Validation of Candidate Genes Associated with Leaf Spot Resistance in Cultivated Peanut (*Arachis hypogea L.*)

By

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Abstract

Peanut is an important oil crop that is grown in several parts of the world including the US, Asia and Africa. Early and Late leaf spot caused by *Cercospora arachidicola* and *Cercosporidium personatum* respectively are the most destructive peanut diseases. In the southeastern US, they cause up to 50% yield losses without any fungicidal spraying. Fungicides are not a sustainable method of controlling leaf spot diseases because they are expensive and cause environmental pollution. It is therefore important to breed for resistant peanut cultivars. Sources of resistance have been identified however the genes responsible have not. The aim of this research was to check for significant differences in the expression of seven candidate genes : chitinase I and II, Defensin, Nucleotide Binding Site (NBS), Phenylalanine ammonia lyase (PalI and Pal II) and thaumatin. Forty two peanut genotypes belonging to the three categories (susceptible, medium and resistant) were inoculated with leaf spot disease pathogens 80 days after planting. Disease evaluation was done and gene expression was quantified using real time q RT-PCR. All the genes were upregulated and there were significant differences in the expression of five of the genes studied except for chitinase II and thaumatin.

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List of Abbreviations

ELS	Early leaf spot
LLS	Late Leaf Spot
MAS	Marker Assisted Selection
TSMV	Tomato spotted wilt virus
US	United States
USDA	United States Department of Agriculture
DNA	Deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
qrtPCR	Quantitative Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Locus
SSR	Simple Sequence Repeats
PDA	Potato Dextrose Agar
PR	Pathogenesis Related
BLAST	Basic Local Alignment Search Tool
ANOVA	Analysis of Variance
NCBI	National Center for Biotechnology Information
ORF	Open Reading Frame

CHAPTER 1

LITERATURE REVIEW

(i) Overview of peanuts

Peanut *(Arachis hypogea L.)* is an important leguminous crop that grows in a wide range of climatic conditions making it one of the major economically important legumes that is cultivated worldwide for its adaptability to grow in semi-arid environments with relatively low inputs of chemical fertilizers (Qin *et al*., 2012). It is an important food crop that is used as a source of oil as well as for confectionary products (Tshilenge-Lukanda *et al*, 2011). It is native to South America and widely distributed in the sub and tropical regions including China, India and Nigeria. In Europe, Africa and Asia it was introduced by traders and explorers. In the U.S., African-American botanist George Washington Carver played an important role in promoting cultivation of peanuts as an alternative crop to cotton, which was declining in productivity due to soil depletion and insect devastation (Ozcan, 2010). The world total peanut production is 45 million metric tons from about 24 million hectares of land. The highest world peanut producers are China, India, United States and Nigeria, producing about 70% of total world peanut produce (Janila *et al*, 2016). The US peanut production is concentrated mainly in three major geographic areas: (i) the southeast, which includes Georgia, Alabama, Florida, and Mississippi; (ii) the southwest, which includes Texas,

New Mexico, and Oklahoma; and (iii) Virginia Carolina, which includes North Carolina, South Carolina, and Virginia (USDA, 2010). Peanut is a valuable crop in many countries, not only for its economic benefits but also for its nutritional benefits.

Biology of Peanut

Peanut *(Arachis hypogea L.)* is a member of the genus Arachis and family Leguminoseae. All members of the genus *Arachis* are distinguishable from other plants in that they flower above ground and produce their seed below the ground (Holbrook and Stalker, 2003). *Arachis hypogaea* is an annual herb of indeterminate growth habit which has been divided into two subspecies *hypogaea* and *fastigiata,* each with several botanical varieties (Stalker, 1997). The subspecies *hypogaea* is characterized by the absence of flower on the mainstem as well as alternate vegetative and reproductive nodes. The subspecies *fastigiata* on the other hand is characterized by flowers on the main stem and sequential reproductive nodes (Holbrook and stalker, 2003). Sub specific and varietal classifications are mostly based on location of flowers on the plant, patterns of reproductive nodes on branches, numbers of trichomes and pod morphology (Stalker, 1997). The ssp. *hypogaea* consists of botanical varieties '*hypogaea*' and '*hirsuta*', whereas the ssp. *fastigiata* consists of botanical varieties '*fastigiata*', '*vulgaris*', '*peruviana*', and '*aequatoriana*' (Krapovickas and Rigoni, 1960). Nearly all *Arachis* species are diploid, but the cultivated peanut is an allotetraploid (genome AABB). *A. duranensis* is the progenitor of the A genome while that of the B genome is *A. ipaensis*. This allotetraploid (2n = 4x = 40) originated through the

hybridization of two ancient diploid species, followed by a rare spontaneous duplication of chromosomes (Stalker, 1997).

Agronomic traits of Peanut

Peanut production and marketing has resulted in designation four market classes which generally correspond to subspecific and varietal groups as follows: runner (subsp. *hypogaea var. hypogaea),* Virginia (subsp. *hypogaea var. hypogaea),* Spanish (subsp. *fastigiata var. vulgaris),* and Valencia (subsp.*fastigiata var. fastigiata).* Runner-type cultivars have medium-sized pods and seeds which range from 550 to 650 mg/seed. They have a relatively long growing season, with 120 or more days needed for maturity, and are highly indeterminate. Runners occupy about 80% of the total peanut acreage in the U.S., with production concentrated in the Southeast (Georgia, Alabama and Florida) (Stalker, 1997). Virginia-type peanuts have large pods and seeds, this makes them a desirable cultivar for gourmet snacks. However, they are generally long-season plants and require more calcium for seed development than smaller-seeded peanuts, and so are not widely grown in arid regions. Peanuts in the Virginia market-class have been historically grown in the Virginia-North Carolina area. This class represents about 15% of the peanuts produced in the U.S.. Spanish types are widely grown around the world. The seeds are similar in size to runner types in terms of size but this market type generally yields lower. One of the main advantages of the Spanish type is that its growing season is short as compared to that of the other two market types discussed above. In the U.S., almost all of the Spanish types are produced under dry-land conditions in

Oklahoma and Texas. The Spanish type accounts for about 5% of the U.S. peanut market. There is only one Valencia market-type cultivar grown in the U.S. and accounts for less than 1% of the market (Stalker, 1997).

Seed chemistry of peanut

Peanut is consumed as whole seeds or processed as peanut oil and peanut butter (Ozcan, 2010). The seed contains 25 to 32% protein, 42 to 52% oil, 10-20% carbohydrates as well as vitamins. The major fatty acids, oleic acid (C18:1) and linoleic acid (C18:2) account for about 80% of peanut oil, the six other fatty acids namely palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), gadoleic acid (C20:1), behenic acid (C22:0), and lignoceric acid (C24:0) make the remaining 20% of the fatty acid profile (Wang et al, 2015).

Even though it is primarily known for oil, peanut is also a good source of protein (20%), minerals, vitamins, antioxidants and secondary metabolites such as folic acid (Chu et al, 2016).

Diseases affecting peanut production

Peanut is susceptible to a variety of diseases. Internationally, the most widely distributed and destructive peanut diseases are early and late spot caused by *Cercospora arachidicola* and *Cercosporidium personatum* respectively. Peanut rust caused by *Puccinia arachidis*, tomato spotted wilt virus, web blotch caused by *Phoma arachidiola* are also widely distributed but are of lesser economic importance. Over the years the severity of rust has increased and caused major

yield loss. Annual crop losses as a result of foliar diseases go from 1% to over 50% (Smith and Litrell, 1980). Web blotch occurs all over the world and is most severe during cool conditions with high humidity.

