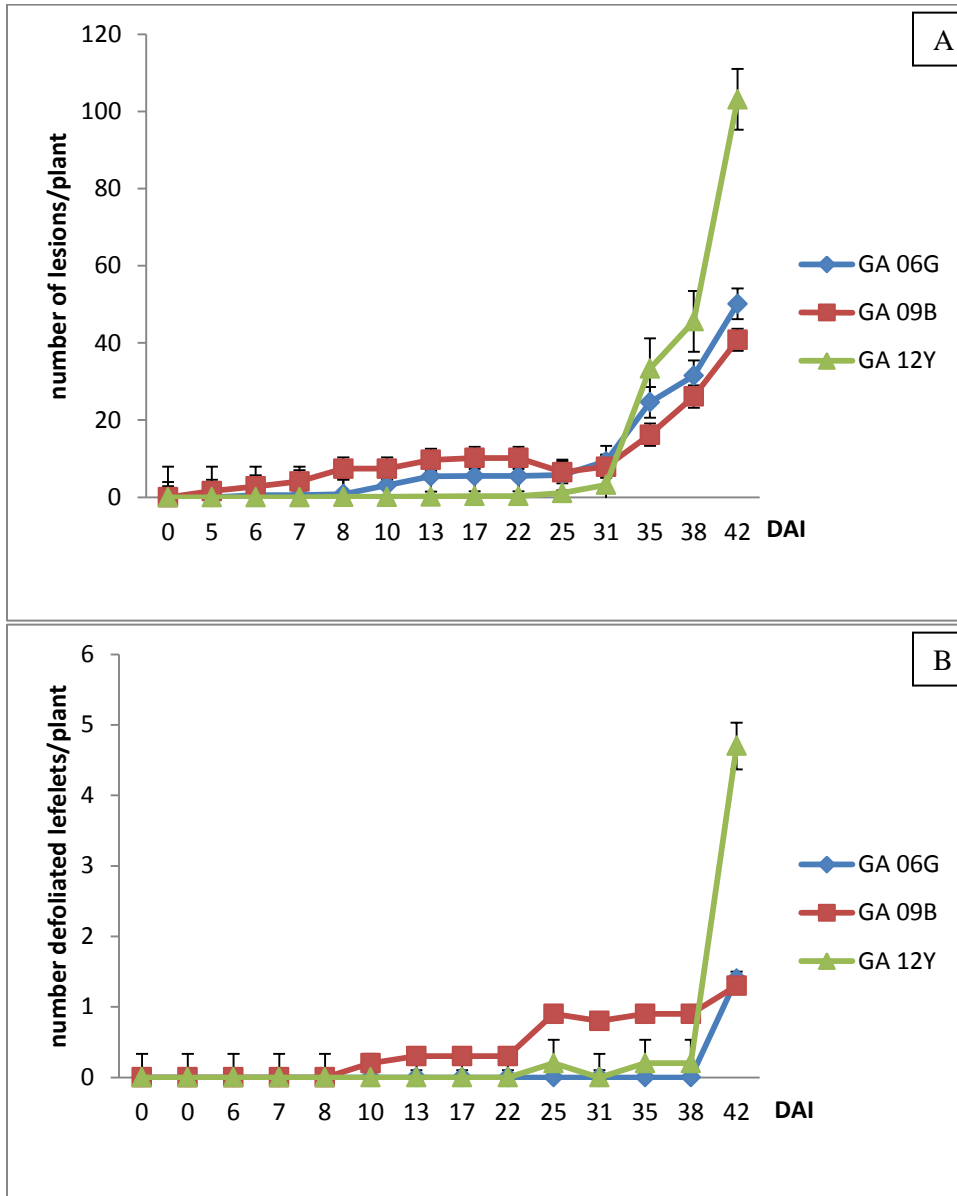


Figure 3. 2. Disease progress curves by inoculation level in a growth chamber: **A.** Disease progress curves based on number of lesions by inoculation rate on whole peanut plants. **B.** Number of defoliation leaflets based on inoculation rate whole peanut plants.

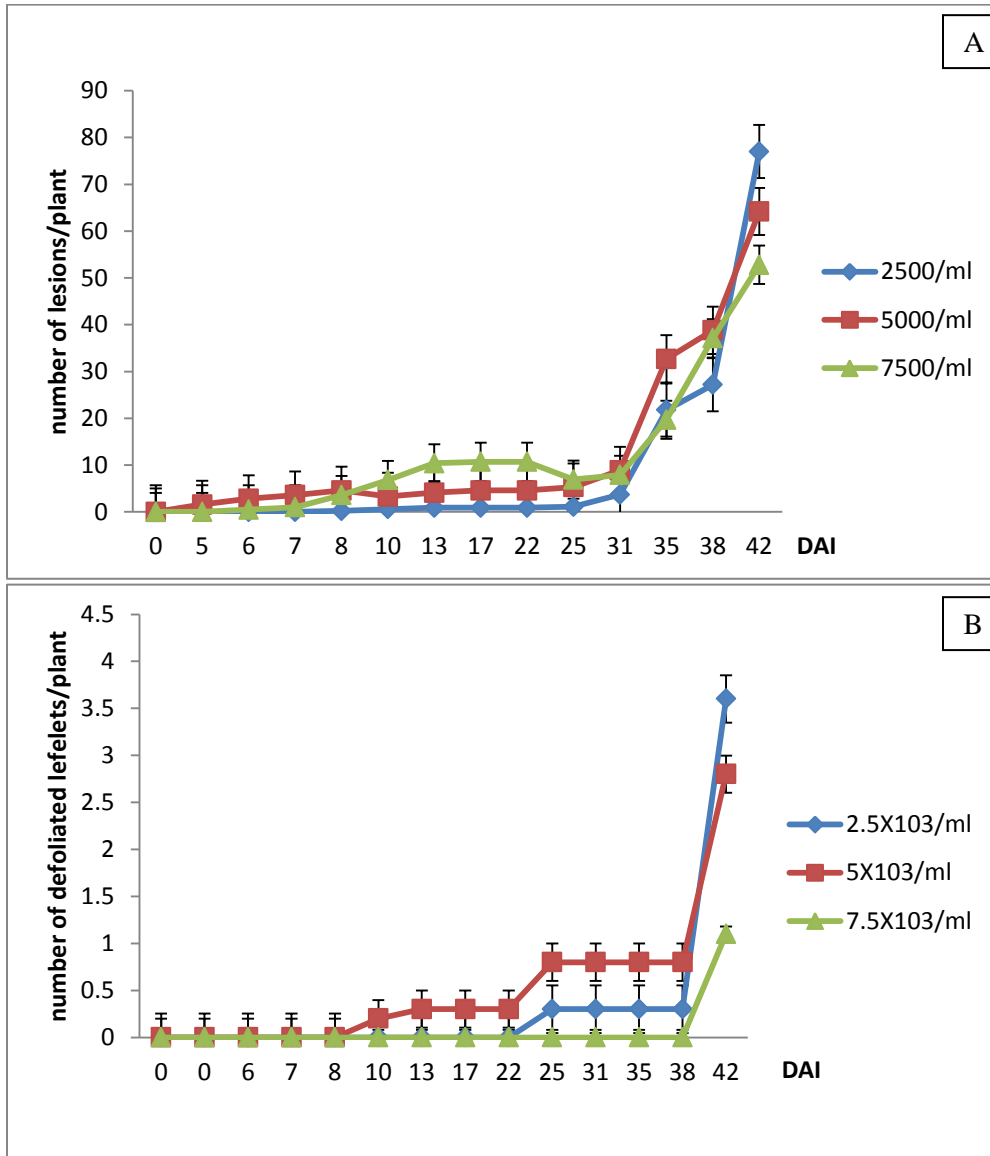
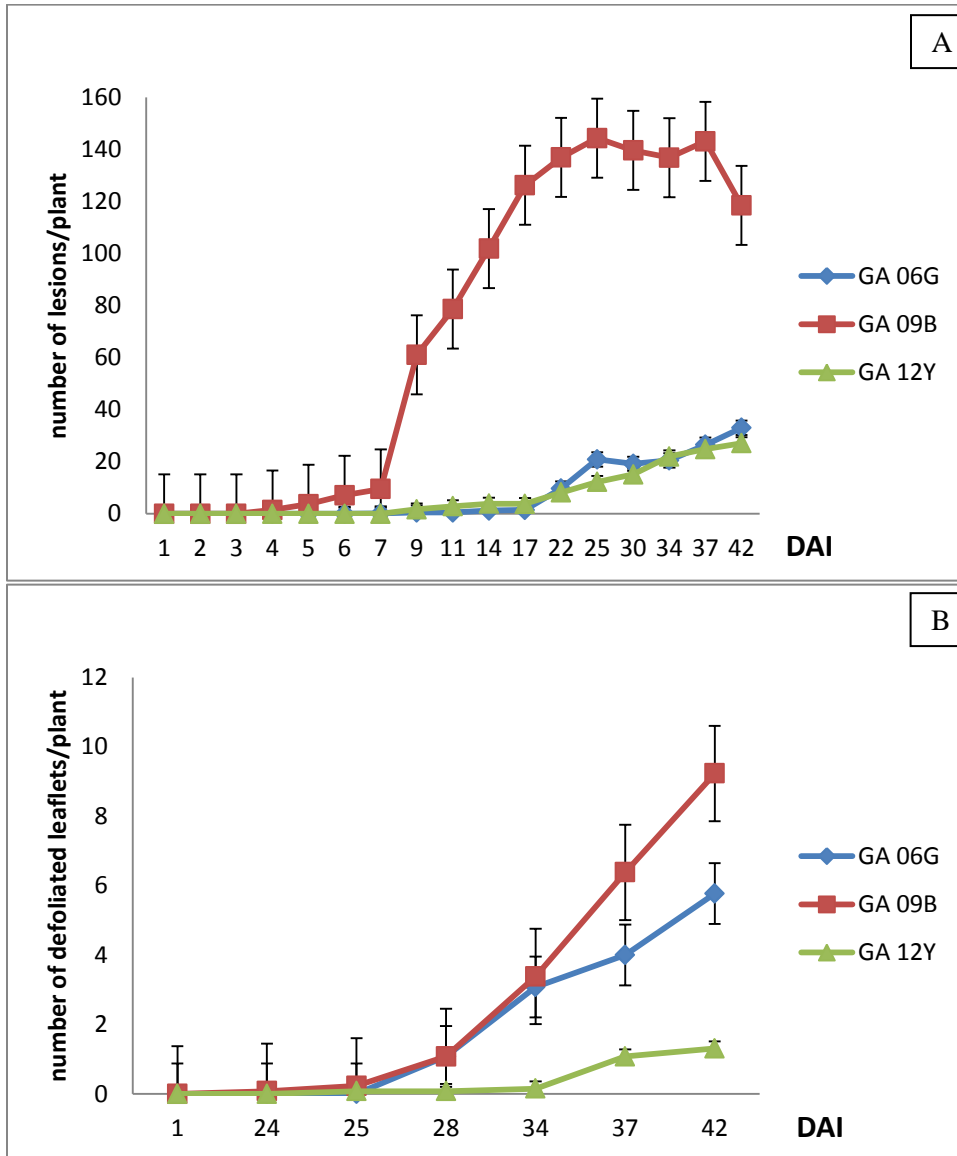


