GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF PEANUT LINES WITH INTERSPECIFIC INTROGRESSIONS CONFERRING LATE LEAF SPOT RESISTANCE

by

SAMUELE LAMON

(Under the Direction of Peggy Ozias-Akins and Scott A. Jackson)

ABSTRACT

Late Leaf Spot (LLS) disease caused by *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash, Videira & Crous affects peanut (*Arachis hypogaea* L.) all around the world. IAC 322 is a breeding line with alien introgressions from *A. cardenasii* Krapov. & W.C. Gregory, a wild diploid relative of peanut, on chromosomes A02 and A03. Progenies from 'TifNV-High O/L' x IAC 322 were genotyped and selected based on the introgressed segments they retained and phenotyped for LLS resistance under both *in vitro* and field conditions. IAC 322-derived progenies were found to have varied levels of LLS resistance. The introgressed segments on the top part of A02 and bottom part of A03 chromosomes accounted for the majority of LLS resistance. Moreover, high correlations between *in vitro* and field experiments, and between late stages of LLS severity and entire LLS infection progression under both field and *in vitro* experiments, were observed.

INDEX WORDS: *Nothopassalora personata*, late leaf spot, peanut, *Arachis hypogaea*, detached leaf method, *Arachis cardenasii*, disease resistance.

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DEDICATION

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CHAPTER 1

INTRODUCTION

Peanut or groundnut, scientifically known as *Arachis hypogaea* L., is a grain legume and cultivated oilseed species belonging to the *Arachis* genus and Fabaceae family. It is preferentially an autogamous species with an outcrossing rate of only 2.5% (Norden 1982). The *Arachis* genus evolved in the southwest of Brazil's Mato Grosso do Sul or northeastern Paraguay (Simpson et al. 2001) and it mostly consists of diploid individuals (2n = 2x = 20). Nevertheless, peanut is an allotetraploid species (AABB-type genome; 2n = 4x = 40). The origin of *A. hypogaea* is probably due to a single event of hybridization between two diploid wild species (*A. duranensis* and *A. ipaensis*) and subsequent polyploidization (Husted 1930; Bertioli et al. 2016). Peanut's A subgenome chromosomal structure shows strong chromosomal centromeric banding (Ramos et al. 2006). This feature, in combination with the presence of one pair of small chromosomes, distinguishes the A from the B subgenomes (Ramos et al. 2006). Peanut has a total genome size of ~2.7 Gb which is almost the sum of *A. duranensis* and *A. ipaensis* genomes (1.25 Gb and 1.56 Gb, respectively) (Samoluk et al. 2015) suggesting that polyploidization did not induce significant change in the genome size (Bertioli et al. 2016).

Peanut can be classified in two subspecies and six botanical varieties based on the presence or lack of flowers on the main stem and other morphological traits and growth habits. *Arachis hypogaea hypogea* subspecies is characterized by the absence of flowers

on the main axis, while the *Arachis hypogaea* ssp. *fastigiata* has flowers on the main stem. The first subspecies is subdivided in two botanical varieties, '*hirsuta'* and *'hypogaea'*, while the second subspecies has four botanical varieties, '*aequatoriana', 'fastigiata', 'peruviana'* and '*vulgaris'* (Moretzsohn et al. 2004).

Peanuts are primarily grown for human consumption and play a key role in human nutrition with a global production of ~29 million metric tons per year (www.nationalpeanutboard.org). In 2017, the United States was the world's third largest producer of peanuts after China and India (www.fao.org), with 718,560 hectares harvested and an average yield of 4,491 kg ha^{-1} (www.nass.usda.gov). Large U.S. producing areas are located mainly in Alabama, Florida, Georgia, Mississippi, North Carolina, Oklahoma, South Carolina, Texas and Virginia (www.nass.usda.gov). Peanut production in Georgia alone accounts for around 50% of U.S. production (www.nass.usda.gov). In 2016, the U.S. peanut exports totaled more than $694.7 million and the peanut oil exports were evaluated up to $11.4 million (Foreign Ag Service (FAS) 2016) (www.fas.usda.gov). It has been estimated that U.S. domestic consumption is around 2 million kilograms (unshelled weight) of peanuts daily. About two-thirds of the total amount of U.S. peanuts is used as a source of food mainly in the form of peanut butter, while the remaining part is used to produce oil, exports or as a source for seeds. A secondary market value comes from peanut-derived, non-food products such as soaps, medicines, cosmetics and lubricants. Noteworthy are the uses of peanuts for livestock nutrition, fuels, mulches and fertilizers (Woodroof 1983).

In 2012, an all-time record was reached by American farmers: peanut average yields overcame all expectations reaching 4,695 kg ha^{-1} (Holbrook et al. 2014). Nevertheless, the first peanut breeding objective is still yield improvement, as it is for most crops. Leaf spot (LS) is a foliar disease that causes peanut yield loss all around the world (Shokes and Culbreath 1997). Yield loss to LS disease ranges from 10% to 80% even in treated fields (Shokes et al. 1983; Knauft et al. 1986; Knauft et al. 1988). Two fungal species, *Cercospora arachidicola* Hori and *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash, Videira & Crous (syn. *Cercosporidium personatum*), cause early and late leaf spot (ELS & LLS) diseases respectively (McDonald et al. 1985). Depending on the location, climate and environmental conditions, year and time of the year, one of the two pathogens can be dominant over the other in infected peanut fields (Shokes and Culbreath 1997). In order to achieve effective control, application of fungicides must be made before and after the manifestation of the symptoms (McDonald et al. 1985). However, chemical control can be difficult to apply in small-scale farms, particularly in developing countries (McDonald et al. 1985). One effective solution to overcome these problems is the employment of resistant cultivars (McDonald et al. 1985).

In 1985, McDonald et al. stated that there was "no agronomically acceptable groundnut cultivar with resistance to either of the leaf spots" in the market. On the other hand, many breeding programs were starting or were already active at that time in several different countries. In fact, in 1987, the Florida Agricultural Experiment Station registered 'Southern Runner' peanut cultivar (Gorbet et al. 1987), which was the first cultivated peanut variety with some levels of LLS resistance. When LS disease was chemically

controlled, Southern Runner was characterized by pod yield similar to 'Florunner' (Norden et al. 1969), while it was superior to Florunner (it averaged 195% of Florunner) when left untreated.

From 1986 to 1991, Holbrook and Anderson screened the entire U.S. peanut germplasm collection for LLS resistance. The collection was comprehensive of 7,432 accessions, of which 112 were identified as sources of resistance (Anderson et al. 1993; Holbrook and Anderson 1995). Three accessions (PI 215695, PI 215696 and PI 215724) were significantly more resistant than Southern Runner to defoliation induced by LLS.