(ii) Leaf spot disease in peanut

Cercospora leaf spots (ELS and LLS) caused by *Cercospora arachidicola* and *Cercosporidium personatum* respectively are the most destructive foliar diseases of peanut in the world (Vasavirama and Kirti, 2012). Both early and late leaf spot diseases are usually present worldwide wherever groundnuts are grown (Subrahmanyam et al, 1985).

Yield loss due to leaf spot is a serious problem in the cultivation of peanuts (Vasavirama and Kirti, 2012). Leaf spot diseases are present every year in the southeastern of the United States and can decrease seed productions by over 50%, if no fungicides are applied for control (Méndez-Natera et al, 2016). Recent studies reveal that in the southern US leaf pot causes direct losses to peanut cultivation through yield reduction and increased management practices associated with multiple fungicide applications (Thiessen, 2012). Recent estimates of combined losses in yield and control measures attributable to these diseases have been as high as $39.7 billion in Georgia (Jordan et al, 2017).

The causal pathogens

The two pathogens (*Cercospora arachidicola* and *Cercosporidium personatum)* that cause leaf spot can be distinguished by the morphology of their conidia and conidiophores. For *C.*

arachidicola conidiophores are arranged in dense fascicles, pale olive or yellowish brown in color and are unbranched. The conidia are subhyaline, obclavate, base rounded to truncate and have 3-12 septae. *C. personatum* on the other hand has numerous conidiophores that are pale to olive brown in color. Conidia is cylindrical, obclavate, usually straight or slightly curved and have 1-9 septae. Diseased sections of infected leaves reveal that *C. personata* produces haustoria inside host cells whereas *C. arachidicola* does not (ICRISAT, 1985).

These fungi cause infection by infection through spores. Once spores are produced they cause initial leaf infection and then under favorable conditions (high humidity and temperatures above 19ºC) the spores develop into germinative tubes and enter the plant cells directly through the stomata or the epidermis allowing mycelia to grow in the cell and obtain nutrients (Shokes and Culbreath, 1997).

Lifecycle of leaf spot diseases in peanut

The pathogens that cause early and late leaf spot are both soil borne and infect the host plant through spores. As the common names imply an attack by *C.arachidicola* normally precedes that of *P.personata* but both diseases may appear 3-5 weeks after sowing. Conidia are produced from mycelium in crop debris in the soil following early rains. The spores are deposited on the young plants through rain splashes or wind. Temperatures between 25 to 30^0C and high humidity favor the development of the disease (ICRISAT, 1985). Once the pathogen has infected the plant it produces many microscopic spores. The production of these spores is favored by high humidity.

Spots typically occur 10-14 days after infection and sporulation occurs thereafter. The resulting spores are then released to the environment through the agency of wind, insects and rain. New spores then infect new leaves and the cycle continues. When conditions are favorable many secondary infections take place causing severe occurrence of the disease. Warm temperatures, high humidity and leaf wetness are important for the progression of the disease cycle throughout the growing season. The pathogens may survive from season to season on crop debris and volunteer peanuts (Tshilenge-Lukanda et al, 2012).

Symptoms

Leaf spot symptoms can appear on any of the plant`s above ground parts such as leaves, stems and petioles (Tshilenge-Lukanda et al, 2012). The damage caused by these two fungi include defoliation, reduction in pod size and yield loss (Debele and Ayalew, 2015). The onset of the disease is characterized by fungal spores entering into the leaf through the stomata resulting in development of lesions on the leaf within a week. The disease usually starts attacking the plant from the lower leaves going up because the fungal spores are normally in soil. Pinhead sized spots appear on the upper surface of leaves and enlarge overtime to become brown or black circular spots of diameters reaching 10mm. Overtime the spots coalesce resulting in defoliation and consequently reducing biomass and yield. Early leaf spots and late leaf spots can be distinguished on the basis of color as well as appearance on the leaf surface. Early leaf spots are brown to reddish brown with a yellow halo and appear on the upper surface and give the leaf surface a raised

appearance as compared to the smooth lower surface. Late leaf spots on the other hand are dark brown to black spots that usually lack a yellow halo and are formed on the lower surface giving it a rough appearance in comparison to a smooth upper surface (Tshilenge-Lukanda et al, 2012). After 20-30 days sporulation occurs, it results in reinfection (Clevenger et al, 2018). Under favorable conditions, warm temperatures and high humidity, the diseases progress continuously (Tshilenge-Lukanda et al, 2012).

Control measures

The control of leaf spot requires an integrated management approach which involves the use of cultural practices, fungicide spraying as well as genetic resistance. Disease management practices such as crop rotation helps to make the disease more manageable . Crop rotations where peanut are grown once every 3 to 4 years allows debris from previous growing seasons to decompose thereby reducing infection. Tillage and eradication volunteer peanut plants and ground keepers also help eliminate fungi from the previous season (Jordan et al, 2012). Adjusting planting date may be beneficial as it prevents infection from outside sources and also avoids environmental conditions that are conducive for the disease. Weeds should be kept under control as they encourage disease development by modifying of the crop climate (ICRISAT, 1985).

Fungicides are effective in agriculturally advanced countries, but require a series of sprays are conducted during the growing season. To obtain effective control of leaf spots, fungicides are

applied before the appearance of symptoms and further applications are made at intervals 10-14 days resulting in up to 8 applications throughout the growing season. Some of the common fungicides that are used to control leaf spot include captafol, carbendazim and chlorothanolil (ICRISAT, 1985). The use of fungicides provides good control of the diseases but they increases the monetary input for peanut production, cause environmental pollution and pose threat to human health (Clevenger et al, 2018). In many less developed countries, the use of fungicides presents some problems for small scale farmers (ICRISAT, 1985). This is especially true for small farmers who lack the technical expertise required to use fungicides efficiently (Zongo et al, 2017). These farmers have difficulties obtaining fungicides and application machinery because of high costs. Low and fluctuating prices of peanut also discourage their use (ICRISAT, 1985).

Resistant cultivars are the best means of minimising crop yield losses from diseases. This strategy is particularly well suited for small scale farmers in semiarid tropics who lack financial resources and technical expertise to use chemical control methods. Disease resistant cultivars are desperately needed in developed countries as they reduce the farmers dependence on fungicides and in turn reduce the cost of peanut cultivation and potential health risks (ICRISAT, 1985).

Using varieties that are resistant to leaf spot will also help reduce the severity of the disease.

Resistant peanut varieties eliminate yield losses caused by disease and reduces costs related to fungicide sprayings and other control methods. The hazard of environmental pollution can also be discarded (Méndez-Natera et al, 2016).