Figure 3.3. Disease progress curves by cultivar in the second greenhouse trial: **A.** Disease progress curves based on number of lesions by cultivars on whole peanut plants. **B.** Number of defoliation leaflets based on cultivars on whole peanut plants.



CHAPTER IV Monocyclic Components for Evaluating Disease Resistance to *Cercospora arachidicola* and *Cercosporidium personatum* in Peanut Plants

4.1 ABSTRACT

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop that is produced in the United States and throughout the world. However, there are two major fungal pathogens of cultivated peanuts, and they each contribute to substantial yield losses of 50% or greater. The pathogens of these diseases are *Cercospora arachidicola* which causes early leaf spot (ELS), and *Cercosporidium personatum* which causes late leaf spot (LLS). While fungicide treatments are fairly effective for leaf spot management, disease resistance is still the best strategy. Therefore, it is important to evaluate and compare different genotypes for their disease resistance levels. Six peanut genotypes (Chit P7, C1001, Exp27-1516, Flavor Runner 458, PI 268868, and GA-12Y) that included two genetically modified lines, a parent line, and commercial standard lines at both vegetative and reproductive growth stages (VGS and RGS, respectively) were inoculated with 5.0×10^3 conidia/ml; both *C. arachidicola* and *C. personatum* were separately evaluated. The monocyclic components evaluated were incubation period, number and size of lesions, and proportion of defoliation. Among all six genotypes, GA-12Y has the highest disease susceptibility to both ELS and LLS; in contrast, Flavor Runner 458 has the best disease resistance. Peanut plants inoculated with *C. arachidicola* at VGS have significantly fewer lesions at 30 days after inoculation than those inoculated at RGS. Peanut plants inoculated with *C. personatum* at VGS have a significantly longer incubation period, fewer lesions at both 30 and 42 DAI, smaller lesions, and less defoliation than those inoculated at RGS. These evaluations of monocyclic components as plant phenotype data could be used as a baseline for any disease resistance study.

Keywords: monocyclic components, disease resistance

4.2 INTRODUCTION

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop that is produced in the United States and throughout the world. The United States ranks fourth in the world for cultivated peanut production averaging 2.03 million metric tons annually over a five-year period (2009-2013) (USDA, 2014). However, globally, there are two major fungal pathogens of cultivated peanuts, and they each contribute to substantial yield losses of 50% or greater, depending on other elements such as climate and peanut cultivar (Cantonwine *et al.*, 2006; Cantonwine *et al.*, 2008; Waliyar *et al.*, 1995). The pathogens of these diseases are *Cercospora arachidicola*, which causes early leaf spot (ELS), and *Cercosporidium personatum*, which causes late leaf spot (LLS). Despite their names, these two diseases can occur at any time of the season. These two diseases cause necrotic lesions on leaves, petioles, stems, and pegs, resulting in eventual defoliation (Porter *et al.*, 1982). While fungicide treatments are fairly effective for leaf spot management, disease resistance is the best strategy for both environmentally benign and economic reasons. In addition, cultivar resistance avoids the risk of the development of resistance to fungicides (Castillo *et al.*, 2000; Gremillion *et al.*, 2011; Porter *et al.*, 1982). Therefore, it is important to evaluate and compare different peanut genotypes for their disease resistance levels.

Besides disease resistant peanut selection, creating transgenic peanut plants that over-express defense proteins is another option. Several studies have found that transgenic peanut plants with enhanced chitinase expression showed higher disease resistance. For example, Rohini and Rao (2001) studied transgenic peanut plants with a tobacco chitinase gene and found better

resistance to *C. arachidicola*. Iqbal *et al.* (2012) introduced a rice chitinase-3 gene using an enhanced version of the CaMV 35S promoter to transgenic peanut plants, and found it showed higher resistance against *C. arachidicola* as well. Prasad *et al.* (2013) reported transgenic peanut plants with an over-expressed rice (*Rchit*) chitinase gene had better resistance to *C. personatum*. All these studies suggest introducing a strong disease resistance gene could be a way to improve disease resistance of peanut plants.

The *Brassica juncea* chitinase *BjCHII* is unique not only because it is the only chitinase to our knowledge with two chitin-binding domains, but also because it has both chitinase and agglutinin activities (Tang *et al.*, 2004; Zhao and Chye, 1999). Several studies reported that tobacco and *Arabidopsis* transgenic plants with enhanced *BjCHII* had higher resistance to infection by *Trichoderma viride* and *Botrytis cinerea*, respectively (Fung *et al.*, 2002; Gao *et al.*, 2014). These findings provided the foundation of the possibility that transgenic peanut plants with the *B. juncea* chitinase gene *BjCHII* would also have the enhanced antifungal abilities.

The overall goal of this study was to determine resistance levels of genetically modified peanuts to ELS and LLS, using whole peanut plants in a greenhouse under intermittent mist. The objectives of this study were: 1) to compare ELS disease reactions of young plants to those of older plants; 2) to compare LLS disease reactions of young plants to those of older plants; and 3) to evaluate and compare leaf spot diseases resistance levels between parental line and transgenic lines.

4.3 MATERIALS AND METHODS

4.3.1 Inoculum production

Peanut leaves with ELS and LLS were collected from a peanut field at the E.V. Smith Research Center in Tallahassee, AL; *C. arachidicola* conidia from a single lesion on these leaves were collected and single spores were cultured on PDA media for three months. *C. personatum* conidia from a single lesion on these leaves were collected and single spores were cultured in peanut oatmeal broth (POB, Smith, 1971) for three months. The identities of the cultures were confirmed, using internal transcribed spacer (ITS) sequence analysis, as *C. arachidicola* and *C. personatum*. One clone was ground using a sterile konte pellet pestle (Kimble Chase, Rockwood, TN) and evenly spread on a V8 agar plate (Dhingra and Sinclair, 1985), left in the hood for 30 min to dry, then parafilmmed and placed under a growth light with a 12 hour photoperiod for one week. A daily microscopy check revealed peak sporulation between five to eight days for *C. arachidicola* and eight to eleven days for *C. personatum*. A solution of 0.005% Tween 20 was used to rinse conidia off the medium using gentle pipetting for higher yield. Four layers of cheese cloth were used to filter any mycelia residue. Conidia were counted with a hemocytometer and then adjusted to 5×10^3 /ml.

4.3.2 Peanut plants

Greenhouse experiments were conducted with six peanut genotypes that included genetically modified lines, a parent line, and commercial standard lines. These six genotypes were Chit P7, C1001, Exp27-1516, Flavor Runner 458, PI 268868, and GA-12Y. Among these six peanut genotypes, Chit P7 and C1001 are genetically modified lines overexpressing *BjCH11* gene and are derived from the parental line, Exp27-1516. Exp27-1516 is a medium-maturity

advanced breeding line, which carries some resistance to *Tomato spotted wilt virus* (TSWV) and leaf spot (Bostick *et al.*, 2010; Hagan *et al.*, 2009). Flavor Runner 458 is a high oleic cultivar and susceptible to most peanut diseases (Anonymous, 2013; Lemon *et al.*, 1999). PI 268868 is a medium-maturity genotype, originally from Sudan, which exhibited resistance to TSWV (Anderson *et al.*, 1996). GA-12Y is a later-maturity commercial cultivar that has good resistance to both TSWV and leaf spots, as well as very good resistance to white mold (Branch, 2013; Kemerait *et al.*, 2016). In order to maintain objectivity, identities of tested genotypes were not revealed to the experimenter prior to the end of all experiments. Reverse transcription-polymerase chain reaction were used to ensure that the inserted chitinase gene was expressed in transgenic lines (Appendix A).

These experiments used a randomized complete block design, with five replications of each treatment, for a total of thirty plants. Two seeds were planted in each one gallon pot containing potting mix (Sunshine mix # 8, Sun Gro Horticulture, Vancouver, Canada) and sand in a 1:1 (vol/vol) mixture, and then thinned to one plant per pot during the first two weeks. Plants at both vegetative and reproductive growth stages (VGS and RGS, respectively) were spray inoculated with 5×10^3 conidia/ml; *C. arachidicola* and *C. personatum* were separately evaluated. Following inoculation, all pots were arranged under Coolnet Pro 4-way Fogger nozzles, each attached to the 61 cm micronet hanging assembly (Netafim, Fresno, CA) and separated by one meter. Intermittent mist was scheduled for 6 s duration every 15 min from 10 p.m. to 10 a.m. daily with a Drip Irrigation Greenhouse 6 (DIG Corp., Vista, CA) station controller. The greenhouse temperature was set at 26 °C from 7 a.m. until 8:30 p.m. and 21 °C from 8:31 p.m. until 6:59 a.m. Plants were watered as needed and fertilized weekly with 20-10-20 Peat-Lite (J.R. Peters Inc., Scotts, Marysville, OH) at the rate of 250 ppm N.