Since then, several other LS resistant cultivars have been released in the U.S. such as Tifrunner (Holbrook and Culbreath 2007), DP-1 (Gorbet et al. 2008) and TifNV-High O/L (Holbrook et al. 2017).

Sources of resistance have been found in many wild *Arachis* species such as *A. cardenasii*, *A. batizocoi*, *A. diogoi*, *A. stenosperma*, etc. (Fávero et al. 2009). However, some difficulties have emerged because of the presence of complex inheritance and the polygenic nature of these traits in addition to the crossing barrier between wild diploids and tetraploid peanut (Sharief et al. 1978; Nevill 1982; Dwivedi et al. 2002; Gill 2013). Nevertheless, success with introgression of leaf spot resistance from *A. cardenasii* to cultivated peanut was achieved by a research team at North Carolina State University (NCSU) (Company et al. 1982) whose lines initially were selected for ELS resistance and later distributed to India and Brazil to further select for LLS resistance (Stalker 2017). The ICRISAT germplasm line (ICGV 86687: CS 16 – B2 – B2) that showed high levels of resistance to LLS (ICRISAT, 1986) was derived from the NCSU materials.

Furthermore, a Brazilian breeding line, IAC 322, is a progeny from the initial cross of

‘Runner IAC 886’, a common cultivar in Brazil, and the ICRISAT germplasm line CS 16 – B2 – B2. In 2017, Clevenger et al. (2017a) detected three major introgressed segments on the top and bottom parts of chromosome A02 and bottom part of chromosome A03 present in the LLS resistant breeding line IAC 322 (chromosome assignment was based on the *A. duranensis* v1 pseudomolecules, peanutbase.org).

Other significant peanut economic losses are due to the presence of root-knot nematodes in peanut fields, particularly if the cultivated areas are characterized by sandy soils and short cycles of rotation (Holbrook et al. 2014). In infested fields, this pest can cause decreased production to less than half of non-infested ones (Minton and Baujard 1990). In 2016, $18.7 million in yield losses were caused by this disease and $7.2 million were spent for its control by farmers (Little 2016). Chemicals are available to deal with this disease but some restrictions are limiting their use (Holbrook et al. 2014). Thus, significant economic gains can be obtained by employing resistant cultivars in those fields where nematode infestation occurs (Chu et al. 2007a).

In addition, tomato spotted wilt virus (TSWV) is a major threat to peanut yield in the southeastern United States. Yield reductions can be higher than 95% (Li et al. 1997) and incidence in Georgia is up to 100% according to a survey that considered fields in ten different counties (Camann 1995). TSWV is vectored by thrips (Thysanoptera) which thrive on over 600 plant species including peanut (Campbell and Waynne 1980; Peter and Goldbach, 1995). Chemicals may be applied to decrease leaf injuries by thrips yet the treatment does not improve yield (Culbreath et al. 1992c; Culbreath et al. 1994).

TifNV- High O/L is a newly released runner-type cultivar. It is characterized by a high level of resistance to both the peanut root-knot nematode and TSWV, and has a high ratio

of oleic acid to linoleic acid (Holbrook et al. 2017). Oleic and linoleic fatty acids comprise almost 80% of the fatty acid in peanut seeds and usually the ratio in standard genotypes ranges from 1.0 to 4.0 (Norden et al. 1987). High O/L mutants identified in the Florida breeding program were used to improve the oleic:linoleic acid ratio up to 40 (Norden et al. 1987). Some segments of the food industry favor cultivars with high ratios since they have longer storage life (Holbrook et al. 2016). This characteristic is due to the fact that oleic acid has ten times higher oxidative stability compared to linoleic acid (O'Keefe et al. 1993). Furthermore, other health benefits from high oleic oil include the reduction of low-density lipoprotein (LDL) levels, repression of tumorigenesis and inflammatory diseases (O'Byrne et al. 1997; Yamaki et al. 2005; Mesa et al. 2006). Recently, molecular markers have been identified that are associated with quantitative trait loci (QTL) for several traits, and marker assisted selection (MAS) technologies have become more frequently used in research programs in peanut (Holbrook et al. 2016; Ozias-Akins et al. 2017). Chu et al. (2016a) summarized the different kinds of molecular markers that have been developed for peanut breeding: "isozymes, storage proteins, randomly amplified polymorphic DNA, restriction fragment length polymorphism, amplified fragment length polymorphism, cleaved amplified polymorphic sequence, simple sequence repeat, single strand conformational polymorphism, single nucleotide polymorphism and miniature inverted repeat transposable element-based markers". Markers have been developed both for high oleic:linoleic fatty acid ratio and peanut root-knot nematode traits (Chu et al. 2011), as well as LLS (Shoba et al. 2012; Clevenger et al. 2018) and rust resistances (Varshney et al. 2014). Due to limited genomic information, only a modest number of traits have benefited from marker assisted selection, mainly

high oleic/linoleic fatty acid ratio and nematode resistance which are qualitatively inherited (Holbrook et al. 2011; Holbrook et al. 2013). The 441_442insA and 665_insMITE mutations control the high oleic:linoleic acid ratio trait in peanut. In 2011, Chu et al. converted the cleaved amplified polymorphic sequence (CAPS) marker 1101/1048 targeting the mutation to a gel-free SNP marker for HybProbe assay applications. Nematode resistance was introgressed from *A. cardenasii* and the genomic regions responsible for the disease resistance are located on chromosome A09 (Nagy et al. 2010). KASPar markers were designed to track the introgression (Simpson and Starr 2001; Nagy et al. 2010; Holbrook et al. 2013; Chu et al. 2016b).

In this project, genotyping and phenotyping was performed on progenies from TifNV-High O/L x IAC 322 crosses to determine the introgressed regions responsible for LLS resistance. Hence, the design determines whether one or more introgressed region can be excluded to reduce linkage drag. Furthermore, the LS infection progression was studied during the phenotyping analysis and different procedures were tested for spore inoculation and management on leaves under *in vitro* and field conditions. The detached leaf method (Melouk and Banks 1978) for LLS resistance assessment under *in vitro* conditions and the Florida scale (1-10) (Chiteka et al. 1988a; Chiteka et al. 1988b) in the field provided reliable data for comparison among introgression lines, their parents, and resistant and susceptible checks.

CHAPTER 2

LITERATURE REVIEW

Leaf Spot Diseases

Leaf Spot (LS) is a foliar disease that affects peanut all around the world since it is present everywhere this species is cultivated (McDonald et al. 1985; Shokes and Culbreath 1997). Yield losses may range from 10% to 80% even in treated fields (Shokes et al. 1983; Knauft et al. 1986; Knauft et al. 1988; Shokes and Culbreath 1997) and pod yield losses increase with delayed harvest, for a given level of defoliation (Shokes and Culbreath 1997). Two fungi may be involved in the disease development, namely *Cercospora arachidicola* Hori and *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash., Videia & Crous (syn. *Cercosporidium personatum*) which cause early and late leaf spot diseases (ELS & LLS), respectively (McDonald et al. 1985). Depending on the location, climate and environmental conditions, year and time of the year, one of the two pathogens can be dominant over the other in peanut infected fields (Shokes and Culbreath 1997). Usually ELS is predominant at the beginning of the peanut growing season while LLS increases late in the season (McDonald et al. 1985). LS symptoms are frequently observable on peanut leaves but they can also emerge on petioles, stipules, stems, and pegs of peanut plants, especially during final stages of disease progression (Shokes and Culbreath 1997).