The role of resistance genes in defense against pathogen attack

Breeding for disease resistance is a more sustainable and cost effective approach to reducing the impact of leaf spot diseases (Zongo et al, 2017). In order to breed for resistance, an understanding of plant defense mechanism is required. Plants possess two major types of disease resistance, basal defense and R-gene mediated defense. Basal defense provides first line of defense to the infection by a wide range of pathogens. R -gene mediated pathogen response is a form of immunity that is based on detection of pathogen proteins by host resistance proteins (Gururani et al, 2012). Chitinases are enzymes that catalyze the hydrolysis of the β-1-4-linkage in the N-acetyl-D-glucosamine polymer of chitin which is a major component of fungal cell walls and arthropod exoskeletons. Plant chitinases also inhibit the growth of fungi by producing hypersensitive reactions and inducing defense responses. Based on their structure chitinases have been phylogenetically categorized into five classes (class I-V). Class I, II, and IV chitinases are found mainly in higher plants and some bacteria. Class I chitinases have an N-terminal cysteine-rich chitin binding domain (CBD) and a catalytic domain (CatD) at the C-terminal. Class II chitinases have high sequence homology with class I chitinases at the CatD but they lack the CBDs (Xu et al, 2016). Defensins are an integral part of the plant innate immune system. Most plant defensins already characterized show a constitutive pattern of expression with up regulation in response to pathogen attack, injury and some abiotic stresses. They interact with the negatively charged molecules present at the cell membrane of pathogens, causing an increase

of its permeabilization, leading to cell leakage and death by necrosis. They also interact with phospholipids producing reactive oxygen species (ROS) which results in programmed cell death (Larceda et al, 2014). Nucleotide-binding site and leucine-rich repeat (NBS-LRR) genes represent the largest and most important type of disease resistance genes in plants. The NBS domain participates in detection of pathogen-associated proteins, most often the effector molecules of pathogens responsible for virulence (DeYoung and Innes, 2006), as well as the transduction of the disease signal which can be used to identify pathogenic effects on host cells (Wu et al, 2017). Phenylalanine ammonia-lyase (PAL) is an enzyme that catalyzes the first step of the phenylpropanoid pathway, which produces precursors to a variety of compounds such as lignin. Lignin is a major component of cell walls of vascular plants and is considered a first line of defense against successful penetration of invasive pathogens. Lignification renders the cell wall more resistant to mechanical pressure applied during penetration by fungal pathogens as well as more water resistant and thus less accessible to cell wall-degrading enzymes (Bhuiyan et al, 2009). The function of thaumatin is not yet clear, though it has strong homology to thaumatin-like proteins which have membrane permeabilizing properties, and thus they have been assumed to play a role in the defense system (Rajam et al, 2007).

Breeding advances for Leaf Spot Resistance

There is a need to develop resistant/tolerant varieties against foliar diseases such as early leaf spot (*Cercospora arachidicola*) and late leaf spot (*Cercosporidium personatum*). There has been no

complete or single-gene resistance to both diseases reported in cultivated peanut. Resistance is partial and rate-reducing. Partial resistance is typically a function of multiple components of resistance that contribute additively to a reduction in the rate of epidemic progress. It is important to continuously screen for peanut genotypes that are resistant to leaf spot as this provides breeders with the necessary information on good varieties that can be used for further disease resistance breeding. Runner varieties are partially resistant, Virginia are intermediate while Spanish varieties are more susceptible (Damicone, 1996).

Components of resistance to ELS and LLS were described for many peanut genotypes under field and greenhouse conditions in the 1980s. Interspecific hybridization has received much attention in peanuts because several wild Arachis species show a very high level of resistance to many biotic stresses, such as rust, ELS, LLS, and stem rot. Conventional breeding has been the major avenue for providing modern peanut cultivars to farmers. Integration of molecular breeding with conventional methods has been successful in some crops but peanut has

lagged behind due to lack of molecular markers linked to traits of interest. However, progress in recent years has made it possible to use marker-assisted selection (MAS) in peanut breeding (Pandey et al, 2017). This method offers great promise for improving the efficiency of conventional plant breeding and also the potential to pyramid resistance genes in peanuts. The two most important steps in any molecular breeding program are the assessment of genetic diversity and identification of QTLs underlying LS resistances. There has been a lot of polymorphisms

observed in wild Arachis but progress in the molecular breeding of cultivated peanuts is greatly constrained due to the low level of detectable molecular genetic variation (Mishra et al, 2015). Despite being an important oilseed crop, very limited work in the area of molecular genetics and breeding of peanuts has been performed. However, over the last decade, there has been significant developments in the various molecular approaches for biotic stress management in peanuts, and

new efforts such as functional genomics are likely to play key roles in the future (Mishra et al, 2015). Recently many DNA markers have been found to be putatively linked with leaf spot diseases, a few of which have been validated. Validation of other linked markers will accelerate the process of introgression of resistance genes into preferred peanut genotypes. Kanyika et al (2015) has identified 376 polymorphic SSR markers in 16 peanut cultivars that are associated with a wide range of disease resistance (Mishra et al, 2015).

An integration of genomic resources and tools such as molecular markers, QTL and marker assisted selection with conventional breeding approaches will enhance the development of ELS and LLS resistant peanut cultivars (Kolekar et al, 2015)

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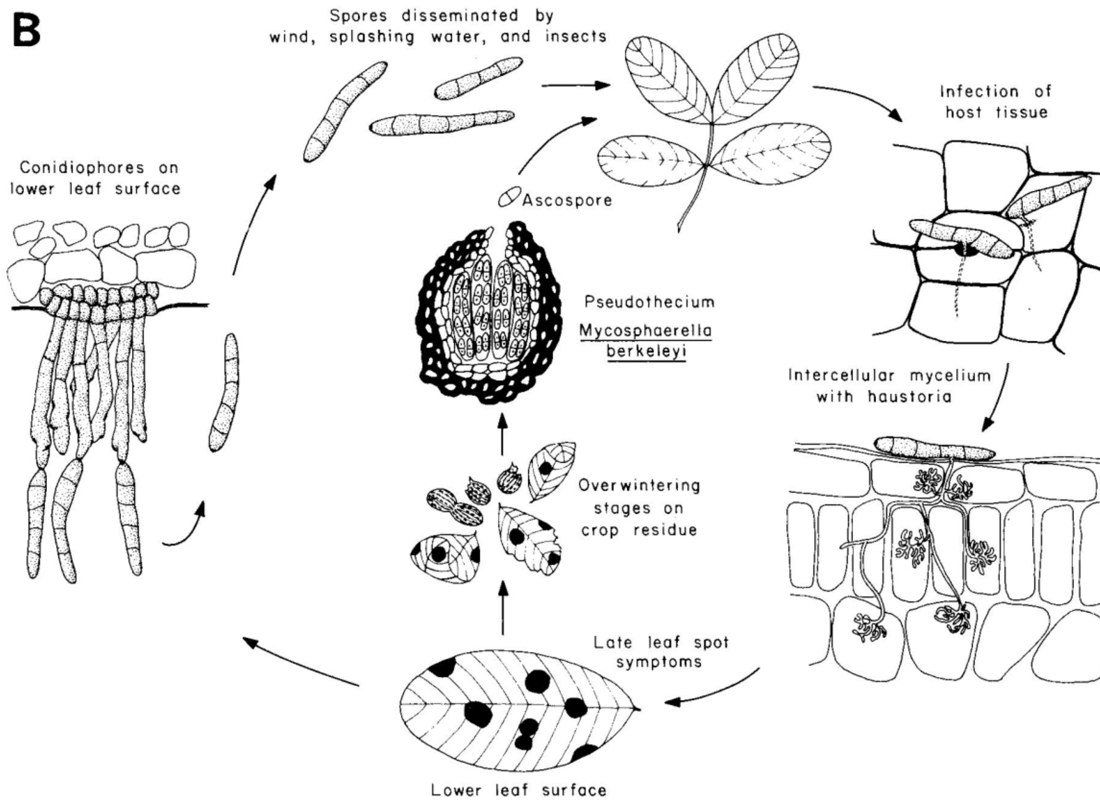

Figure 1 ; Disease cycle of late leaf spot, caused by Cercosporidium personatum (Berk. and Curt.) Deighton (reprinted with permission from Shokes and Culbreath, 1997).