4.3.2.1 *C. arachidicola* VGS

A total of four experiments (GH 1, 2, 4, and 10) were carried out in the greenhouse for evaluating ELS disease reactions of young plants, which were at VGS. Seeds were planted on July 30, 2015, September 10, 2015, November 16, 2015, and April 19, 2016. Three weeks after planting, all peanut plants had reached V6-V8 growth stages (Boote, 1982). All pots were then moved into the mist chamber, and a total volume of 0.5 ml *C. arachidicola* conidial suspension of 5×10^3 /ml was sprayed on each peanut plant in the evening.

4.3.2.2 *C. arachidicola* RGS

A total of three experiments (GH 6, 8, and 11) were carried out in the greenhouse for evaluating ELS disease reactions of older plants, which were at RGS. Seeds were planted on December 15, 2015, January 25, 2016, and March 29, 2016. Six weeks after planting, all peanut plants had reached R1-R2 growth stages (Boote, 1982). All pots were then moved into the mist chamber, and a total volume of 0.5 ml *C. arachidicola* conidial suspension of 5×10^3 /ml was sprayed on each peanut plant in the evening.

4.3.2.3 *C. personatum* VGS

Two experiments (GH 3 and 9) were carried out in the greenhouse for evaluating ELS disease reactions of young plants, which were at VGS. Seeds were planted on November 9, 2015 and January 26, 2016. Three weeks after planting, all peanut plants had reached V6-V8 growth stages (Boote, 1982). All pots were then moved into the mist chamber, and a total volume of 0.5

ml *C. personatum* conidial suspension of 5×10^3 /ml was sprayed on each peanut plant in the evening.

4.3.2.4 *C. personatum* RGS

Two experiments (GH 5 and 7) were carried out in the greenhouse for evaluating ELS disease reactions of older plants, which were at RGS. Seeds were planted on December 3, 2015 and January 15, 2016. Six weeks after planting, all peanut plants had reached R1-R2 growth stages (Boote, 1982). All pots were then moved into the mist chamber, and a total volume of 0.5 ml *C. personatum* conidial suspension of 5×10^3 /ml was sprayed on each peanut plant in the evening.

In order to compare ELS and LLS disease reactions of young plants to those of older plants, GH10 (*C. arachidicola* VGS) and 11 (*C. arachidicola* RGS) were inoculated at the same time (May 10, 2016), and GH 7 (*C. personatum* RGS) and 9 (*C. personatum* VGS) were inoculated at the same time (February 18, 2016).

4.3.3 Disease evaluation

Five oldest leaves of each plant (GH 1-9 experiments) and five leaves of each plant at the same leaf age (GH 10 and 11 experiments) were examined daily after *C. arachidicola* / *C. personatum* inoculation for evaluating monocyclic components (incubation period, number of lesions per leaflet, size of lesions, and defoliation) of ELS and LLS. Incubation period was defined as the number of days from inoculation to the first day on which lesions were observed. Number of lesions per leaflet was the total number of lesions observed from the evaluated five leaves divided by the total number of leaflets (20 leaflets). Size of lesions was determined as the

average diameter of the three largest lesions on the five leaves of each plant. Defoliation was determined by the number of defoliated leaflets from these same five leaves of each plant.

4.3.4 Data analysis

For these greenhouse studies, effects of cultivar, plant growth stage, and the two-way interaction on monocyclic components were determined with mixed model analysis (Proc Glimmix, SAS 9.4). A *P*-value less than or equal to 0.10 was considered significantly different. Means are significantly different according to Fisher's protected least significant difference (LSD) test ($P \leq 0.1$). All letters from the statistical results were given numerical values for ranking; for example, 1 was given to "A", 2 was given to "B", 1.5 was given to "AB", *etc.*, for all monocyclic components except incubation period, which was given the opposite value to correlate its contribution to the overall disease resistance level. Numerical values from the same treatment were added together from each repeat experiment and divided by the number of repeats for an average value. Average values for all monocyclic components were added together as a total value that represents the disease resistance level for each treatment.

4.4 RESULTS

4.4.1 Comparison ELS disease reactions of young plants to those of older plants

4.4.1.1 *C. arachidicola* inoculated VGS plants

In GH 1, genotype had no significant effect on incubation period or number of lesions per leaflet. However, the genotype had a significant effect on largest lesion sizes and number of defoliated leaflets at 30 and 42 DAI. PI 268868 had the largest lesions and GA-12Y had the highest number of defoliated leaflets (Table 4.1). In GH 2, genotype had a significant effect on

incubation period, number of lesions per leaflet, and largest lesion sizes at 30 and 42 DAI; the genotype also had a significant effect on number of defoliated leaflets at 42 DAI. For example, PI 268868 showed significantly longer incubation period compared to C1001 and GA-12Y (Table 4.2). In GH 4, the genotype had no significant effect on incubation period, but had a significant effect on number of defoliated leaflets at 42 DAI, number of lesions per leaflet and largest lesion sizes at both 30 and 42 DAI. For example, GA-12Y had more lesions per leaflet, more defoliation and larger lesions than the others (Table 4.3). In GH 10, the genotype had a significant effect on incubation period, number of lesions per leaflet at both 30 and 42 DAI, largest lesion sizes and number of defoliated leaflets at 42 DAI. For instance, Flavor Runner 458, Chit P7, and C1001 had significantly longer incubation period compared to Exp 27-1516 and GA-12Y (Table 4.4). When combined over repeated experiments, the ranking of ELS resistance of these six tested genotypes at VGS from least susceptible to most susceptible were: Flavor Runner 458, PI 268868, Exp27-1516, C1001, Chit P7, and GA-12Y (Table 4.10).

4.4.1.2 *C. arachidicola* inoculated RGS plants

In GH 6, the genotype had no significant effect on largest lesion sizes throughout the duration of the experiment. However, the genotype had a significant effect on incubation period, number of lesions per leaflet and number of defoliated leaflets at 42 DAI. PI 268868 and C1001 showed significantly longer incubation period compared to the other genotypes, GA-12Y had significant more lesions at 30 DAI and more defoliated leaflets at 42 DAI than most of the other genotypes (Table 4.5). In GH 8, the genotype had no significant effect on incubation period, largest lesion sizes at the experimental duration. However, genotype had a significant effect on number of lesions per leaflet at 30 DAI and number of defoliated leaflets at 30 and 42 DAI, with

GA-12Y having significantly more lesions than other genotypes at 30 DAI. GA-12Y had significantly more defoliation than Flavor Runner 458, Exp27-1516, PI 268868, and Chit P7 at 30 and 42 DAI (Table 4.6). In GH 11, the genotype had a significant effect on incubation period and defoliated leaflets at 42 DAI. Flavor Runner 458, Chit P7, and C1001 had significantly longer incubation period compared to GA-12Y. No other significant difference was observed in this experiment (Table 4.4). Combined over all repeated experiments, the ranking of ELS resistance of these six tested genotypes at RGS from least susceptible to most susceptible were: Flavor Runner 458, PI 268868, Exp27-1516, Chit P7, C1001, and GA-12Y (Table 4.10).

4.4.1.3 *C. arachidicola* inoculated VGS plants compared to RGS plants

In the GH 10 and 11 experiments, when both VGS and RGS plants were inoculated with *C. arachidicola* at the same time, significantly different incubation periods were observed between growth stages (VGS and RGS). Plants inoculated at RGS had significantly shorter incubation periods than those inoculated at VGS (Table 4.4). Furthermore, growth stage had a significant effect on number of lesions per leaflet and defoliated leaflets at both 30 and 42 DAI, plants inoculated at VGS had fewer lesions and less defoliation than those inoculated at RGS (Table 4.4).

In the GH 10 and 11 experiments, despite the growth stage, Chit P7 and C1001 had significantly longer incubation periods compared to Exp27-1516, PI 268868 and GA-12Y, with Flavor Runner 458 being intermediate. Chit P7, C1001, and Flavor Runner 458 had significantly fewer lesions per leaflet compared to GA-12Y at 30 and 42 DAI. Flavor Runner 458 had significantly fewer defoliated leaflets than Chit P7 and GA-12Y at 42 DAI. However, Flavor

Runner 458 developed significantly larger lesions compared to the other tested genotypes at 42 DAI (Table 4.4).

No significant difference in lesion size was observed in RGS experiments (Tables 4.4, 4.5, and 4.6). These results indicated that GA-12Y, PI 268868 and Flavor Runner 458 developed larger lesion sizes than other genotypes at VGS; however, this difference vanished for plants inoculated with *C. arachidicola* at RGS.

4.4.2 Comparison LLS disease reactions of young plants to those of older plants

4.4.2.1 *C. personatum* inoculated VGS plants

In GH 3, the genotype had no significant effect on incubation period or number of defoliated leaflets throughout the duration of the experiment. However, the genotype had a significant effect on number of lesions per leaflet at 42 DAI and largest lesion sizes 42 DAI. For instance, GA-12Y had significantly larger lesions compared to Flavor Runner 458, Exp27-1516, PI 268868, and Chit P7 at 30 DAI (Table 4.7). In GH 9, the genotype had no significant effect on incubation period and largest lesion sizes at the experimental duration; the genotype had a significant effect on number of lesions per leaflet and number of defoliated leaflets at 42 DAI, which is GA-12Y had significantly more lesions per leaflet and defoliation than Flavor Runner 458, Exp27-1516, PI 268868, and Chit P7 (Table 4.8). Combined over all experimental repeats, the ranking of LLS resistant levels of these six tested genotypes at RGS from least susceptible to most susceptible was PI 268868, Flavor Runner 458 or Exp27-1516, C1001, Chit P7, and GA-12Y (Table 4.11).