The main source of inoculum is overwintered conidia in peanut crop residues (McDonald et al. 1985; Shokes and Culbreath 1997). Other possible sources of inoculum may be

chlamydospores, mycelial fragments and ascospores, even though the teleomorph phase is rarely detected on peanut (Shokes and Culbreath 1997). Wind, water and insects are preferential vectors for conidia dispersal. Human transfer of infected crop residuals is the most plausible way of long-distance distribution, although it is not common (McDonald et al. 1985). Thus, infection usually starts with conidia release from already infected material. Conidia release from lesions mainly occurs in the morning when leaf surfaces dry and before precipitation events. Optimal conditions for disease progression are high relative humidity and temperatures between 25 to 30 ℃. With these favorable conditions, the LS infection may progress throughout the peanut growing season resulting in almost the total defoliation of peanut plants (McDonald et al. 1985).

LS symptoms start as chlorotic flecks around 10 d after spores first contact peanut leaves. Lesions usually appear first on the oldest leaves near the soil surface and, as long the disease progresses, lesions become darker and may reach a diameter of 10 mm (McDonald et al. 1985; Shokes and Culbreath 1997). Sporulating lesions can become visible around 15 d after spores contact leaves. Fungal symptoms look similar on both surfaces of peanut leaves, although a prominent yellow halo may develop in the case of ELS lesions which is frequently reduced or absent for LLS (Shokes and Culbreath 1997). This feature is not fully reliable, however, since it is influenced by genotype and environmental factors (McDonald et al. 1985). Some differences can be highlighted also in terms of sporulation behavior between the two fungi. Although they are both amphigenous, ELS sporulation primarily occurs on adaxial leaf surfaces while LLS sporulates predominantly on abaxial surfaces (McDonald et al. 1985; Shokes and Culbreath 1997). In some peanut varieties ELS lesions appear light tan to reddish brown

on the abaxial surfaces, whereas they usually range from dark brown to black in case of LLS and they could be smaller in size (McDonald et al. 1985; Shokes and Culbreath 1997). Sometimes, some phytotoxic pesticides create symptoms on peanut leaves that look very similar to LS. They are usually lighter in color in the center and obviously do not show sporulation. For these reasons, the most appreciated method by scientists for fungal discrimination is the LS conidia identification by microscopic examination (Shokes and Culbreath 1997). At the microscope level, lack of conidia may be observed on ELS lesions, while this is not generally true for LLS. When present, conidia are often rare and light in color on ELS lesions, whereas they are darker and clustered in concentric rings as regards LLS (Shokes and Culbreath 1997).

Different approaches can be used to control LS disease such as cultural measures, fungicides and resistant peanut cultivars. Cultural approaches try to reduce the contact of the peanut crop with fungal inoculum as much as possible. Among those approaches, the crop rotation technique plays a primary role. A 2-3 year rotation out of peanut is recommended since it can provide a delay of 2-3 weeks in symptom appearance. The sowing time should be adjusted by the length of the growing season and cultivar maturity to avoid overlapping periods of crop permanence in the field as much as possible and conidia dispersal from already infected peanut plants. Peanut residues should be removed from the field, buried with a moldboard plow, burned in situ or used for animal feeding (if it is allowed given the probable previous pesticide applications) (McDonald et al. 1985; Shokes and Culbreath 1997). Surrounding volunteer *Arachis* plants should be removed as well in order to avoid overwintering of diseases or formation of suitable microclimates for disease development (McDonald et al. 1985; Shokes and Culbreath

1997). Cultural approaches are an effective tool to delay disease progression, but they are not able to eliminate fungal inoculum that can be wind-borne from neighboring fields. Given the great potential for a secondary spread that characterizes LS fungi, dangerous epidemics may develop from just small amounts of initial inoculum (Monfort et al. 2004).

Chemical treatments have been effective and economical in the past when they were widely adopted on large-scale farms in developed countries. On the other hand, this approach showed complications for application on small-scale farms in developing countries. Fungicide application equipment can be expensive and requires regular maintenance and expertise in pesticide handling by farmers. Lack of clean water or water transportation limitations are a serious problem in certain areas of the world and yield advantages are not easily predictable even with fungicide applications. Even in cases where increased yield is possible, price fluctuations may discourage farmers from risk-taking investments (McDonald et al. 1985; Shokes and Culbreath 1997). Furthermore, chemical treatments need to be applied before or just right after the appearance of the first symptoms to be effective, and further applications are required with a 10-14 d interval until 2-3 weeks before harvest. Generally, 6-8 applications per season are necessary but this number must be increased in those years with favorable environmental conditions for LS development. Moreover, undesirable effects on target and nontarget organisms must be considered before fungicide application. Tolerant strains of LS fungi appeared in the southeastern United Stated three years after benomyl registration (McDonald et al. 1985). For cultivars growing under water stress conditions, this may result in reduced yield since they more likely face permanent wilting compared to those

plants that lose most of their leaves due to LS disease progression (McDonald et al. 1985). Focusing on nontarget organisms, increased levels of *Sclerotium rolfsii* Sacc. and *Sclerotinia sclerotiorum* (Lib. de Bary) were found in peanut fields sprayed with benomyl and chlorothalonil, respectively (Backman et al. 1977; Porter 1980). In short, alternative control measures are necessary worldwide to mitigate the impact of this devastating disease and reduce cultivation costs.

McDonald et al. (1985) stated that resistant cultivar breeding is one of the best ways to reduce yield losses due to LS diseases. This strategy is particularly effective for small-scale farmers who may lack resources and expertise to apply the previously described approaches but, at the same time, it is also advantageous for large-scale farmers and could reduce input costs of the peanut crop (McDonald et al. 1985). Resistant cultivars combined with other varieties, more susceptible but high-yielding, can also play a role in epidemic arrest or slowdown. For this purpose, LS resistances that can be effective in field conditions are extended latent periods, decreased sporulation, smaller lesions, reduced infection frequency, necrotic area of leaves and defoliation, and fewer lesions on stems (Shokes and Culbreath 1997).

Peanut breeding programs focusing on ELS and LLS resistances started to appear in the U.S. since the early 1980s and progress toward greater resistance has been made since then (Norden 1982; Gill 2013). On the other hand, the complex inheritance and polygenic nature of these resistance traits make their introgression in high performing cultivars laborious (Sharief et al. 1978; Nevill 1982; Dwivedi et al. 2002; Gill 2013).