CHAPTER 2; VALIDATION OF CANDIDATE GENES ASSOCIATED WITH LEAF SPOT RESISTANCE IN CULTIVATED PEANUT (*Arachis hypogea* L.)

INTRODUCTION

Background of peanut

The cultivated peanut (*Arachis hypogea* L.) is one of the most important oilseed crops worldwide (Han et al, 2017). It occupies the sixth position in the world among oilseed crops (Vasavirama and Kirti, 2012) and has very high nutritive as well as commercial value (Wang et al, 2013). It provides high quality edible oil and high protein content in the seed (Vasavirama and Kirti, 2012; Han et al, 2017). It is grown throughout the tropics and warm temperate regions of the world including Asia, America and Africa. In 2014, peanut was grown in 115 countries covering a total area of about 26.54 million (M) hectares (ha) with a global production of about 43.91 M tons and an average yield of about 1655 kg/ha (Zongo et al, 2017).

A wide gap exists between the genetic potential of modern peanut cultivars and their actual yield. This is due to several biotic and abiotic stress factors. Among the biotic factors are diseases such

as early leaf spot (ELS) and late leaf spot (LLS), rust and tomato spotted wilt virus (TSWV). Abiotic factors include fluctuating climatic and environmental conditions for example low rainfall resulting in drought (Wang et al, 2013).

Cercospora leaf spots are the most serious foliar diseases in peanut in the world (Vasavirama and Kirti, 2012). These two diseases caused by *Cercospora arachidicola* and *Cercosporidium personatum* respectively, cause significant yield loss ranging from 10-80% without adequate control measures. Even though leaf spot can be controlled effectively by spraying fungicides, they are very expensive and cause environmental pollution. The most economic and effective method of controlling these diseases is through the development of resistant crop varieties. Sources of resistance have been identified in several wild peanut species and these have been used to develop resistant varieties (Han et al, 2017). Conventional breeding has been used to develop resistant cultivars (Wang et al, 2013). The only drawback is that the conventional breeding methods used are time consuming (Han et al, 2017). Identification of resistant resources and knowledge of components and mechanisms of resistance is a prerequisite in disease resistance breeding programs (Dwivedi et al, 2002). Integration of conventional breeding and genomic tools to develop diseases resistance has been employed successfully in many crops but peanut is lagging behind. With the recent advancement of genetic research in peanut, there are many possibilities for development of resistant varieties. Identification of linked markers is the base for implementation

of marker assisted selection (MAS). This has already been successfully used to develop high oleic peanut (Wang et al, 2013).

Statement of the problem

Early leaf spot and LLS are the most destructive diseases of peanut worldwide, causing yield losses of up to 50%. In an effort to control these diseases over the years farmers have been using multiple fungicides throughout the growing season. These fungicides are not only expensive but are also health hazards and cause environmental pollution. The most effective and economically viable management option to control leaf spot diseases is through the use of resistant peanut cultivars. Currently there are cultivars that have partial or moderate resistance to leaf spot such as `Tifguard` and AU-NPL 17(Chen et al , 2017). There hasn't been any cultivar that has absolute resistant to the disease. This is mainly due to inadequate genetic information on the sources of resistance which limits efforts to breed resistance into the crop. One of the many natural defense mechanisms plants use to resist pathogen attack is to accumulate proteins (e.g., chitinases) active against disease causing organism (Iqbal et al, 2017). The mechanism of plants response to the challenge by pathogens is complex and requires activating integrated pathways to defend pathogen attacks. One of such responses is to activate an inducible protein-based defense system that includes 17 families of pathogenesis-related (PR) protein (Han *et al*, 2017).

Significance of the study

This study will broaden the knowledge on the sources of resistance (candidate genes) to leaf spot resistance in peanut as well as pave a way for development of resistant peanut cultivars. This will reduce production costs associated with fungicidal spraying and eliminate loss of yields associated with leaf spot disease.

Objectives

The objectives of the study are to validate candidate resistance genes that are associated with early and late leaf spot in peanut and to check if there is a significant difference in the relative expression of the seven candidate genes between the susceptible, medium and resistant peanut genotypes.

MATERIALS AND METHODS

A total of 41 genotypes of *Arachis hypogaea* L. from the U.S. peanut mini core collection (Table 1) were included in the experiment. These genotypes included four botanical varieties, *fastigiata, hypogaea, peruviana,* and *vulgaris* and can be classified as three categories of resistant (R), Medium (M), and susceptible (S), respectively. The line 'SPT06-6' was selected as a resistance control. It was from an interspecific hybridization between *A. hypogaea* and wild species *A. cardenasii*, and has multiple disease resistances including ELS (Tallury et al., 2014).

Preparation of inoculum

Peanut leaves showing leaf spot symptoms were sampled from a peanut field at the National Peanut Research Lab in Dawson, Georgia. Spores from a single lesion on the leaves were collected and cultured on PDA medium for three months. The identity of the cultures was confirmed to be *C.arachidiola* using internal transcribed spacer (ITS) sequence analysis. One clone was ground using a 16ml 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 minutes and the plate was parafilmed and placed under a grow light with a 12hr photoperiod for one week. 0.005% Tween 20 solution was used to flush conidia off medium using gentle pipetting for higher yield. Eight layers of cheese cloth were used to filter any mycelia residue from the inoculum. A hemacytometer was used to count conidia then concentration of conidia in the inoculum was adjusted to 5×10^3/ml.

Phenotyping of leaf spot in the field plastic chamber

A total of 41 genotypes were used as a genetic diversity panel with a wide range of variation of disease responses. The selected genotypes were grown in the fine-loamy and siliceous soil at the USDA-ARS National Peanut Research Laboratory at Dawson, Georgia (31o 44' 44" N by 84o 36' 30"W) in 2016 for late leaf spot evaluation. The genotypes were planted in late May using a randomized complete block (RCB) design with three replications. Each experiment plot was hand-planted in a field plastic chamber (Fig. 2); consisting of single rows of 0.91 m long and 0.91 m between rows at a seeding rate of 12 seeds m^{-1}. Before planting, the field area was cultivated and irrigated with 15 mm of water as needed to ensure adequate moisture for uniform seedling stands. Crop management for all tests was according to extension reccommendations for soil nutrients, herbicides, and insecticide but received no fungicide. In order to create a favorable condition for leaf spot disease development, a moisture misting system was installed within the field plastic chamber. Eighty days after planting, the plants were inoculated and 3 days later, the misting system was turned on 5 minutes every hour. The evaluation of LS took place 40 days after inoculation. The most common symptom of LLS was detected by dark brown lesions showed on the underside of affected leaves. Fully expanded young leaves were collected from a prominent stem from 4 randomly selected plants in 1 m linear row. Round punches (2 cm) of each leaf from 4 plants were pooled, placed into a 2 mL tube, frozen and stored at -80^{0} C until processed. A brief description of the candidate gene and their function is in Table 2.