4.4.2.2 *C. personatum* inoculated RGS plants

In GH 5, the genotype had no significant effect on incubation period and largest lesion sizes at the experimental duration. However, the genotype had a significant effect on number of lesions per leaflet at 30 and 42 DAI, such as GA-12Y had significantly more number of lesions than other genotypes at 30 DAI (Table 4.9). Defoliation was not observed in this GH 5 experiment. In GH 7, the genotype had a significant effect on incubation period, number of lesions per leaflet 30 DAI, and number of defoliated leaflets at 30 and 42 DAI. For example, PI 268868 had a significantly longer incubation period compared to C1001 and GA-12Y. The genotype also had a significant effect on largest lesion sizes at 30 DAI. For instance, GA-12Y had significantly larger lesions compared to Flavor Runner 458, Exp27-1516, and PI 268868 (Table 4.8). Combined over all repeats and monocyclic components, the ranking of LLS resistant levels of these six tested genotypes at RGS from least susceptible to most susceptible was PI 268868, Flavor Runner 458 or Exp27-1516, Chit P7, C1001, and GA-12Y (Table 4.11).

4.4.2.3 *C. personatum* inoculated VGS plants compared to RGS plants

In GH 7 and 9 experiments, when both VGS and RGS plants were inoculated with *C. personatum* at the same time, significantly different incubation periods were observed between both growth stages (VGS and RGS). Plants inoculated at RGS had significantly shorter incubation periods than those inoculated at VGS (Table 4.8). Furthermore, growth stage had a significant effect on number of lesions per leaflet and largest lesions at both 30 and 42 DAI. Plants inoculated at VGS had fewer lesions and less defoliation than those inoculated at RGS. Growth stage also had a significant effect on number of defoliated leaflets at 42 DAI; lower defoliation was observed on plants inoculated at VGS than others inoculated at RGS (Table 4.8).

In the GH 9 and 7 experiments, despite the growth stage, GA-12Y had a significantly shorter incubation period and larger lesions at 42 DAI compared to Flavor Runner 458, Exp27-1516, and PI 268868; Flavor Runner 458, Exp27-1516, and PI 268868 had significantly fewer number of lesions per leaflet compared to C1001 and GA-12Y at 42 DAI; GA-12Y had significantly more defoliation than the rest tested genotypes at 42 DAI (Table 4.8).

The two-way interaction had a significant effect on number of defoliated leaflets. GA-12Y inoculated at RGS had significantly more defoliated leaflets compared to other genotypes. No other significant differences were observed for the two-way interaction (Table 4.8).

4.4.3 Comparison of leaf spot diseases resistance levels between parental line and transgenic lines.

Both transgenic peanut lines, Chit P7 and C1001, were tested in this study along with their parental line, which is Exp27-1516. Both transgenic lines had significantly longer incubation periods compared to their parental line in GH 10 (Table 4.4). C1001 also had significantly longer incubation period compared to the parental line in GH6 (Table 4.5). Both transgenic lines developed significantly fewer numbers of lesions per leaflet than the parental line at 30 DAI in GH 10 (Table 4.4). Chit P7 had significantly more lesions per leaflet than parental line at 30 DAI in GH 2 (Table 4.2); C1001 had significantly more lesions per leaflet compared to its parental line at 30 DAI in GH 7, and at 42 DAI in GH 5 and 9 (Table 4.8, 4.9). Both transgenic lines showed significantly higher defoliation compared to their parental line at 42 DAI in GH 2 (Table 4.2); C1001 had significantly higher defoliation compared to its parental line at 42 DAI in GH 7, 8 and 9 (Table 4.6, 4.8).

4.5 DISCUSSION

In all the *C. arachidicola* inoculated vegetative growth stage peanut plants, Flavor Runner 458, PI 268868, and transgenic line Chit P7 consistently had long incubation periods. In all the *C. arachidicola* inoculated reproductive growth stage peanut plants, PI 268868 and transgenic lines, C1001 and Chit P7, had longer incubation periods than other lines. As for disease cycle, the longer incubation period indicates better disease resistance of the host (Agris, 2014). These results suggest that PI 268868 and transgenic line Chit P7 consistently displayed better disease resistance than other tested lines; in contrast, GA-12Y consistently was least resistant. GA-12Y also had more lesions per leaflet, more defoliation, and larger lesions compared to most of the other tested genotypes, which suggests it is least resistant against ELS regardless of growth stage. In contrast, Flavor Runner 458 had fewest numbers of lesions per leaflet, less defoliation, and smaller lesions compared to most of the other tested genotypes despite growth stages, which suggest it is the least susceptible to ELS.

Similarly, in all the *C. personatum* inoculated vegetative growth stage peanut plants, Flavor Runner 458, PI 268868, and Chit P7 (transgenic line) consistently had long incubation periods. In all the *C. personatum* inoculated reproductive growth stage peanut plants, PI 268868 and transgenic lines, C1001 and Chit P7, had longer incubation periods than remaining lines. In contrast, GA-12Y consistently had a shorter incubation period regardless of growth stage. In all the *C. personatum* inoculated peanut plants, GA-12Y also consistently had a significantly higher number of lesions per leaflet and defoliated leaflets. In contrast, Flavor Runner 458 had consistently less number of lesions per leaflet and defoliated leaflets. Furthermore, GA-12Y had the largest lesions when inoculated with *C. personatum* at VGS. Considering the combination of

growth stage and genotype, GA-12Y at RGS had the lowest LLS resistance, while Flavor Runner 458 at VGS had the best LLS resistance based on defoliation.

This finding regarding Flavor Runner 458 suggests that it is the least susceptible genotype to ELS and the second least susceptible genotype to LLS among six tested genotypes. However, this finding appears to be in conflict with unpublished field data (C. Y. Chen, personal communication) in which Flavor Runner 458 was found to be susceptible to most peanut diseases. A possible reason for this discrepancy could be that peanut plants in the field can be affected by multiple diseases, in contrast to the case of plants evaluated in a greenhouse where only one disease was introduced by inoculation. It is also possible that certain attributes of Flavor Runner 458, such as its leaf spot resistance, may be overlooked by researchers because of its susceptibility to other peanut diseases such as TWSV and white mold, among others.

In conclusion, growth stages had a significant effect on most of the monocyclic components; plants at VGS have better disease resistance, despite leaf age. This conclusion is in line with the finding reported by Zhang (2001) in which peanut plants inoculated at 3-weeks had significantly lower disease severity than plants inoculated at 4, 5, or 6 weeks of age. The conclusion from this study differs from those of Pretorius *et al.* (1988) and Reuveni *et al.* (1986) who noted that older plants showed increased resistance in wheat against rust, caused by *Puccinia recondita* f.sp. *tritici* and tobacco against *Peronospora tabacina*, respectively. This difference could be because exhibiting age related resistance requires salicylic acid (SA) accumulation, which mostly shows in plants expressing the salicylate hydroxylase gene (NahG gene, Kus *et al.*, 2002). Regarding this SA accumulation in peanut, there are contrasting results in previous studies (Cardoza *et al.*, 2003; Chitra *et al.*, 2008; Kobeasy *et al.*, 2011; Yan *et al.*, 2013; Zhang *et al.*, 2001). Among all six genotypes, GA-12Y has the lowest disease resistance to

both ELS and LLS; in contrast, Flavor Runner 458 has the best disease resistance to ELS and PI 268868 has the best disease resistance to LLS. Transgenic lines did not show improved disease resistance in this study.

Previous evaluation studies focused on comparing two of the monocyclic components: incubation period and latent period; all data from repeated experiments were combined as replications before analysis (Broers, 1997; Czaja et al., 2016; Mersha *et al.*, 2014). The main reason for this is the lack of practical methods to combine results from experimental repeats to develop comprehensive results. A different method is needed because other monocyclic components should also be included when evaluating disease resistance levels for cultivars, and there might be inconsistent results from each repeat, mainly due to the fact that plants react differently to environmental conditions, such as time of the year, temperature variations, light variations, *etc.* In this study, a ranking method was adopted based on statistical results. All repeats were given the same weight (final results were based on average values) and statistical significance carried comparable weight (more significance led to a higher numerical value). This method solved the data analysis problem of evaluating treatments with several variables and repeats.

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Table 4.1. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on vegetative growth stage peanut plants as GH 1.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	7.0 A ^c	4.0 A	5.5 A	1.2 B	7.2 C	1.6 B ^c	3.2 B
Exp27-1516	4.4 A	6.0 A	8.2 A	4.6 AB	13.4 B	2.4 AB	2.3 B
PI 268868	6.2 A	4.5 A	5.7 A	4.4 AB	9.6 BC	4.2 A	6.0 A
Chit P7	5.6 A	3.5 A	8.0 A	5.0 AB	14.0 AB	0.6 B	1.2 B
C1001	5.0 A	5.2 A	6.6 A	8.2 A	14.6 AB	1.3 B	1.9 B
GA-12Y	4.2 A	2.2 A	5.3 A	8.0 A	19.4 A	0.5 B	1.0 B
<i>P</i> -value	0.624	0.316	0.604	0.080	0.003	0.011	0.001

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter of three largest lesions from each plant's five evaluated leaves.