Late Leaf Spot Disease in Peanut

Nothopassalora personata (Berk. & M.A. Curtis) U. Braun, C. Nakash., Videira & Crous (syn. *Cercosporidium personatum*) is a devastating disease that affects peanut everywhere this species is cultivated (McDonald et al. 1985; Shokes and Culbreath 1997). However, many sources of resistance have already been identified in cultivated peanut and wild *Arachis* species (Chalal and Sandhu 1972; Abdou et al. 1974; Sowell et al. 1976; Hassan and Beute 1977; Monasterios de la Torre 1980; Subrahmanyam et al. 1982). Since the level of disease resistance in cultivated peanut is relatively low, breeders are keen to find new sources of resistance and introgress them into elite material. As a general trend, Subrahmanyam et al. (1985) found that the wild *Arachis* species in sections *Erectoides, Triseminalae, Extranervosae, Rhizomatosae* and *Caulorhizae* typically show small and non-sporulating lesions when infected by LLS, while those in section *Arachis* are either without sporulating lesions or with variably sporulating ones. However, defoliation and frequency of infection seem to be variable resistance components within each section and species. Contradicting reports indicating either negative correlation or independent inheritance between the resistances to ELS and LLS were published in the past and all involved gene actions are still not clear (Anderson et al. 1986a; Anderson et al. 1986b). Historically, when breeders have a choice between wild species and adapted germplasm as a source of resistance, the latter is preferred (Nevill 1982). In one of the first attempts to study LLS resistance, Sharief et al. (1978) found that the resistance in diploid *Arachis* species was controlled by a multifactorial genetic system. Unfortunately, a similar behavior in tetraploid cultivated *Arachis* species cannot simply be assumed (Nevill 1982).

Nevill (1982), studying five F_2 progenies derived from crosses between two resistant and three susceptible peanut cultivars found strong association among incubation period, latent period and lesion diameter. He proposed a multi-locus genetic system controlling the expression of LLS pathogen development components where the resistance to LLS is completely recessive. Five loci are controlling LLS resistance and the partial resistance is defined by the number of recessive genes present (Wall and Wynne 1985).

According to Wall and Wynne (1985), the Nevill (1982) model cannot completely explain the results they achieved crossing five resistant peanut lines and four cultivars crossed in a M x N mating design (Simmonds 1979). They propose that the genes at loci controlling the resistance are affected by the presence of modifier genes which influence the phenotypic expression. On the other hand, as they reported, the presence of pleiotropic or linkage effects could explain the close association of the studied resistance components among the individuals as proposed by Subrahmanyam et al. (1983) for peanut rust. Besides, they concluded that LLS may be controlled by large additive effects since, from their results, general combining ability effect (GCA) is far more important than specific combining ability (SCA) in explaining the variation among crosses for resistance to LLS.

Jogloy et al. (1987) studied the resulting F_2 population from Walls and Wynne (1985) M x N mating design and reported that all the components of the resistance were significantly positively correlated among them, with the only exception of latent period which was negatively correlated. Analogous results were obtained by other authors (Nevill 1982; Wall and Wynne 1985; Anderson et al. 1986a; Anderson et al. 1986b). This suggests that breeding lines may be selected with decreased lesion number per leaf, lesion

size, defoliation and sporulation, and increased latent period. In this case, the latent period was defined as the number of days from the inoculation until 50% of leaf lesions were sporulating and sporulation determination was done using a 1-5 score scale according to Subrahmanyam et al. (1985), while the disease index was calculated as sum of the weighted products of each considered resistance component. Jogloy et al. (1987) discovered that the GCA was highly significant for all the considered agronomic traits of peanut (pod yield, pod length, seed yield, seed size and shelling percentage) and for some of the late leaf spot resistance measurements (lesion size, lesion number per leaf, sporulation and disease index). On the other hand, the SCA was not significant for all the considered parameters of LLS resistance. This confirmed what was proposed by Wall and Wynne (1985), that the population responses were controlled by additive genes. Similar results were also obtained by previous studies (Hamid et al. 1981; Anderson et al. 1986a; Anderson et al. 1986b).

Chiteka et al. (1988b) evaluated 116 genotypes for LLS resistance in three different tests. Two tests were made in the greenhouse (Gainesville and Quincy, FL) and the other in the field (Marianna, FL). Evaluated components of the resistance were incubation period, percent leaf necrotic area, latent period, lesion number per leaf, lesion size, and sporulation. The incubation period was defined as the number of days from inoculation to the appearance of the first lesion. Three different measurements were made for the latent period which was calculated as the number of days from inoculation to the first lesion, second lesion and 50% of leaf lesions sporulating. They found that lesion size, latent period and sporulation showed the most consistency when used for rating in different environments. They stated that lesion size, latent period and sporulation may be more

effective tools for genotype evaluations compared to the other components of resistance (Chiteka et al. 1988a). They also claimed that latent period measured as the number of days from inoculation to the first and second sporulating lesions is a more practical approach under field conditions than the number of days from inoculation to 50% of primary sporulating lesions (Chiteka et al. 1988a). This assertion received further support by other authors (Aquino et al. 1995; Dwivedi et al. 2002). Correlations were calculated in this experiment, both for components of the resistance within the single study and between greenhouse and field studies (Chiteka et al. 1988b). High correlation within tests was recorded among lesion size, latent period and sporulation, such as between those components and the percent necrotic area per leaf. In the case of lesion size, latent period and sporulation, the Kendall's tau B genotypic rank of genotypes in the field was significantly correlated with the rank in the greenhouse ($r = 0.46$, $r = 0.57$, and $r = 0.59$, respectively). On the other hand, comparison of the Marianna field study with the greenhouse test in Gainesville did not show a significant rank correlation for any of the resistance components indicating that selection for field resistance must not just be based on the greenhouse tests only. The greatest negative correlation was between latent period and sporulation, suggesting that selection of peanut genotypes with longer latent periods would also imply selection for reduced sporulation (Chiteka et al. 1988b).