Selection of candidate disease resistance genes

Transcriptomic project targeting peanut leaves infected with foliar diseases were utilized to identify candidate disease resistance genes (Guo et al., 2009). Sequence analyses were performed using Sequencher DNA analysis software (Gene Codes, Ann Arbor, MI). Unique sequences with potential open reading frame (ORF) and with low E-value in BLASTx search (NCBI) results were selected for further analysis. Sequences were searched against all *Arachis* EST and TSA NCBI databases. Sequences of each EST and TSA were downloaded and re-assembled to verify uniformity of each alignment and to obtain longer ORFs.

RNA Extraction and cDNA Synthesis

Total RNAs from fresh-frozen peanut leaves were extracted utilizing Direct-zol RNA Mini-Prep Kit (Zymo Research, Irvine, CA) according to manufacturer's instruction. RNA was quantified using Nanodrop 2000 spectrophotometer (ThermoFisher Sci., Waltham, MA) and quality was determined based on agarose gel electrophoresis analysis. RNA was DNase-treated with Turbo DNA-free (Ambion) prior to cDNA synthesis. One µg total RNA was used as template and cDNAs were produced according to Dang et al. (2013). cDNAs were diluted 1:10 with sterile water and used as template in standard PCR reaction. Primers were designed using Clone Manager (Sci-Ed Software, Denver, CO) to obtain the largest ORF sequence possible for each predicted RGA. The 20 µL PCR reaction consisted of 3 µL of diluted cDNAs, 10 µL GoTaq Green Master mix (Promega, Madison, WI) and 0.4 µM of each primer, with cycling conditions

of 2 min at 94°C to completely denature cDNAs, followed by 40 cycles of 20 s at 94°C, 20 s at 55°C and 50 s at 72°C, and a final cycle 10 min at 72°C to produce complete PCR products. PCR products were resolved on 1% TAE gel-electrophoresis, single bands at the predicted molecular weight were isolated and purified utilizing QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and 70 ng of purified-PCR products were sent for dideoxy-chain termination method sequencing (Eurofins MWG Operon, Louisville, KY) with the Forward or Reverse specific primer.

Quantitative (q) RT-PCR

Diluted cDNAs were used as template in real-time fluorescence qRT-PCR with specific gene primers (Table 2). Data was generated on QuantStudio7 Flex real-time PCR system (ThermoFisher Sci.) utilizing Relative Quantitation (RQ) method as described by manufacturer. The 20 µL reaction consisted of 3 µL of diluted cDNAs, 10 µL PowerUp SYBR green master mix and 0.4 mM of each primer, with PCR cycling conditions of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 58°C, and a dissociation curve analysis cycle of 15 sec at 95°C, 20 s at 58°C and 15 s at 95°C. The threshold cycle (Ct) was automatically calculated by QuantStudio Real-Time PCR software (ThermoFisher Sci.) and relative expression was calculated based on $2^{-\Delta\Delta Ct}$ described by Livak and Schmittgen (2001). All samples were first normalized to *Actin* (EZ723877) as an internal control then transformed data were normalized with FR458 $2^{-\Delta\Delta Ct}$ values and compared with the other 3 peanut genotypes to determine relative fold changes in gene-expression. Student T-Test was used to determine significant differences.

RESULTS AND DISCUSSION

There is a significant difference in the expression of NBS gene between the resistant genotypes compared to the medium and susceptible (Table 3). NBS genes are considered the most abundant and most important resistance genes in plants (Young and Innes, 2006). According to our results the NBS gene did not have a high fold increase relatively to the other genes. This could possibly be the reason why the other genes also do not show relatively high fold changes because the plant depends on pathogen recognition to send signals for expression of other genes involved in pathogen attack.

For the expression of Def, the resistant genotypes show a significantly higher fold increase of 27 compared to about 20 for the medium and the susceptible genotypes. Bala et al (2014) found out that in a study where transgenic lines of peanut containing defensin gene were studied for resistance to ELS and LLS, all the transgenic lines showed significantly elevated resistance to *C. arachidicola* and *P. personata* when measured for number of lesions and lesion size. Out of all the seven genes studied, expression of Def shows the highest fold increases across all genotypes (Table 3). Defensins are part of the plant`s innate immunity and are therefore considered the first line of defense in the first few critical hours and days after pathogen attack (Alberts et al, 2002). Also, bioinformatical studies have found out that defensin-encoding genes are over-represented in some plants species and may contribute around 3 % of all genetic material in *Arabidopsis.* This accentuates the significance of these peptides in general plant biology, including plant defense

(Bala et al, 2014). Defensins make good candidate genes for breeding resistance not only because of their abundance but also because their interaction with pathogens is non specific therefore pathogens cannot acquire resistance towards them (Alberts et al, 2012).

The resistant genotypes have the greatest fold increase in the expression of ChitinaseI (ChiI) gene as compared to the medium and susceptible genotypes Table 3). The resistant genotypes show a 3.5 fold increase as compared to a 2.4 fold increase in both the medium and susceptible genotypes. The observed significant upregulation of chiI in the event of leafspot disease in the resistant peanut genotypes suggests that chiI codes for proteins that are important in resistance to the fungal pathogen. This is to be expected because chiI gene codes for chitinases which hydrolyse chitin, which is part of the cell wall of fungi (Xu et al, 2016). In a study where high yielding peanut lacking leaf spot resistance was transformed with a chitinase gene, transgenic strains exhibited a higher resistance to leaf spot disease as compared to the non-transgenic plants. When chitinase gene expression in highly resistant transgenic strains were compared to that of a susceptible non transgenic plants, a good correlation was observed between chitinase activity and resistance to fungal pathogen. The study demonstrated that the transgenic expression of chi1 inhibits the activity of the leaf spot pathogens in peanut. Iqbal et al., (2012) reported resistant transgenic lines showed a 5 fold increase in chitinase enzyme activity. This validates the notion that chitinase genes play an important role in disease resistance.

There is no significant difference in expression of ChitinaseII (ChiII) between the resistant, medium and susceptible genotypes. The fold increase in expression of ChiII is much higher compared to that of ChiI (Table 3). This is not unusual because it has been demonstrated in peanut that the regulation of expression for class I and II chitinases is not coordinated. In an experiment where peanut cell cultures were used, the mRNA representing one chitinase class was induced following elicitor treatment while the expression of the other class remained unaffected (Collinge et al,1993). This supports our results that the two enzyme classes are not directly correlated (data not shown).

PalI and PalII increased significantly in the resistant genotypes as compared to the medium and susceptible genotypes (Table 3). Expression of PalII is much higher than that of PalI. For PalII the resistant genotypes had a fold increase of 14 which is ten times more than that of PalI. PalII also has the second highest fold increase recorded after Def gene. This may be telling of its importance in the plant`s defense system. Shadle et al (2003) showed that tobacco plants overexpressing Pal1 exhibited markedly reduced susceptibility to infection with the fungal pathogen *Cercospora nicotianae*. Pal is involved in the production of phenylpropanoid compounds which are precursors for lignin which strengthens the structural barriers to pathogen invasion. This is an important response because it is a direct method of pathogen control (Walter,1992)

There is no significant difference between the expression of thaumatin between all the three

genotype groups (Table 3). The fold change for this gene is the lowest for all the genes studied, with values close to 1. This suggests that there is very little regulation of expression of this gene. Han *et al* (2017) reports a similar phenomenon in his study and suggests that there is another mechanism involved to reduce the capacity of the host defense to the challenge by pathogen in the resistant genotype. This may involve integrated pathways that negatively mediated the defense responses and resulted in a failure of defense after pathogen infection.Genotype PI 158854 has the highest fold increase for all candidate genes excluding NBS (Table 4) implying that this variety can recognize the pathogen and respond by increasing chitinase activity and lignification. It is in the genus *fastigiata* and native to China (Table1). This makes it an excellent parent in breeding for leaf spot disease.