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Table 4.2. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on vegetative growth stage peanut plants as GH 2.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	7.8 ABC ^c	0.9 D	1.4 B	0.0 A	0.4 CD	0.2 CD	0.7 C
Exp27-1516	7.6 ABC	1.7 CD	2.6 B	0.0 A	0.0 D	0.8 BC	2.0 ABC
PI 268868	9.4 A	2.5 BC	2.7 B	1.0 A	1.8 BCD	0.2 D	1.2 BC
Chit P7	8.2 AB	3.2 AB	3.9 B	0.8 A	3.2 B	1.4 AB	2.4 AB
C1001	5.0 C	2.6 BC	2.8 B	0.4 A	2.4 BC	0.9 B	1.3 BC
GA-12Y	6.2 BC	4.4 A	8.8 A	0.2 A	10.6 A	1.7 A	3.1 A
<i>P</i> -value	0.055	0.001	0.001	0.2161	<0.001	<0.001	0.016

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter of three largest lesions from each plant's five evaluated leaves.

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

Table 4.3. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on vegetative growth stage peanut plants as GH 4.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number		- - - - -		- - mm ^b - -	
Flavor Runner 458	16.8 A ^c	0.5 B	0.7 B	. ^d	7.2 C	0.9 AB	1.7 B
Exp27-1516	20.0 A	0.2 B	0.6 B	.	13.4 B	0.3 B	0.4 B
PI 268868	16.8 A	0.2 B	0.6 B	.	9.6 BC	0.5 B	0.7 B
Chit P7	21.0 A	0.2 B	0.9 B	.	14.0 AB	0.4 B	1.1 B
C1001	22.3 A	0.2 B	1.2 B	.	14.6 AB	0.5 B	1.8 B
GA-12Y	17.4 A	1.2 A	5.1 A	.	19.4 A	1.5 A	4.2 A
<i>P</i> -value	0.269	0.044	0.017	.	0.003	0.028	0.003

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter of three largest lesions from each plant's five evaluated leaves.

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

^dNo defoliation was observed at 30 day after inoculation.

Table 4.4. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on both vegetative and reproductive growth stage peanut plants as GH 10 and 11.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter			
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI		
VGS ^a	days ^b	- - - - - number - - - - -						- - mm ^c - -	
Flavor Runner 458	18.0 A ^d	1.1 AB	2.5 AB	0.4 A	3.0 C	2.2 A	7.1 A		
Exp27-1516	9.0 B	1.9 A	1.8 B	0.0 A	6.5 BC	0.7 A	2.1 B		
PI 268868	15.5 AB	1.3 AB	1.4 B	1.5 A	3.2 C	0.7 A	1.1 B		
Chit P7	21.3 A	0.5 B	2.3 AB	1.8 A	10.2 AB	0.4 A	1.4 B		
C1001	21.5 A	0.8 B	1.3 B	1.0 A	6.5 BC	1.2 A	1.9 B		
GA-12Y	11.0 B	1.8 A	5.7 A	2.0 A	13.0 A	0.5 A	2.0 B		
<i>P</i> -value	0.009	0.069	0.002	0.648	0.011	0.385	0.052		
RGS									
Flavor Runner 458	14.6 AB	2.7 A	4.9 A	1.4 A	6.0 C	1.7 A	5.1 A		
Exp27-1516	12.4 BC	2.8 A	5.7 A	5.6 A	10.0 BC	0.7 A	2.3 A		
PI 268868	13.0 ABC	2.7 A	8.5 A	3.7 A	12.5 B	0.7 A	1.2 A		
Chit P7	17.8 A	2.3 A	5.1 A	5.4 A	10.6 BC	1.2 A	2.6 A		
C1001	17.6 AB	2.4 A	3.2 A	3.2 A	9.4 BC	1.7 A	2.6 A		
GA-12Y	8.8 C	4.6 A	7.5 A	7.0 A	16.8 A	1.2 A	0.6 A		
<i>P</i> -value	0.026	0.253	0.268	0.164	0.067	0.217	0.342		
Source of variation	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter			
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI		
- - - - - <i>P</i> -value - - - - -									
Growth stage	0.098	0.005	0.016	<0.001	0.085	0.433	0.902		
Cultivar	<0.001	0.073	0.061	0.142	0.001	0.081	0.011		
Two way interaction	0.567	0.824	0.514	0.545	0.614	0.724	0.842		
Growth stage	days	- - - - - number - - - - -						- - mm - -	
VGS	16.0 A	1.2 B	2.5 B	1.1 B	7.1 B	0.9 A	2.6 A		
RGS	14.0 B	2.9 A	5.8 A	4.4 A	10.9 A	1.2 A	2.4 A		
Genotype									
Flavor Runner 458	16.3 AB	1.9 B	3.6 B	0.9 B	4.5 C	1.9 A	6.1 A		
Exp27-1516	10.7 C	2.3 AB	3.7 AB	2.8 AB	8.3 BC	0.7 B	2.2 B		
PI 268868	14.3 BC	2.0 AB	4.9 AB	2.6 AB	7.8 BC	0.7 B	1.1 B		
Chit P7	19.5 A	1.4 B	3.6 B	3.6 A	10.4 B	0.8 B	2.0 B		
C1001	19.6 A	1.6 B	2.2 B	2.1 AB	7.96 BC	1.42	2.3 B		
GA-12Y	9.9 C	3.2 A	6.6 A	4.5 A	14.91 A	0.85 B	1.4 B		

^aVGS and RGS stands for Vegetative growth stage and Reproductive growth stage, respectively.

^bLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^cLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves.

^dMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values, non-significant data is not presented in the table.

Table 4.5. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on reproductive growth stage peanut plants as GH 6.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	12.2 B ^c	0.2 A	0.70 C	0.0 A	0.8 B	0.3 A	0.7 A
Exp27-1516	14.2 B	0.3 A	0.75 BC	0.4 A	2.2 B	0.9 A	3.1 A
PI 268868	25.3 A	0.1 A	1.11 BC	0.0 A	0.4 B	0.5 A	2.8 A
Chit P7	15.4 B	0.3 A	1.83 AB	0.2 A	3.4 AB	1.3 A	4.0 A
C1001	27.7 A	0.1 A	0.75 BC	1.6 A	2.6 AB	0.8 A	3.6 A
GA-12Y	11.4 B	0.5 A	2.83 A	2.0 A	6.9 A	1.4 A	6.9 A
<i>P</i> -value	0.001	0.166	0.003	0.1046	0.024	0.3121	0.2378

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values, non-significant data is not presented in the table.

Table 4.6. Factor significance and factor means in evaluation of monocyclic components of *Cercospora arachidicola* on reproductive growth stage peanut plants as GH 8.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	10.0 A ^c	0.4 B	0.4 A	0.0 B	0.3 C	0.60 A	1.0 A
Exp27-1516	6.0 A	0.6 B	0.6 A	0.0 B	0.7 C	0.64 A	0.7 A
PI 268868	13.0 A	0.4 B	0.6 A	1.3 B	1.3 BC	0.59 A	0.7 A
Chit P7	12.3 A	0.6 B	0.9 A	2.0 B	2.7 BC	1.27 A	1.3 A
C1001	10.7 A	0.6 B	0.8 A	4.0 AB	6.7 B	1.56 A	1.7 A
GA-12Y	7.3 A	1.8 A	2.3 A	9.0 A	13.3 A	1.93 A	2.0 A
<i>P</i> -value	0.5654	<0.001	0.154	0.058	0.002	0.123	0.177

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values, non-significant data is not presented in the table.

Table 4.7. Factor significance and factor means in evaluation of monocyclic components of *Cercosporidium personatum* on vegetative growth stage peanut plants as GH 3.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	number				mm ^b	
Flavor Runner 458	12.4 A ^c	0.6 A	0.9 B	0.0 A	0.0 A	. ^d	0.62 BC
Exp27-1516	13.8 A	0.7 A	0.9 B	0.2 A	0.2 A	.	0.50 BC
PI 268868	12.4 A	0.4 A	0.5 B	0.2 A	0.8 A	.	0.27 C
Chit P7	9.0 A	0.6 A	1.0 B	0.0 A	1.25 A	.	0.89 AB
C1001	13.6 A	0.7 A	0.8 B	0.6 A	4.0 A	.	0.62 BC
GA-12Y	11.2 A	0.8 A	2.1 A	0.0 A	4.0 A	.	1.16 A
<i>P</i> -value	0.347	0.563	0.063	0.461	0.287	.	0.006

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values, non-significant data is not presented in the table.

^dLesions were to small to measure at 30 day after inoculation.