Aquino et al. (1995) confirmed these results, which has drawn breeders' attention to the existing relationship between latent period and sporulation of LLS disease. In 1988 and 1989, Aquino et al. (1995) studied the components of partial resistance to LLS and disease progression on 14 peanut genotypes with known disease resistance characteristics in different field experiments in Marianna, FL. The studied components of the resistance

were incubation period, latent period, maximum percentage of lesions that sporulated, lesion size, lesion number, sporulation and percent necrotic area per leaf. Maximum percentage of lesions that sporulated was calculated at 35 DAI by dividing the number of lesions that had sporulated by the lesion number of ten days before, while sporulation was evaluated with a 1-5 scale at 35 DAI (Smith and Littrell 1980). Both the apparent infection rate and the area under the disease progress curve (AUDPC) were calculated for the components of the resistance and comparisons between the two methods were made. Latent period and maximum percentage of lesions that sporulated were highly correlated with each other with r = -0.84. They were also most highly correlated with LLS disease development. As for Chiteka et al. (1988a), the incubation period was not significantly different among genotypes. This component seemed to be more affected by environmental factors rather than genotype (Aquino et al. 1995). In contrast, latent period, lesion size, maximum percentage of lesions that sporulated and sporulation exhibited a strong genotypic effect. Latent period, maximum percentage of lesions that sporulated and sporulation were also the components that had the greatest effect in reducing the disease development. Aquino et al. (1995) stated that sporulation and maximum percentage of lesions that sporulated were the most valuable components for identifying genotypes with high levels of resistance and besides, their evaluation is rather easy to perform. Since sporulation and maximum percentage of lesions that sporulated were closely correlated, the authors concluded that both could provide a measure of the sporulation capacity on a given genotype. Referring to the AUDPC, Aquino et al. (1995) claimed that it provided a more precise measure of genotypic effects on disease progress compared to apparent infection rate.

Dwivedi et al. (2002) evaluated 15 interspecific derivatives for LLS under greenhouse conditions. In agreement with previous studies, they found that LLS resistance was determined by the longer incubation period and latent period, reduced lesion number, smaller lesion diameter, lower sporulation index (defined as intensity of sporulation and calculated using a 1-9 scale), and lesser leaf area damage and disease score (Hassan and Beute 1977; Subrahmanyam et al. 1982). They found that the percentage of defoliation, which has the strongest effect on photosynthesis, was not correlated with other components of the resistance, except for damaged percentage leaf area. This may imply that defoliation under artificial conditions and inoculum could be influenced by other factors rather than components of the resistance. In fact, some of the 15 genotypes had high sensitivity to LLS since even a few lesions on leaves caused defoliation (Dwivedi et al. 2002).

LLS phenotyping to date has been widely performed both *in vitro* and under field conditions (Melouk and Banks 1978; Nevill 1982; Wall and Wynne 1985; Anderson et al. 1986a; Anderson et al. 1986b; Jogloy et al. 1987; Knauft et al. 1988; Anderson et al. 1993; Aquino et al. 1995; Dwivedi et al. 2002; Leal-Bertioli et al. 2009). The most accredited phenotyping technique under *in vitro* conditions is the detached leaf method developed by Melouk and Banks (1978) for ELS phenotyping. The method has been modified and advanced to date and applied to other disease phenotyping such as LLS, rust, etc. (Subrahmanyam et al. 1980; Nevill 1982). The original procedure directs to isolate conidia from peanut genotypes susceptible to *C. arachidicola* and culture them on oatmeal-agar medium. A conidial suspension is prepared by flooding 15-20 d-old cultures in Petri-plates with distilled water and filtering it through four layers of cheesecloth in

order to remove mycelial fragments. Subsequently, detached peanut leaf petioles, reinforced by a foam plug, must be immersed in a Hoagland'solution (Hoagland and Arnon 1950) in 1 x 14 cm test tubes. Adaxial and abaxial peanut leaf surfaces are misted with a 2 x 10^4 conidia/ml *C. arachidicola* suspension by means of an atomizer. The inoculated tubes are then placed in racks in a clear polyethylene chamber in the greenhouse. Temperatures should be maintained at 26 ± 2 ºC and 31 ± 2 ºC, night and day, respectively, while humidity should range from 80-90% by hanging wicks of cheesecloth with their bases immersed in water on both sides of the chamber (Melouk and Banks 1978). Recent applications of an advanced detached leaf method have been performed by Leal-Bertioli et al. (2009) on F_1 peanut hybrids and Guimaraes et al. (2017) on cultivated varieties for LLS phenotyping and hairy root induction, respectively. Test tubes where substituted by Petri dishes in order to create moist chambers and peanut leaf petioles were covered with moistened cotton wool. These modifications have been applied in order to favor rooting of petioles and prolong leaf viability (Leal-Bertioli et al. 2009; Guimaraes et al. 2017). In the latter case, moist chambers were placed in growth chambers at 25 ± 2 ºC with 16 h of photoperiod instead of the greenhouse. Culture media and techniques are available to grow *N. personata* conidia (Abdou and Coopers 1974), although other procedures, such as direct spore collection from already infected peanut leaves allows rapid availability of spores over a prolonged period of time since they can be preserved in a refrigerator at 4 ºC (Gill 2013). The detached leaf method has been claimed to be efficient, cheap and requiring little space (Favero et al. 2004; Sharma et al. 2005; Leal-Bertioli et al. 2009; Guimaraes et al. 2017).

The Florida scale (1-10) is the most used rating scale for LS field phenotyping in Georgia since its first introduction in 1988 due to its efficiency and convenience (Chiteka et al. 1988a; Chiteka et al. 1988b). It considers the number of lesions, lesion positions and degree of defoliation per plot. Scores range from 1 to 10 where, 1 = No leaf spot disease; 2 = Very few lesions on the leaves with none on upper canopy; 3 = Few lesions with very few lesions on upper canopy; 4 = Some lesions with more on upper canopy and slight defoliation (5%); 5 = Noticeable lesions even on upper canopy with noticeable defoliation (20%); 6 = Numerous lesions and very evident on upper canopy with significant defoliation (50%); 7 = Numerous lesions on upper canopy with much defoliation (75%); 8 = Upper canopy covered with lesions with high defoliation (90%); 9 = Very few leaves remaining and covered with lesions, and some plants completely defoliated; 10 = Dead plants (Chiteka et al. 1988a; Chiteka et al. 1988b; Li et al. 2012). At the present time, further studies are needed to better clarify all the gene actions involved in LLS resistance in *Arachis* species. Especially, it will be important to understand the reasons leading to contrasting results in the literature indicating either negative correlation or independent inheritance between the resistances to ELS and LLS (Anderson et al. 1986a; Anderson et al. 1986b). New and different sources of resistance are required from screening of wild *Arachis* species since LLS is still one of major foliar diseases for peanut. In the short term, pyramiding of the different components of resistance could be a smart approach to rapidly improve resistance in partially resistant cultivated material.