CONCLUSION

The presence of leaf spot disease causes an upregulation of all of the seven candidate genes.

Five genes showed significant fold increases in expression between the resistant, medium and susceptible lines. Of all the genotypes, the resistant had the highest fold increases for all genes except for Thaumatin. There was no significant difference in the expression of chitinase II and thaumatin across all genotypes. PI158854, in the resistant family, showed the highest level of expression across 5 of the 7 genes. Future breeding efforts for leaf spot may be beneficial by using this genotype as a parent. It may be more beneficial to study these gene expressions under the individual diseases.

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Table 1; Fourty peanut genotypes used in the leaf spot study; Resistance (R), Susceptible (S), Medium resistance (M)

PI Number	**Botanical Variety**	**Origin**	**Disease Class**
PI158854	*fastigiata*	China	R
PI268868	*hypogaea*	Sudan	R
PI576614	*hirsuta*	Mexico	R
PI471954	*fastigiata*	Zimbabw	R
PI290566	*fastigiata*	India	R
PI162857	*hypogaea*	Sudan	R
PI371521	*hypogaea*	Israel	R
PI196622	*hypogaea*	Cote	R
PI288210	*vulgaris*	India	R
PI290536	*hypogaea*	India	R
PI274193	*hypogaea*	Bolivia	R
PI259658	*hypogaea*	Cuba	R
PI290560	*vulgaris*	India	M
PI296550	*hypogaea*	Israel	M
PI493356	*fastigiata*	Argentina	M
PI295309	*hypogaea*	Israel	M
PI648245	*aequatoriana*	Ecuador	M
PI343398	*fastigiata*	Israel	M
PI240560	*hypogaea*	South	M
PI403813	*vulgaris*	Argentina	M
Grif12579	*aequatoriana*	Ecuador	M
PI576634	*hirsuta*	Mexico	M
PI259617	*fastigiata*	Cuba	M
PI493329	*fastigiata*	Argentina	M
PI493717	*fastigiata*	Argentina	M
PI494018	*vulgaris*	Argentina	M
PI493880	*fastigiata*	Argentina	M
Grif12545	*aequatoriana*	Ecuador	S
PI482189	*fastigiata*	Zimbabw	S
PI493693	*fastigiata*	Argentina	S
PI268586	*hypogaea*	Zambia	S
PI319768	*hypogaea*	Israel	S
PI200441	*fastigiata*	Japan	S
PI270786	*hypogaea*	Zambia	S
PI271019	*vulgaris*	Zambia	S
PI482120	*hypogaea*	Zimbabw	S
PI461427	*hypogaea*	China	S
PI155107	*vulgaris*	Uruguay	S
PI337399	*hypogaea*	Morocco	S
PI337406	*fastigiata*	Paraguay	S

Table 3; Real time PCR primers

ID	Forward	Reverse	Prod Size
ahDefensin1	CTGCATCTTCTTCCTCGTTCTC	GATCATCGCAGCTTGCATTG	125bp
ahNBS-LRR1	ACCTGAACTTGGGAAGCTTGAG	AGGGATGGTGCCAGATATGTTG	145bp
ahPAL1	GCCAGAGTTCACTGACCATTTG	AACTTCTTGGCGGCTTTGAC	120bp
ahPAL2	CTTGCGAATCCGGTAACCAG	TGGCAAAGCGCCATCAAG	149bp
ahThaumatin1	CTTAGCCCAGTTCGAGGTCCAC	CCAGATCGCTCCGACAACCTAC	146bp
ahChitI	TGGCTCATGCCGCTGGATTC	TGGCACCAACGTGTCCAAGG	141bp
ahChitII	GCAATATTATGGCCGTGGTCC	CGCATCTGTTGCTACCAAGTC	115bp

Table 3; Statistical significance from t-test results for relative expression of candidate genes associated with leaf spot resistance

GENE	GROUP	MEANS	T STATISTIC
ChiI	R	3.568	a
	M	2.389	b
	S	2.536	b
ChiII	R	15.017	a
	M	10.792	a
	S	10.774	a
Def	R	27.37	a
	M	18.934	b
	S	20.416	b
NBS	R	2.877	a
	M	2.437	b
	S	2.493	b
PalI	R	3.929	a
	M	2.779	b
	S	3.416	b
PalII	R	14.007	a
	M	8.928	b
	S	8.099	b
Thau	R	1.048	a
	M	1.78	a
	S	1.118	a

Significance level = 0.001

Table 4; Relative fold changes in the resistant geneotypes

Genotype	**Leafspot rating**	**ChiI**	**ChiII**	**Def**	**NBS**	**PalI**	**PalII**	**Thau**
PI 290560	R	2.65463	4.55215	17.7277	2.78252	2.4445	9.25588	4.51488
Grif 12545	R	1.20022	6.08872	28.0894	1.98857	1.1381	3.12846	1.88686
PI 296550	R	1.20733	8.01633	33.2773	2.01706	2.16302	4.35974	1.97698
PI 158854	R	**5.79705**	**22.2117**	**65.6675**	3.01316	**10.4894**	**53.5617**	**3.97582**
PI 493356	R	2.42607	13.7341	15.9663	2.35344	3.46857	11.4033	0.07797
PI 295309	R	3.51057	9.54724	24.4903	3.17898	2.46067	7.75965	0.79262
PI 648245	R	1.03772	4.34034	13.2179	1.603	0.82988	3.47348	0.73412
PI 268868	R	2.81148	12.3692	8.7893	3.3773	2.72502	14.8221	0.28611
PI 482189	R	3.04226	4.83098	20.5499	2.50581	2.05786	7.16888	0.48086
PI 343398	R	4.63618	7.14009	19.0212	3.74086	2.52878	10.9115	1.36677
PI 493693	R	2.86881	3.0553	11.2284	2.40762	2.96517	8.28063	0.44242
PI 200441	R	2.18126	14.2494	35.501	2.98144	2.16808	4.16689	3.94991
PI 403813	R	2.22274	9.49798	21.866	3.45475	3.31775	8.4394	2.90777
PI 270786	R	2.79227	6.64364	13.1787	2.88287	2.63906	6.4302	2.56943
PI 271019	R	3.95131	9.81158	21.6815	2.93882	3.7306	7.74235	0.41482
PI 461427	R	2.91391	6.26299	12.4076	3.25469	6.75444	19.1504	0.15685
SPT06-6	R	2.31582	11.8382	13.831	3.23044	5.92436	19.0275	0.08218

Table 5; Candidate genes and their functions

Candidate Gene	Function
Chitinase I	encodes chitinase which hydrolyses chitin
Chitinase II	encodes Chitinase which hydrolyses chitin
Defensin	Function in membrane permeabilisation Interact with specific lipids, produce reactive oxygen species and cause cell wall stress Ubiquitous in plant`s innate immunity
Nucleotide Binding Site	Encodes proteins that mediate pathogen recognition in plants by binding pathogen-derived molecules directly or by sensing the modification of host proteins by pathogen-derived molecules
Phenylalanine ammonia lyase I	Encodes an PAL enzyme which catalyzes the formation of phenyalpropanoid compunds which are precursors for lignin
Phenylalanine ammonia lyase II	Encodes an PAL enzyme which catalyzes the formation of phenyalpropanoid compunds which are precursors for lignin
Thaumatin	Defense protein,function not clear