Table 4.8. Factor significance and factor means in evaluation of monocyclic components of *Cercosporidium personatum* on both vegetative and reproductive growth stage peanut plants as GH 9 and 7.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
VGS	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	31.0 A	0.0 A	0.47 C	.	0.0 C	0.0 A	0.5 A
Exp27-1516	30.2 A	0.0 A	0.59 C	.	1.0 C	0.1 A	0.6 A
PI 268868	26.8 A	0.1 A	0.56 C	.	0.4 C	0.5 A	1.4 A
Chit P7	27.6 A	0.1 A	0.65 BC	.	2.8 BC	0.9 A	0.7 A
C1001	32.6 A	0.1 A	1.14 AB	.	6.0 AB	0.4 A	0.8 A
GA-12Y	22.4 A	0.3 A	1.58 A	.	8.2 A	0.7 A	1.7 A
<i>P</i> -value	0.2891	0.328	<0.001	.	0.002	0.312	0.161
RGS							
Flavor Runner 458	17.6 AB ^d	0.7 B	1.0 A	0.0 B	0.0 C	1.9 B	1.7 A
Exp27-1516	15.8 ABC	0.7 B	1.2 A	0.0 B	1.8 C	2.4 B	2.6 A
PI 268868	22.2 A	0.4 B	0.9 A	0.0 B	1.4 C	1.7 B	1.2 A
Chit P7	14.2 ABC	1.0 AB	1.5 A	0.6 B	3.2 BC	4.0 AB	1.5 A
C1001	10.2 BC	1.6 A	1.7 A	0.2 B	6.2 B	3.7 AB	2.1 A
GA-12Y	8.6 C	1.2 AB	1.8 A	4.4 A	17.2 A	7.4 A	2.5 A
<i>P</i> -value	0.035	0.074	0.176	0.007	<0.001	0.093	0.110
Source of variation	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
		- - - - - <i>P</i> -value - - - - -					
Growth stage	<0.001	<0.001	0.022	.	0.097	<0.001	0.003
Cultivar	0.053	0.044	0.002	.	<0.001	0.037	0.130
Two way interaction	0.152	0.117	0.805	.	0.007	0.142	0.129
Growth stage	days	- - - - - number - - - - -				- - mm - -	
VGS	28.4 A	0.9 A	0.8 B	.	3.1 B	0.5 B	0.9 B
RGS	14.8 B	0.1 B	1.4 A	.	5.0 A	3.5 A	1.9 A
Genotype				.			
Flavor Runner 458	24.3 A	0.36 C	0.8 C	.	0.0 D	1.0 B	1.1 A
Exp27-1516	23.0 A	0.38 BC	0.9 C	.	1.4 CD	1.2 B	1.6 A
PI 268868	23.0 A	0.27 C	0.7 C	.	0.9 CD	1.1 B	1.3 A
Chit P7	20.9 AB	0.55 ABC	1.1 BC	.	3.0 C	2.5 AB	1.1 A
C1001	21.4 AB	0.81 A	1.4 AB	.	6.1 B	2.1 AB	1.5 A
GA-12Y	15.5 B	0.78 AB	1.7 A	.	12.7 A	4.0 A	2.1 A
Two way interaction							

V	Flavor Runner	31.0 A	0.0 A	0.5 A	.	0.0 D	0.0 A	0.5 A
G	458							
S	Exp27-1516	30.2 A	0.0 A	0.6 A	.	1.0 D	0.1 A	0.6 A
	PI 268868	26.8 A	0.1 A	0.6 A	.	0.4 D	0.5 A	1.4 A
	Chit P7	27.6 A	0.1 A	0.6 A	.	2.8 CD	0.9 A	0.7 A
	C1001	32.6 A	0.1 A	1.1 A	.	6.0 BC	0.4 A	0.8 A
	GA-12Y	22.4 A	0.3 A	1.6 A	.	8.2 B	0.7 A	1.7 A
R	Flavor Runner	17.6 A	0.7 A	1.0 A	.	0.0 D	1.9 A	1.7 A
G	458							
S	Exp27-1516	15.8 A	0.7 A	1.2 A	.	1.8 D	2.4 A	2.6 A
	PI 268868	22.2 A	0.4 A	0.9 A	.	1.4 D	1.7 A	1.2 A
	Chit P7	14.2 A	1.0 A	1.5 A	.	3.2 CD	4.0 A	1.5 A
	C1001	10.2 A	1.6 A	1.7 A	.	6.2 BC	3.7 A	2.1 A
	GA-12Y	8.6 A	1.2 A	1.8 A	.	17.2 A	7.4 A	2.5 A

^aVGS and RGS stands for Vegetative growth stage and Reproductive growth stage, respectively.

^bLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^cLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves.

^dMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values.

^eNo defoliation was observed during the experimental period.

Table 4.9. Factor significance and factor means in evaluation of monocyclic components of *Cercosporidium personatum* on reproductive growth stage peanut plants as GH 5.

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter	
		30 DAI	42 DAI	30 DAI	42 DAI	30 DAI	42 DAI
	days ^a	- - - - - number - - - - -				- - mm ^b - -	
Flavor Runner 458	25.6 A ^c	0.5 B	1.1 BC	.	.	1.5 A	3.7 A
Exp27-1516	29.5 A	0.3 B	0.7 C	.	.	0.6 A	3.0 A
PI 268868	32.2 A	0.2 B	0.9 BC	.	.	0.6 A	4.6 A
Chit P7	26.0 A	0.3 B	1.7 BC	.	.	1.0 A	2.3 A
C1001	30.5 A	0.2 B	1.9 AB	.	.	1.1 A	2.7 A
GA-12Y	23.0 A	1.2 A	2.7 A	.	.	1.9 A	3.7 A
<i>P</i> -value	0.121	0.026	0.005	.	.	0.145	0.573

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of three largest lesions from each plant's five evaluated leaves

^cMeans in each column with the same letter do not differ at $P \leq 0.1$ based on Fisher's protected LSD values, non-significant data is not presented in the table.

^dNo defoliation was observed during the experimental period.

Table 4.10. Ranks of *Cercospora arachidicola* inoculated plants for disease resistance levels based on monocyclic components at both vegetative growth stage (VGS) and reproductive growth stage (RGS).

Genotype	Incubation period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter		Actual total	Rank
		30	42	30	42	30	42		
		DAI	DAI	DAI	DAI	DAI	DAI		
VGS		----- rank ^a -----							
Flavor Runner 458	3	1	4	1	1	1	2	13	1
Exp27-1516	4	2	1	2	3	3	2	17	3
PI 268868	1	4	1	2	2	1	4	15	2
Chit P7	1	5	4	2	5	5	4	26	5
C1001	4	2	1	5	4	3	1	20	4
GA-12Y	6	6	6	5	6	6	6	41	6
RGS									
Flavor Runner 458	4	1	1	1	1	1	1	10	1
Exp27-1516	5	1	2	1	2	1	1	13	3
PI 268868	2	1	2	1	3	1	1	11	2
Chit P7	2	1	5	1	3	1	1	14	4
C1001	1	1	2	5	5	1	1	16	5
GA-12Y	6	6	6	6	6	1	1	32	6

^aGenotypes were ranked from least susceptible (1) to most susceptible (6).

Table 4.11. Ranks of *Cercospora personatum* inoculated plants for disease resistance levels based on monocyclic components at both vegetative growth stage (VGS) and reproductive growth stage (RGS).

Genotype	Incuba tion period	Lesions per leaflet		Defoliated leaflets		Largest lesions diameter		Actural total	Rank
		30	42	30	42	30	42		
		DAI	DAI	DAI	DAI	DAI	DAI		
VGS									
Flavor Runner 458	1	1	1	1	1	1	2	8	2
Exp27-1516	1	1	1	1	1	1	2	8	2
PI 268868	1	1	1	1	1	1	1	7	1
Chit P7	1	1	4	1	4	1	5	17	5
C1001	1	1	5	1	5	1	2	16	4
GA-12Y	1	1	6	1	6	1	6	22	6
RGS									
Flavor Runner 458	2	1	2	1	1	1	1	9	2
Exp27-1516	3	1	1	1	1	1	1	9	2
PI 268868	1	1	2	1	1	1	1	8	1
Chit P7	3	4	2	1	4	4	1	19	4
C1001	5	5	5	1	5	4	1	26	5
GA-12Y	6	6	6	6	6	6	1	37	6

^aGenotypes were ranked from least susceptible (1) to most susceptible (6).

CHAPTER V An evaluation of peanut early leaf spot monocyclic components following salicylic acid treatment of cultivated peanut (*Arachis hypogaea* L.)

5.1 ABSTRACT

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop for the United States and globally. *Cercospora arachidicola*, the causal agent of early leaf spot (ELS) on cultivated peanut, can threaten the yield with up to 50% losses. Salicylic acid (SA) has been a research focus for its effects to regulate disease resistance mechanisms on plants. Furthermore, previous studies indicated exhibiting age related resistance requires SA accumulation. However, in our studies, age related disease resistance was not observed. Previous work reported inconsistent results with SA relative to peanut pathogens; there is a need to examine the ability of SA to induce resistance against ELS on a high-oleic cultivar. We evaluated the effect of spraying three rates of SA (100, 200, and 300 μ M) at 3, 5, and 7 days prior to ELS inoculation to whole peanut plants (cultivar GA-09B) in a growth chamber. At 30 days after inoculation (DAI), most SA treatments had significantly fewer lesions compared to the control. At 42 DAI, all SA treatments had significantly fewer conidia compared to the control. Results of this study suggest that SA plays at least a transient role in ELS resistance in peanut cultivar GA-09B.

Keywords: Salicylic acid, monocyclic components, disease resistance

5.2 INTRODUCTION

Cultivated peanut (*Arachis hypogaea* L.) is an economically important crop that is produced not only in the United States, but worldwide. The United States ranks fourth in the world for cultivated peanut production with 2.03 million metric tons as a five-year (2009-2013) average production (USDA, 2014). Globally, one of the most important foliar fungal diseases of

peanut is early leaf spot (ELS), caused by *Cercospora arachidicola* S. Hori. This disease causes necrotic lesions on leaves, petioles, stems, and pegs, and eventually leads to defoliation (Porter *et al.*, 1982). ELS causes yield losses of 10 to 50% annually, depending on other factors such as climate and peanut cultivar (Cantonwine *et al.*, 2006; Cantonwine *et al.*, 2008; Waliyar *et al.*, 1995). While fungicide treatments are fairly effective for ELS management, a potential complementary approach would be to use induced systemic acquired resistance (SAR) to activate host defenses (Zhang *et al.*, 2001).

Salicylic acid (SA), a critical defense signal, regulates both local disease resistance mechanisms, such as hypersensitive response (HR) and defense gene expression, as well as SAR (Vlot *et al.*, 2009). Applications of SA have been shown to enhance plant resistance to viral, bacterial, and fungal pathogens, including *Tobacco mosaic virus*, *Agrobacterium tumefaciens*, and *Alternaria alternata*, in many plant species, for example in tobacco, *Nicotiana benthamiana*, *Arabidopsis*, and peanut, respectively (Anand *et al.*, 2008; Vlot *et al.*, 2009; White, 1979).