Nematodes in Peanut

All around the world, nematodes are classified as a main parasite of peanut (Timper et al. 2018). In 1987, worldwide estimates claimed that annual losses in the peanut crop due to nematodes were 12%, which corresponded to roughly $1.03 billion (Sasser and Freckman 1987). In the southern area of the U.S., the *Meloidogyne arenaria* species is the dominant one and the most destructive among all the others. There are two races reported for *M. arenaria*: the race 1 infects peanut and race 2 does not (Taylor and Sasser 1978). Economic losses caused by the presence of *M. arenaria* in peanut fields are still significant, particularly if the cultivated areas are characterized by sandy soils and short cycles of rotation (Holbrook et al. 2014). Irregular incidence has been recorded also in North Carolina, South Carolina and Virginia (Timper et al. 2018). In infested fields, this pest can cause decreases in production down to less than half of non-infested ones (Minton and Baujard 1990). At times, 100% losses have been recorded in areas of severely infested fields (Timper et al. 2018). In 2016, $18.7 million yield losses were caused by this disease and $7.2 million were spent for its control by farmers (Little 2016).

Organs of peanut plants damaged by *M. arenaria* parasitism are roots, pegs and pods. The second-stage juveniles cause mechanical injury entering in the root tips. Usually, this injury is not strongly detrimental for the peanut plants even though it can be severe when several nematodes penetrate in a restricted area. *M. arenaria* females are globose, white in color, 800 µm long x 500 µm wide, and have pointy necks and prominent heads. They have a primary role in peanut plant infection since they deposit the egg masses near to the peanut root surfaces. Positioning egg masses outside of the galled tissue facilitates the

hatch and secondary infection of the roots. Egg masses hold from 300 to 500 eggs each, they are brown in color and around 1 mm in width (Timper et al. 2018). The presence of juveniles in vascular cells that will be active in eight days from egg deposition is a severe problem (Minton 1963). Juveniles cause increased cell number and cell size, phenomena known as hyperplasia and hypertrophy, respectively. The latter is particularly detrimental because it causes deformation of the xylem and phloem tissues, which involves impaired nutrient and water uptake of infected roots (Timper et al. 2018). Moreover, *M. arenaria* causes vascular tissues disorder and galling in infected peanut plants, which slow down root growth resulting in a stunted root system (Timper et al. 2018).

Infected peanut plants show rusty, yellowish and mottled patches of varying size, chlorosis, incipient wilting, nutrient deficiencies or even death (Zhang 1985; Timper et al. 2018). Symptoms are visible both above and below ground around 45-75 d after planting (DAP) and start to become more frequent and severe around 90-120 days. Even though symptoms may be visible above ground in early season, galling and egg masses do not appear on roots until 55-90 DAP. Galls are visible to the naked eye, but they may not be easily recognized by amateurs (Timper et al. 2018).

On the other hand, several methods are available for nematode identification, which can be separated into morphological, biochemical (multilocus enzyme electrophoresis and enzyme-linked immunosorbent assay) and PCR-based methods (Seesao et al. 2016).

Nematode dispersal can happen in many ways such as by humans, animals, water, agricultural machinery, wind and any other means that involve movement of soil or infected portions of plants (Timper et al. 2018). Among these, water and wind play a key role. In western Texas, Orr and Newton (1971) collected *Meloidogyne* juveniles from

traps positioned 2 m above the ground, while Meagher (1967) and Sauer (1968) detected nematodes dispersed by irrigation and surface run-off (Timper et al. 2018).

Dickson et al. (1994) claimed that once root-knot nematodes are detected in peanut fields, they will continue to multiply unless they are repressed by antagonists or other causes. This means that from an agricultural point of view, it is essential to determine population densities in the field and relative treatment thresholds. Most of the techniques for population-density determination are based on juvenile densities in the soil since it is difficult to extract eggs (Rodriguez-Kabana et al. 1986; Timper et al. 2018). Thus, the best method to determine densities is to collect soil core samples (Barker et al. 1986). McSorley et al. (1992) estimated that in Florida the damage threshold is 1 juvenile/100 cm^3 soil. Rodriguez-Kabana et al. (1982) analyzed data from 16 experiments in Alabama and found that yields of peanut were negatively correlated to *M. arenaria* juvenile density in the soil measured near harvest. Similar results were reported by Wheeler and Starr (1987) in peanut microplot tests (Timper et al. 2018).

There are several management methods for nematode control. One of the most effective is crop rotation which should be employed as a general rule in agriculture. Rotating peanut with other crops such as cotton, maize, small grains and pasture grasses may reduce nematode pressure in the field (Bailey 1988; Hagan 1988; Dickson and Melouk 1995; Dunn and Dickson 1995; Timper et al. 2018). Rotations of 3 years or more are preferred (Dickson and Hewlett 1989) and tropical forage is the best rotation for preceding peanut (Norden et al. 1977; Rodriguez-Kabana et al. 1994; Timper et al. 2018). The purpose of crop rotation is not to completely exterminate the nematode population but to keep it suppressed (Dunn and Dickson 1995; Timper et al. 2018).

Mechanical disruption of the soil may be a useful tool to reduce nematode infection. Disturbance of host plant roots that precede peanut will stop reproduction and help to reduce potential damage. Moreover, tillage disperses juveniles from roots and exposes eggs and juveniles to high temperatures and adverse conditions close to the soil surface (Timper 2009). Plowing and tillage have stimulated the deterioration of live plant roots that protect nematodes from antagonists or from nematicides (Dunn and Dickson 1995; Timper et al. 2018).

Nematicides are considered one of the most reliable methods of managing important nematode diseases of peanut but some restrictions limit their use (Holbrook et al. 2014). Nematicides can be divided into fumigants and non-fumigants. Fumigants kill nematodes by gas contact. They are highly volatile, thus special safety precautions must be taken during application (Rodriguez-Kabana and Robertson 1987; Riegel et al. 2000a; Riegel et al. 2000b). Non-fumigants are liquids or granules that are totally dependent on water for redistribution. This means that excessive rainfall or irrigation can cause loss of the active ingredient into the surrounding environment, which results in economic losses for farmers and environmental pollution (Timper 2009; Timper et al. 2018). Therefore, significant economic gains can be obtained by employing resistant cultivars in those fields where nematode infestation occurs.

In 1989, Nelson et al. identified *M. arenaria* resistance in 21 *Arachis* spp. and two interspecific hybrids. In 1992, Holbrook and Noe completed a systematic search of the *A. hypogaea* U.S. germplasm collection for sources of nematode resistance (Timper et al. 2018). Results showed that *M. arenaria* resistance was able to reduce nematode population densities by 40-60% (Noe et al. 1992; Timper et al. 2018). Resistance

introgression from wild species is a complex procedure since most wild relatives are diploids while cultivated peanut is an allotetraploid. Timper et al. (2018) summarized the introgression of the resistance genes in three pathways. The first pathway described by Simpson et al. (1993) entails the formation of a complex hybrid ('TxAG-6') of three wild species (*A. batizocoi*, *A. cardenasii* and *A. diogoi*) nematode resistant that was cross-compatible with *A. hypogaea* (Nelson et al. 1989). Among the three wild species, *A. cardenasii* was the major donor for nematode resistance and its genes were dominantly inherited (Burow et al. 1996; Choi et al. 1999; Timper et al. 2018).