Figure 2; Image of field plastic chamber

APPENDIX

Means and standard deviations of fold changes of seven candidate genes studied

Level of genotype	Level of group	N	**ChiI** Mean	Std Dev
Grif1257	M	2	2.7395	1.63978063
PI240560	M	2	1.964	0.35779603
PI259617	M	2	1.831	1.01681955
PI290560	M	2	2.6105	1.38239376
PI296550	M	2	1.3165	0.47729708
PI343398	M	2	4.722	1.71544105
PI403813	M	2	2.286	1.00974848
PI493329	M	2	1.055	0.68447936
PI493356	M	2	2.488	1.69847049
PI493717	M	2	2.14	1.04651804
PI493880	M	2	1.291	0.4695189
PI494018	M	2	4.6775	4.03970104
PI576634	M	2	1.911	0.76226111
PI648245	M	2	0.972	0.35638182
Pi295309	M	2	3.845	2.23445743
PI158854	R	2	6.527	4.67397582
PI162857	R	2	0.9405	0.39385848
PI196622	R	2	2.052	1.24167951
PI259658	R	2	5.0305	2.0753584
PI268868	R	2	4.261	3.30360288
PI274193	R	2	2.3465	1.19288914
PI288210	R	2	1.9505	0.87186266
PI290536	R	2	2.935	1.37178716
PI290566	R	2	4.032	1.98272741
PI371521	R	2	2.686	1.64473037
PI471954	R	2	4.673	2.21183001
PI576614	R	2	6.7325	4.86560176
SPT06-6	R	2	2.2275	0.60033366
Grif1254	S	2	1.371	0.36910974
PI155107	S	2	2.1375	0.94823019
PI200441	S	2	2.09	0.74953319
PI268586	S	2	2.714	1.17945411
PI270786	S	2	3.0645	1.54785674
PI271019	S	2	4.1255	2.09657161
PI319768	S	2	1.639	0.68023672

PI337399	S	2	0.8295	0.37971634
PI337406	S	2	3.299	1.51320851
PI461427	S	2	3.811	2.5653834
PI482120	S	2	1.8955	0.86196317
PI482189	S	2	3.212	2.04071017
PI493693	S	2	2.7905	0.20576807

Level of genotype	Level of group	N	chill Mean	Std Dev
Grif1257	M	2	8.542	8.8982317
PI240560	M	2	9.954	8.9703566
PI259617	M	2	11.6345	12.500941
PI290560	M	2	6.7705	7.0647039
PI296550	M	2	11.3165	10.801056
PI343398	M	2	11.776	9.509172
PI403813	M	2	8.0305	6.0705117
PI493329	M	2	14.6605	15.383108
PI493356	M	2	22.572	25.304523
PI493717	M	2	3.9505	4.2914311
PI493880	M	2	11.7915	11.604329
PI494018	M	2	20.1845	23.705755
PI576634	M	2	4.1785	3.7794857
PI648245	M	2	6.371	6.8942911
Pi295309	M	2	10.1485	9.0361176
PI158854	R	2	34.2595	36.433677
PI162857	R	2	23.557	24.284875
PI196622	R	2	10.354	10.404369
PI259658	R	2	18.048	15.632717
PI268868	R	2	17.7675	17.471902
PI274193	R	2	9.387	9.9970757
PI288210	R	2	19.645	21.418264
PI290536	R	2	7.341	7.4331065
PI290566	R	2	11.148	11.691304
PI371521	R	2	9.79	11.638978
PI471954	R	2	3.8575	3.8346401
PI576614	R	2	12.364	12.720851
SPT06-6	R	2	17.709	17.799292
Grif1254	S	2	8.927	9.1485475
PI155107	S	2	16.386	14.450434
PI200441	S	2	20.911	21.484732
PI268586	S	2	20.223	20.893591
PI270786	S	2	9.2005	9.4151268
PI271019	S	2	14.586	15.279163
PI319768	S	2	9.1535	9.6244304
PI337399	S	2	4.1695	4.5445753
PI337406	S	2	13.0975	14.000007
PI461427	S	2	9.138	9.442704
PI482120	S	2	3.572	3.628872
PI482189	S	2	6.897	6.9565165
PI493693	S	2	3.813	3.4421958

Level of genotype	Level of group	N	------------ Mean	-Def---------- Std Dev
Grif1257	M	2	26.592	7.9690934
PI240560	M	2	11.339	3.0165175
PI259617	M	2	10.4045	0.1704127
PI290560	M	2	17.964	2.2839549
PI296550	M	2	34.9535	5.708473
PI343398	M	2	24.7635	7.8255507
PI403813	M	2	21.779	0.0254558
PI493329	M	2	7.915	0.3606245
PI493356	M	2	16.0995	0.3514321
PI493717	M	2	12.0155	2.1899097
PI493880	M	2	17.941	2.1085924
PI494018	M	2	33.0105	29.233916
PI576634	M	2	9.9075	1.8618122
PI648245	M	2	13.015	0.3719382
Pi295309	M	2	26.313	13.761712
PI158854	R	2	69.0355	27.995065
PI162857	R	2	29.4655	2.6085169
PI196622	R	2	17.4605	3.5150278
PI259658	R	2	42.054	19.598172
PI268868	R	2	8.7855	1.0839947
PI274193	R	2	37.1085	5.4256303
PI288210	R	2	11.744	0.0028284
PI290536	R	2	24.449	1.8653477
PI290566	R	2	33.948	8.0101056
PI371521	R	2	16.7335	13.47392
PI471954	R	2	20.75	1.397243
PI576614	R	2	30.3015	3.0087394
SPT06-6	R	2	13.983	0.066468
Grif1254	S	2	28.2885	5.5670517
PI155107	S	2	43.904	29.602318
PI200441	S	2	36.9155	3.1897587
PI268586	S	2	29.4935	0.4928534
PI270786	S	2	13.2615	0.2298097
PI271019	S	2	22.7875	2.529321
PI319768	S	2	14.525	2.2797123
PI337399	S	2	10.4085	2.6566002
PI337406	S	2	9.4475	0.820951
PI461427	S	2	12.211	0.4780042
PI482120	S	2	12.0725	0.7841814
PI482189	S	2	20.7375	0.2566798
PI493693	S	2	11.3595	1.0033845