There is conflicting evidence regarding the role of SA in disease resistance in peanut. For instance, Yan *et al.* (2013) studied the role of SA-induced resistance against peanut web blotch and found SA appeared to play a significant role in peanut web blotch resistance and signal transduction based on the lower number and size of spots at 20 days after inoculation compared to the control. Chitra *et al.* (2008) also studied the effect of SA in inducing resistance in peanut against *A. alternata*, and reported that SA-treated peanut plants had significantly reduced disease intensity of the leaf blight. In addition, Kobeasy *et al.* (2011) reported that the effect of spraying SA improved peanut plant resistance to *Peanut mottle virus*. Despite these positive effects of SA-induced disease resistance, there are several conflicting reports including Cardoza *et al.* (2003), who found that SA levels in white mold, caused by *Sclerotium rolfsii*, infected peanut plants

were not significantly different than in control plants. Zhang *et al.* (2001) found SA did not induce systemic resistance to peanut late leaf spot; the percentage of leaflets per plant with lesions was not significantly different than on nontreated plants. Even though Zhang *et al.* (2001) concluded SA had no effect on inducing systemic resistance to peanut late leaf spot, which is caused by a biotrophic fungus (*Cercosporidium personatum*), there is a possibility that the causal agent of ELS, *C. arachidicola*, which is a hemibiotrophic fungus, could have different outcome. Therefore, there is a need to examine the ability for SA to induce resistance against ELS for a better disease control strategy.

In this study, the recently released peanut cultivar Georgia-09B (Branch, 2010) was chosen as a plant host, because it is a high-oleic cultivar. In addition, from our previous studies, this cultivar has been shown to be highly susceptible to peanut ELS, which indicates this cultivar could be a suitable host for this study.

The overall objective of this study was to evaluate and compare ELS monocyclic components of SA treated peanut high-oleic cultivar Georgia-09B to examine the ability for SA to induce resistance against ELS. The two specific objectives of this experiment were: 1) to evaluate optimal SA application time; 2) to evaluate the optimal SA application concentration.

5.3 MATERIALS AND METHODS

5.3.1 Inoculum production

Peanut leaves with ELS were collected from a peanut field at the E.V. Smith Research Center in Tallahassee, AL; *C. arachidicola* conidia from a single lesion on these leaves were collected and single spores were isolated and cultured on PDA media for three months. The identity of cultures was confirmed as *C. arachidicola* using internal transcribed spacer (ITS)

sequence analysis. One clone was ground with a clean konte pellet pestle (Kimble Chase, Rockwood, TN) and evenly spread on a modified (without CaCO₃) V8 agar plate (Dhingra and Sinclair, 1985). The plate was left open under laminar flow in the hood until dry, then closed, sealed with parafilm (Bemis, Oshkosh, WI) and placed under a grow light with a 12-hour photoperiod for one week. A daily microscopy check revealed peak sporulation between three and seven days. A solution of 0.005% Tween 20 was used to rinse conidia off the medium using gentle pipetting for higher yield. Eight layers of cheese cloth were used to filter any mycelia residue. Conidia were counted with a hemocytometer then adjusted to 5×10^3 /ml, and spray applied to peanut plants. A total volume of 0.5 ml conidial suspension was applied to each plant. After inoculation, each culture box was covered with another clear plant culture box to retain high relative humidity.

5.3.2 Peanut plants

Cultivar Georgia-09B was used in this growth chamber trial. Forty seeds were individually planted on March 19, 2015 in 40 Magenta GA-7 plant culture boxes ($7.62 \times 7.62 \times 10.16$ cm³) containing potting mix (Sunshine mix # 8, Sun Gro Horticulture, Vancouver, Canada) and sand in a 1:1 (vol/vol) mixture, with 100 ml mixture in the bottom of the box, seed in the middle, and 50 ml mixture on the top. Thirty ml of water was applied onto the media in each culture box at planting; water was added as needed. Three weeks after germination, peanut plants had reached the V4-V5 growth stages (Boote, 1982); thirty out of forty plants were chosen for use in this study based on their size uniformity. Thirty two days after planting, when plants reached V8-10 stage, *C. arachidicola* was applied.

5.3.3 SA application

Three SA solution concentrations were used for this trial: 100, 200, and 300 μM (SA1, SA2, and SA3, respectively). Half ml per plant SA solution was spray applied to peanut plants at three, five, and seven days before *C. arachidicola* inoculation (D3, D5, and D7, respectively). There were three SA concentrations and three time points, for a total of nine SA treatments plus a non-treated control, with three replications, for a total of thirty plants. These treatments were arranged in randomized complete blocks. The growth chamber was set at 26°C with light from 7 a.m. until 8:30 p.m., and 21°C without light from 8:31 p.m. until 6:59 a.m.

5.3.4 Disease evaluation

Five leaves of each plant were examined daily after *C. arachidicola* inoculation for evaluating the monocyclic components (incubation period, number and size of lesions, defoliation, sporulating lesions, and reproductive ability) of ELS. Incubation period was defined as the number of days from inoculation to the first day on which lesions were observed. Size of lesion was determined as the diameter of the largest lesion on the five leaves of each plant. Defoliation was determined by the number of defoliated leaflets from these same five leaves of each plant. Sporulating lesions was the number of sporulating lesions from these five leaves of each plant at 30 DAI. Reproductive ability was the number of conidia formed by sporulating lesions. Double sided clear tape was used to gently press onto conidia of each sporulating lesion. The tape was then fixed onto a glass slide. A light microscope was used to count the number of conidia on the tape.

5.3.5 Data analysis

For this growth chamber trial, monocyclic components effects of all treatments were determined with mixed model analysis (Proc Glimmix, SAS 9.4). A *P*-value less than or equal to 0.05 was considered significantly different. Means are significantly different according to Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

5.4 RESULTS AND DISCUSSION

Number of lesions at 30 DAI and number of conidia at both 30 and 42 DAI were significantly affected by treatment. No significant difference was observed on incubation period, largest lesion sizes, number of defoliated leaflets, and sporulating lesions.

SA application at 100 μM applied at 5 DBI, all SA applications at 200 μM , and SA at 300 μM applied at 5 and 7 DBI led to fewer lesions compared to the control at 30 DAI. These results indicate that SA applications at 200 μM could improve resistance to ELS at all application times tested in the current study.

Higher SA concentrations (200 and 300 μM) applied at longer intervals before inoculation (5 and 7 DBI) led to significantly fewer conidia from sporulating lesions compared to control. In contrast, all SA applications at 100 μM and the higher SA concentrations (200 and 300 μM) applied at the shortest interval between SA treatment and inoculation (3 DBI) led to significantly higher numbers of conidia compared to the control. However, at 42 DAI, all SA treatments resulted in significantly fewer conidia than the non-treated control.

There are several previous studies with *Arabidopsis* that have indicated that SA treatment reduces plants' fitness, since SA induces disease resistance mechanisms, causing the plants to focus energy on defense rather than growth (Cipollini, D. F., 2002; Heil, M., 2002; Vos *et al.*,

2013). This could be a reason why certain SA treatments did not enhance peanut resistance to ELS in the current study.

SA application concentrations may play a role in our results. All SA concentrations used in this experiment, 100, 200, and 300 μM , were chosen based on previous work by Kobeasy *et al.* (2011). The intermediate SA concentration had slightly better results than the lower or the higher SA concentration in the current study. Fu *et al.* (2012) reported that basal SA concentrations are required to maintain basal levels of non-expressor of pathogenesis related genes 1 (NPR1) for basal resistance; moderate SA concentrations enable NPR1 to accumulate and therefore to establish systemic acquired resistance for cell survival; highest SA concentrations lead to degradation of NPR1 and triggered a hypersensitive response for localized cell death. This could be a reason the intermediate SA concentration used in this study had consistently better performance. However, the SA concentrations used in this study did not cover a wide range. The exact moderate range of SA concentration for inducing peanut plants' SAR is not clear. Further study could focus on identifying the basal, moderate, and highest SA concentrations for SAR solicitation in peanut plants.

The SA application time points used in this experiment were three, five, and seven days before inoculation. Time of application could also affect the results, but the effect was complicated by the combination with SA concentrations. At 42 DAI, the lowest SA concentrations with the longest interval between SA treatment and inoculation, led to significantly fewer conidia; with the middle SA concentration, the shortest interval led to a significantly fewer conidia; among the highest SA concentration applications, middle interval led to a significantly lower conidia production compared to the others. Zhang *et al.* (2001) similarly applied SA one week before inoculation, and saw the higher and the lower SA concentrations led

to numerical lower percent disease compared to a non-treated control in their first experiment. Yan *et al.* (2013) reported that SA content in leaflets of nearby pathogen-treated-leaflets showed two peaks, at four hours after pathogen inoculation and a second between 40 and 48 hours after pathogen inoculation. These results indicate that when a plant leaflet is attacked by a pathogen (*Phoma arachidicola*), SA content increases significantly in nearby leaflets at two time points: 4 and 40 to 48 hours. In the current study, the interval between SA applications and pathogen inoculation was reduced compared to that used by Zhang *et al.* (2001); however, the reduction might not be enough. Even shorter intervals, such as two, four, eight, etc. until 56 hours before inoculation, could be used in future studies, and may reduce the complication from different SA concentrations.

SA treatments evaluated in this study had a significant effect in inducing resistance in peanut by reducing the number of lesions and conidia. This finding is in line with results of Yan *et al.* (2013), Chitra *et al.* (2008), and Kobeasy *et al.* (2011). However, results here in contradicte with the report Zhang *et al.* (2001) with peanut plant against late leaf spot, caused by a biotrophic fungus (*C. personatum*). Even though SA as a signaling molecule plays a role in plant defense against both biotrophic and hemibiotrophic pathogens (Boatwright and Pajerowska-Mukhtar 2013), it is possible that the causal agent of ELS, *C. arachidicola*, which is a hemibiotrophic fungus, elicited a different result.