A. cardenasii was the donor of *M. arenaria* resistance also for the second pathway but in this case the breeding approach was different (Stalker et al. 1994). A hexaploid hybrid was created from the cross *A. hypogaea* x *A. cardenasii* and subsequent colchicine chromosome doubling. Then the hybrid was self-pollinated, and the resulting progeny selected for presence of tetraploid individuals.

This procedure allowed the release of two germplasm lines, GP-NC WS5 and GP-NC WS6 (Stalker et al. 2002a). In this case, two linked dominant genes were proposed to determine the resistance. The first acts on egg production, while the second on gall development (Garcia et al. 1996; Timper et al. 2018).

The third pathway followed a procedure for induced allotetraploid hybrid production (Favero et al. 2006) using the starting cross, *A. batizocoi* and *A. stenosperma* (Leal-Bertioli et al. 2015). In this case, the resistance to *M. arenaria* was mainly determined by *A. stenosperma* (Leal-Bertioli et al. 2016). In fact, fewer juveniles enter the roots of *A. stenosperma* than other plants and those entered were repressed by hypersensitive

response in cells surrounding the nematode feeding site (Proite et al. 2008; Timper 2009; Guimaraes et al. 2010; Guimaraes et al. 2015; Morgante et al. 2013; Timper et al. 2018). The first two nematode-resistant cultivars, COAN and NemaTAM, were derived from the crossing of TxAG-6 and 'Florunner' as the recurrent parent (Simpson and Starr 2001a). COAN showed reduction in nematode reproduction (Simpson and Starr 2001a) and, at the end of the growing season, it exhibited less than 10% of the final nematode population density in comparison to Florunner (Starr et al. 1995). Nevertheless, given the low yield potential, COAN is no longer present in the market (Timper et al. 2018). Tifguard is a runner type cultivar of peanut released in 2007 by the USDA-ARS and the Georgia Agricultural Experiment Stations (Holbrook et al. 2008). It derives from COAN and 'C-99R' (Gorbet et al. 1987; (Holbrook et al. 2008) and has high resistance to both the peanut root-knot nematode (similar to COAN and NemaTAM) and TSWV, which causes tomato spotted wilt disease. Furthermore, it shows higher yield than COAN (Holbrook et al. 2008). TifNV-High O/L was released by the USDA-ARS and the Georgia Agricultural Experiment Station, Tifton, GA in 2014 (Holbrook et al. 2017). Breeding populations were developed by hybridizing the nematode resistant cultivar Tifguard (Holbrook et al. 2008) with the high oleic cultivar Florida-07 (Gorbet and Tillman 2009). Marker assisted selection was used to select for nematode resistance and the high oleic/linoleic fatty acid ratio (O/L) characteristics (Chu et al. 2011).

TxAG-6 was developed to facilitate introgression of nematode resistance from wild diploid species into cultivated peanut cultivars. Resistance is determined by the presence of *Rma*, dominant resistance gene, located on a large chromosomal region spanning one-third to one-half of linkage group A09 (Nagy et al. 2010). The *Rma* locus determines

nematode resistance also in TifNV-High O/L cultivar. During breeding for TifNV-High O/L, marker assisted selection was employed in the F_2 population derived from Tifguard by Florida-07 to identify homozygous plants for nematode resistance and high oleic acid content (Chu et al. 2011; Holbrook et al. 2017). The markers employed for screening were the sequence characterized amplified region (SCAR) dominant marker 197/909 (resistant allele) (Chu et al. 2007a), the dominant cleaved amplified length polymorphism sequence (CAPS) marker 1169/1170 (susceptibility allele), and the simple sequence repeat (SSR) marker GM565 (Nagy et al. 2010). Other markers linked to the *Rma* locus were available, such as R2430E and R2545E restriction fragment length polymorphism (RFLP) markers (Choi et al. 1999) and Z3/265 (Garcia et al. 1996) and RKN440 (Burow et al. 1996) random amplified polymorphic DNA (RAPD) markers. However, Chu et al. (2007a) claimed that these markers do not give a reproducible level of correlation with the collected phenotyping data. SCAR marker 197/909 was shown to be a reliable predictor for the nematode resistance able to identify a high percentage of *Rma* carriers and reduce the risk of false negatives caused by failed reactions (Chu et al. 2007a; Nagy et al. 2010). This marker was able to reduce screening costs from ~$4.50 per plant to <$0.80 per plant (Chu et al. 2007a). Unfortunately, it does not completely distinguish resistant heterozygote from resistant homozygote plants (Nagy et al. 2010). On the other hand, CAPS 1169/1170 and SSR GM565 markers are codominant (Nagy et al. 2010; Chu et al. 2011).

In 2012, Nagy et al. produced the first high-density linkage map for *A. duranensis*, 1236 EST-SNP markers were polymorphic between two *A. duranensis* accessions as were 300 SNP markers from genomic sequences representing conserved legume orthologs. 1054 of

the 1536 generated SNP markers were placed onto a genetic map. These markers were effectively used in a study to further delineate the large chromosomal region in linkage group A09, which demonstrates that one portion of the region gives moderate resistance while a minor, distal portion confers strong resistance (Chu et al. 2016b).

Tomato Spotted Wilt Virus in Peanut

Tomato spotted wilt, caused by *Tomato spotted wilt tospovirus* (TSWV), is a severe disease that affects many cultivated plant species such as peanut (*Arachis hypogaea* L.), potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), tobacco (*Nicotiana tabacum* L.), lettuce (*Lactuca sativa* L.), papaya (*Carica papaya* L.), spinach (*Spinacia oleracea* L.), pineapple (*Ananas comosus* (L.) Merr.), eggplant (*Solanum melongena* L.), etc. (Best 1968; German et al. 1992). Overall, TSWV and related viruses infect over 650 species across 50 plant families (German et al. 1992; Ullman et al. 2002). The first description of TSWV was on tomato in Australia in 1915 (Brittlebank 1919), while the first report identifying it as a virus was published in 1930 (Samuel et al. 1930; Culbreath et al. 2003). Tomato spotted wilt disease was originally described on peanut in Brazil in 1941 (Costa 1941), while in the U.S., it was observed for the first time in Texas in 1971 (Halliwell and Philley 1974). Afterward, several epidemics occurred within Texas peanut-producing counties between 1985 and 1991 with peanut yield reductions up to 95% (Culbreath et al. 1992c; Camann 1995). From Texas, TSWV rapidly spread all over the southeast of the U.S. establishing itself in the peanut producing areas of Alabama, Florida, Georgia and Mississippi (Culbreath et al. 1992b; Culbreath et al. 2003).