Level of genotype	Level of group	N	NBS Mean	Std Dev
Grif1257	M	2	1.8205	0.0671751
PI240560	M	2	2.99	0.4044651
PI259617	M	2	2.291	0.1216224
PI290560	M	2	2.6735	0.4277996
PI296550	M	2	1.8965	0.2948635
PI343398	M	2	3.4025	0.5055814
PI403813	M	2	3.372	0.8881261
PI493329	M	2	1.6575	0.0954594
PI493356	M	2	2.2795	0.0502046
PI493717	M	2	1.7585	0.2708219
PI493880	M	2	2.3405	0.2283955
PI494018	M	2	2.7225	0.0247487
PI576634	M	2	2.7925	0.4362849
PI648245	M	2	1.5355	0.1011163
Pi295309	M	2	3.0305	0.6512454
PI158854	R	2	2.965	0.3931514
PI162857	R	2	1.6225	0.2199102
PI196622	R	2	1.922	0.2022325
PI259658	R	2	4.944	1.436841
PI268868	R	2	3.254	0.3535534
PI274193	R	2	2.2465	0.2001112
PI288210	R	2	2.2875	0.4391133
PI290536	R	2	2.242	0.079196
PI290566	R	2	3.2465	0.2524371
PI371521	R	2	3.2245	1.0613673
PI471954	R	2	2.7	0.3917372
PI576614	R	2	3.6835	0.6173042
SPT06-6	R	2	3.068	0.1173797
Grif1254	S	2	1.9365	0.1223295
PI155107	S	2	3.282	1.7465538
PI200441	S	2	2.594	0.1244508
PI268586	S	2	3.1085	0.2326381
PI270786	S	2	2.7545	0.0558614
PI271019	S	2	2.783	0.3054701
PI319768	S	2	2.4475	0.0827315
PI337399	S	2	1.318	0.1117229
PI337406	S	2	2.845	0.0622254
PI461427	S	2	2.9995	0.5451793
PI482120	S	2	1.609	0.1103087
PI482189	S	2	2.447	0.2729432
PI493693	S	2	2.287	0.2983991

Level of genotype	Level of group	N	**PalI** Mean	Std Dev
Grif1257	M	2	3.944	0.06363961
PI240560	M	2	2.1395	0.58053467
PI259617	M	2	5.7915	0.55507882
PI290560	M	2	2.5455	0.22839549
PI296550	M	2	2.1795	0.18172644
PI343398	M	2	2.564	0.24465895
PI403813	M	2	3.444	1.29400541
PI493329	M	2	2.2455	0.03606245
PI493356	M	2	3.479	0.04666905
PI493717	M	2	1.877	0.83721443
PI493880	M	2	2.6085	0.00636396
PI494018	M	2	2.568	0.24183052
PI576634	M	2	3.0215	0.6257895
PI648245	M	2	0.812	0.01414214
Pi295309	M	2	2.471	0.28991378
PI158854	R	2	10.854	2.26557013
PI162857	R	2	5.0335	1.10379369
PI196622	R	2	3.683	0.46527626
PI259658	R	2	3.589	0.33234019
PI268868	R	2	2.7655	0.47446865
PI274193	R	2	2.431	1.30673333
PI288210	R	2	5.129	2.3249671
PI290536	R	2	3.1715	1.51815826
PI290566	R	2	1.9015	0.25102291
PI371521	R	2	2.186	0.55861436
PI471954	R	2	2.2065	0.63003214
PI576614	R	2	1.795	0.02545584
SPT06-6	R	2	6.3405	2.39921331
Grif1254	S	2	1.151	0.25173001
PI155107	S	2	5.363	0.30971277
PI200441	S	2	2.4845	0.95388705
PI268586	S	2	2.231	0.18950462
PI270786	S	2	2.622	0.39173716
PI271019	S	2	3.7775	0.09828784
PI319768	S	2	2.104	0.86832713
PI337399	S	2	3.683	1.75645324
PI337406	S	2	5.0215	1.70059181
PI461427	S	2	7.3	0.65478088
PI482120	S	2	3.66	0.15980613
PI482189	S	2	2.0255	0.1774838
PI493693	S	2	2.985	0.1767767

Level of	Level of		------------	**Pall1--**
genotype	group	N	Mean	Std Dev
Grif1257	M	2	9.0895	1.4318912
PI240560	M	2	10.5815	0.1774838
PI259617	M	2	13.8285	1.4516902
PI290560	M	2	8.5635	0.3585031
PI296550	M	2	5.697	1.6206887
PI343398	M	2	11.3095	0.2298097
PI403813	M	2	8.6295	0.0516188
PI493329	M	2	9.565	0.7254916
PI493356	M	2	11.6345	1.0698526
PI493717	M	2	4.3985	0.1025305
PI493880	M	2	7.1485	0.5140666
PI494018	M	2	12.3145	0.0700036
PI576634	M	2	9.3685	1.966464
PI648245	M	2	3.72	0.0537401
Pi295309	M	2	8.0775	0.3627458
PI158854	R	2	54.332	10.30396
PI162857	R	2	11.7235	2.4826519
PI196622	R	2	14.4395	1.1462201
PI259658	R	2	13.711	0.2828427
PI268868	R	2	14.8175	0.2128391
PI274193	R	2	5.9355	1.284813
PI288210	R	2	9.935	0.8259007
PI290536	R	2	14.165	0.0919239
PI290566	R	2	4.7165	0.5508362
PI371521	R	2	10.0425	2.3186031
PI471954	R	2	4.3805	0.284964
PI576614	R	2	4.178	0.8796408
SPT06-6	R	2	19.7225	1.7104913
Grif1254	S	2	3.398	1.2600643
PI155107	S	2	15.4155	2.8390337
PI200441	S	2	4.1745	0.2694077
PI268586	S	2	5.3805	0.4320422
PI270786	S	2	6.5505	0.7290271
PI271019	S	2	7.619	1.629174
PI319768	S	2	3.142	0.2672864
PI337399	S	2	7.037	0.7650895
PI337406	S	2	11.152	0.1018234
PI461427	S	2	19.3775	0.1294005
PI482120	S	2	6.745	0.6844794
PI482189	S	2	6.938	0.165463
PI493693	S	2	8.3605	0.7063997

Level of genotype	Level of group	N	------------ Mean	Thau Std Dev
Grif1257	M	2	0.213	0
PI240560	M	2	0.886	0.0905097
PI259617	M	2	0.1515	0.0714178
PI290560	M	2	5.1965	2.9762124
PI296550	M	2	1.977	0.1272792
PI343398	M	2	1.469	0.8061017
PI403813	M	2	2.95	0.496389
PI493329	M	2	0.149	0.1088944
PI493356	M	2	0.0965	0.0077782
PI493717	M	2	0.2345	0.006364
PI493880	M	2	0.4025	0.1958686
PI494018	M	2	10.4255	14.673173
PI576634	M	2	0.1215	0.026163
PI648245	M	2	0.76	0.3238549
Pi295309	M	2	1.6815	0.7629682
PI158854	R	2	4.479	2.7959002
PI162857	R	2	0.097	0.0735391
PI196622	R	2	0.0395	0.013435
PI259658	R	2	0.34	0.0989949
PI268868	R	2	0.2595	0.0813173
PI274193	R	2	0.1835	0.0049497
PI288210	R	2	0.4525	0.0473762
PI290536	R	2	0.279	0.0721249
PI290566	R	2	1.38	0.9376236
PI371521	R	2	2.954	3.7405949
PI471954	R	2	2.026	0.7523616
PI576614	R	2	1.0365	0.0601041
SPT06-6	R	2	0.0975	0.0417193
Grif1254	S	2	1.883	0.5897271
PI155107	S	2	1.553	0.6363961
PI200441	S	2	3.7605	0.8110515
PI268586	S	2	0.861	0.4256783
PI270786	S	2	2.5825	1.2593572
PI271019	S	2	0.3705	0.033234
PI319768	S	2	0.88	0.185262
PI337399	S	2	0.2935	0.2213244
PI337406	S	2	0.114	0.0735391
PI461427	S	2	0.199	0.0098995
PI482120	S	2	0.991	0.5006316
PI482189	S	2	0.534	0.22486
PI493693	S	2	0.521	0.0212132