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Table 5.1. Evaluation of monocyclic components of *Cercospora arachidicola* on salicylic acid (SA) treated peanut plants in growth chamber.

Treatment		Incubation period ^a	Largest lesion diameter ^b	Lesions	Defoliated leaflets	Sporulating lesions	Conidia ^c	
SA concentration	Days before inoculation						30 DAI	
		days	mm	-----		number	-----	
100 μ M	3	11.00 A	2.40 A	3.22 CD ^d	0.00 A	0.00 A	13900 D	11550 E
	5	7.00 A	4.97 A	3.15 D	0.00 A	0.00 A	16500 A	14275 B
	7	9.00 A	3.93 A	3.97 A	0.00 A	0.18 A	15075 B	3950 H
200 μ M	3	11.00 A	2.37 A	2.28 E	0.00 A	0.00 A	14225 C	4900 H
	5	7.00 A	4.33 A	2.32 E	0.29 A	0.18 A	12575 F	13975 C
	7	11.00 A	3.40 A	2.46 E	0.51 A	0.00 A	10250 H	10350 F
300 μ M	3	13.00 A	3.90 A	3.77 AB	0.00 A	0.00 A	14325 C	11825 E
	5	9.67 A	1.83 A	2.41 E	0.00 A	0.00 A	11950 G	8300 G
	7	10.33 A	2.87 A	2.63 E	0.51 A	0.00 A	8100 I	13400 D
Control		13.00 A	1.87 A	3.50 BC	0.00 A	0.00 A	13400 E	15450 A
<i>P</i> -value		0.391	0.164	<0.001	0.519	0.833	<0.001	<0.001

^aLeast square means from Proc Glimmix of days after inoculation (DAI) until first lesion observed.

^bLargest lesions diameter is the mean diameter (mm, mini meter) of the largest lesion from each plant's five evaluated leaves.

^cHarvested conidia/ ml with total volume of 15 ml.

^dMeans in each column with the same letter do not differ at $P \leq 0.05$ based on Fisher's protected LSD values.

APPENDIX A

***BjCH11* gene expression**

Reverse transcription-polymerase chain reaction (RT-PCR) was used to ensure that the inserted chitinase gene was expressed in genetically modified genotypes.

1. RNA Extraction

Approximately 250 mg of tissue from four leaflets were weighed and placed into a 2 ml impact resistant tube, and frozen at -80°C. Ice-cold Trizol Reagent (500 µl; Invitrogen, Carlsbad, CA) was added to the tube, and then the tube was put into Bead Ruptor 24 (Omni International, Kennesaw, GA) to homogenize the tissue. The sample was immediately put on ice for 5 min after homogenization. An additional 300 µl of ice-cold Trizol Reagent was added to the tube, and then the tube was rotated for 5 min. Then 180 µl of chloroform was added into the tube, and mixed well by physical inversion for another 5 min. The sample was then put into centrifuge for 5 min at 8°C at maximum speed (14,800 × g).

Five hundred µl of clear supernatant was transferred to a new standard 2 ml tube (tube a), and 500 µl of 1.2 M NaCl was added. After pipetting to mix, 500 µl of the mix was transferred to another new standard 2 ml tube (tube b). Three hundred and seventy five µl of 100% ethanol was added to each tube, mixed by inversion, placed at room temperature for 10 min, and then centrifuged at maximum speed for 10 min at 8 °C. A white pellet was visible at the bottom of each tube after centrifugation.

The liquid was completely removed by pipetting and the pellet was kept in the tube. Three hundred and seventy five µl of diethylpyrocarbonate (DEPC) treated water was added into the tube, and the pellet was resuspended completely by mixing at room temperature.

The liquids were combined from these two tubes (tube a and b), and then 750 μ l of 1.2 M NaCl was added, and mixed by inversion. Then 100% ethanol was added until full. Liquid in the tube was mixed by inversion, placed on ice for 10 min, and then centrifuged for 10 min at 8 °C. Supernatant was discarded and the pellet was washed with 750 μ l of ice-cold 70% ethanol. The sample was centrifuged for 5 min at 8 °C, then liquid was completely removed by pipetting and the pellet was dried at 37°C incubator for 10 min.

Two hundred μ l of DEPC water was added into the sample to resuspend the pellet. The nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to determine the final product concentration.

2. Gel Electrophoresis for RNA samples

From the sample, 1 μ g and 2 μ g of extracted RNA were added into two tubes, each with DEPC water up to 15 μ l total volume, then mixed with 4 μ l of 5X loading dye and 1 μ l of 0.2 μ g/ μ l ethidium bromide (EB). The samples were denatured at 65 °C for 5 min, cooled down to 4 °C, and then loaded into the 1.2% agar RNA denaturing gel (Biotium., Hayward, CA) to run at 80V for 24 min. Gel images from samples were taken with Gel Logic 2200 (Kodak, Rochester, NY).

3. Complementary DNA (cDNA) synthesis and reverse transcription PCR (RT-PCR)

cDNA was synthesized using SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, CA) by reverse transcription with oligo (dT)₁₅ (Promega, Madison, WI). RT-PCR was carried out with plant actin primers to ensure the quality of cDNA. PCR was carried out with Kanamycin resistant gene-specific primers Kan2 (Table A.1).

4. Results

cDNA quality was ensured by RT-PCR with plant actin primers (Fig. A.1). Bright band presents good quality RT-PCR product, this indicate that RNA is qualified to be used as template. Then, Kanamycin resistance gene-specific primers Kan2 were used with this qualified RNA, a band indicate this Kanamycin resistance gene was expressed in the plant (Fig A.2). Because the *Brassica juncea* chitinase gene *BjCHI1* was introduced with Kanamycin resistance gene, the expression of this ensured the successful transformation of introduce *BjCHI1* gene to peanut.

Table A.1. Primer sequences for Kanamycin resistant gene.

Primer	Length	Aim	Sequence (5'-3')
Kan_2F	20	Conserved region	CGGCCACAGTCGATGAATCC
Kan_2R	20	Conserved region	GGATTGCACGCAGGTTCTCC

Figure A.1. Gel image of Actin RT-PCR from six peanut genotypes (E27, Chit P7, GA12Y, Chit P4, C5, C1001). Actin bands were indicated by the arrow.

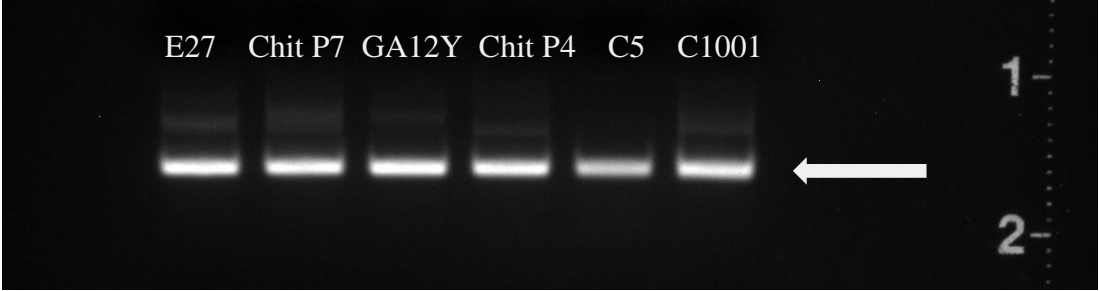
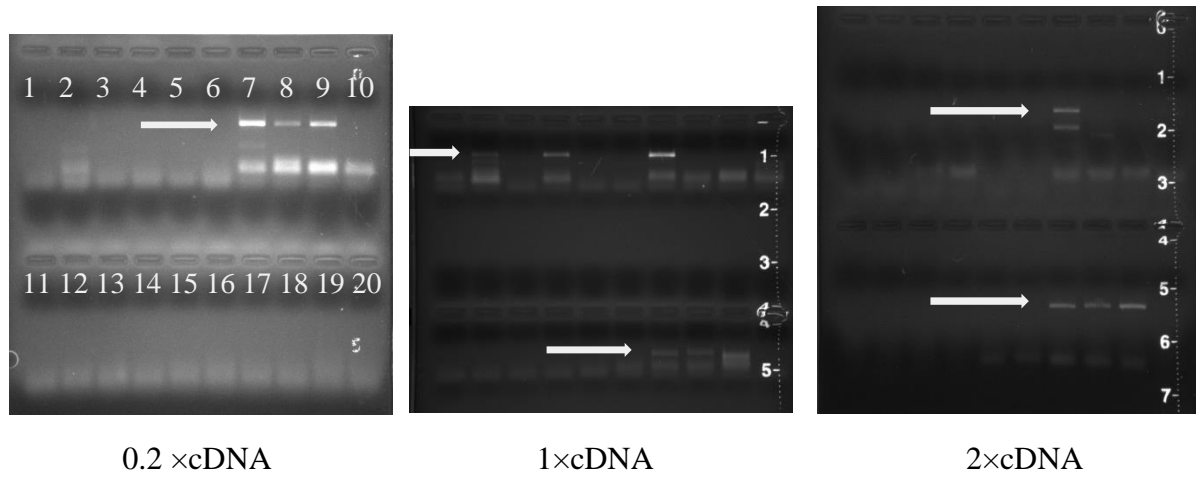


Figure A.2. Gel image of Kanamycin resistant gene RT_PCR from 20 transgenic peanut samples (1-20): 0.2 \times cDNA, 1 \times cDNA, and 2 \times cDNA. Different cDNA concentration affects final RT-PCR products, indicated by arrows.



C1001: sample # 3, 4, 6, 7, 8, 9, 13, 19

Chit P4: sample # 2, 5, 12, 20

Chit P5: sample # 6, 10, 14, 17

Chit P7: sample # 1, 11, 15, 18