In Georgia, TSWV incidence on peanut started to be significant in 1986. In 1989, symptomatic peanut plants were found in almost every peanut field inspected (Culbreath et al. 1992c). In 1997, estimated peanut crop losses due to TSWV were 12% of peanut yield, approximately $40 million (Bertrand 1998). Nowadays, TSWV infection arises in

peanut fields every growing season with severity fluctuations determined by year and location (Culbreath et al. 2003).

TSWV is characterized by a wide range of symptoms in systemic hosts, while it shows at the most few lesions, necrosis and chlorosis in non-systemic ones (Mumford et al. 1996; Culbreath et al. 2003). In systemic hosts, symptoms comprise concentric ring spots, various lesions and patterns, stunting, wilting, silvering, mottling, bronzing, chlorosis and necrosis (German et al. 1992; Culbreath et al. 2003). Symptom severity can vary from very few lesions on the plant canopy to severe stunting and death. Particularly, in the latter case the pod yields and kernel size in peanut plants significantly decrease. As a general rule, plants that are symptomatic early in the season normally are less productive than those that are symptomatic later (Culbreath et al. 2003). Most tomato spotted wilt epidemics seem to follow a pattern of primary and secondary infection even though it seems that most of the virus spread among plants is due to the primary infection, while the secondary is somehow limited (Culbreath et al. 2003). The element that mainly affects the symptom expression is the specific virus-host combination, even though environmental factors such as temperature still have a central role (Allen et al. 1991; Mumford et al. 1996). On the other hand, asymptomatic infections of plants may occur as frequently as symptomatic ones (Culbreath et al. 1992a; Culbreath et al. 2003). Unexpectedly, physical contact of peanut plants is not a relevant circumstance for tomato spotted wilt epidemic spread as well as for the presence of TSWV infected seeds in cultivated fields. In fact, mechanical inoculation of TSWV does not occur easily and infected seed germination does not produce growth of infected plants (Pereira 1993; Mandal et al. 2001; Culbreath et al. 2003). In contrast, thrips (order Thysanoptera) act as

the main vector of tomato spotted wilt in peanut field epidemics. *Frankliniella fusca* Hinds (Sakimura 1963) and *Frankliniella occidentalis* (Pergrande) (Sakimura 1962), commonly known with the name of tobacco and western flower thrips, respectively, are the two confirmed ones, while *Thrips tabaci* (Lindeman) or onion thrip has not been yet identified as a relevant vector even though it arises in the southeastern U.S. (Ullman et al. 1997; Culbreath et al. 2003).

Culbreath et al. (2003) summarized the four points that a peanut plant, volunteer or not, needs to meet to be recognized as significant source of inoculum: (a) the plant needs to be already infected by the TSWV; (b) larvae of at least one vector species need to be hosted by the plant for reproduction; (c) the plant virus needs to be assimilated by the thrips; and (d) the disease cycle needs to be completed with the plant already present.

A TSWV risk index it has been developed to help peanut farmers to identify high risk situations and take proper precautions. It involves the application of a predictive model that combines cultural practices with local weather conditions information. A coefficient of determination (R^2) of 61% explaining the variation in spotted wilt severity have been identified by the best fitting model. On the other hand, significant variation in accuracy from year to year was present (Olatinwo et al. 2008). The model would be further optimized through additional evaluations and field validations during the following years in order to make it predictive also in cases of low disease incidence and different environments (Olatinwo et al. 2008).

A field scale (0-5) is available for TSWV phenotyping. It was adapted from Baldessari (2008) and most recently used by Li et al. (2012). It is based on visual estimation of the degree of stunting (plant height reduction, width and combination of the two) of infected

plants, where 0 = No TSWV symptoms, 1 = TSWV symptoms, with no or minor stunting (80-100% plant size relative to typical healthy plants), 2 = Noticeable stunting (60-79% plant size relative to typical healthy plants), 3 = Marked stunting (40-59% plant size relative to typical healthy plants), 4 = Very marked stunting (30-40% plant size relative to typical healthy plants) and 5 = Severe stunting (0-20% plant size relative to typical healthy plants).

Unfortunately, a single management technique able to adequately control TSWV in peanut is still lacking. On the other hand, integrated management with the employment of chemical treatments for thrips control, cultural practices and resistant cultivars is able to minimize peanut yield losses (Culbreath et al. 2003).

Among chemicals, organophosphorus insecticides are the only ones able to offer an adequate rate of suppression, although they are not able to fully control TSWV epidemics (Todd et al. 1996; Todd et al. 1998; Wiatrak et al. 2000; Baldwin et al. 2001; Culbreath et al. 2003). On the contrary, the application of other chemicals such as imidacloprid resulted in a substantial increase of tomato spotted wilt incidence (Todd et al. 1994; Culbreath et al. 2003).

Cultural practices for controlling TSWV epidemics include sowing density, sowing date, planting layout and tillage system management, and weed control.

Incidence of TSWV is significantly higher in sparsely planted peanuts compared to densely sown (Branch et al. 2003); a sowing rate of >13 plants/m is suggested in Georgia (Culbreath et al. 2003). The recommended planting date in the southeastern U.S. is during the first two weeks of May, which generally results in a lower occurrence of tomato spotted wilt, while sowing in early April increased frequency. Furthermore, late

sowing during the month of June frequently caused severe epidemics (Hagan et al. 1991; Brown et al. 1997; Todd et al. 1998; McKeown et al. 2001; Brown et al. 2003; Culbreath et al. 2003). Scientific explanations for TSWV epidemic development due to early or late planting dates are still missing. The most widely-accepted theory claims that there are yearly variations of thrips populations as well as across planting dates (Culbreath et al. 2003). Plants sown in twin-rows spaced 18-24 cm apart is another practice with unknown mechanism that reduces incidence of tomato spotted wilt in peanut fields. Speculations state that it may be due to visual interference of migrating thrips to recognize host plants. Moreover, even though epidemics occur, the healthy plants are able to compensate for the stunted ones resulting in higher yields compared to a single row planting pattern (Culbreath et al. 2003). Planting also plays a key role in peanut fields with presence of directional winds. In fact, there is evidence of increased TSWV incidence in fields where peanut plants were sequentially planted down-wind to earlier planted ones (Black 1990). Anyway, most of the peanut production areas of Georgia are characterized by winds of variable direction, so particular attention is not required by farmers in most of the cases (Culbreath et al. 2003). Conservation practices may be another effective tool for TSWV control. In fact, lower thrips densities have been recorded in minimum and no-tillage fields compared with conventional tillage (Campbell et al. 1985; Campbell 1986; Brown et al. 1995). Moreover, Minton et al. (1991) detected less thrips feeding injury on peanut plants in fields cultivated with minimum practices. These techniques establish new host-vector-environment interactions in the specific cultivated areas, which may explain the better results in terms of tomato spotted wilt suppression in comparison to conventional practices. In contrast, the choice of conservation tillage practices should not only be

based on TSWV infection incidence but also consider the overall agronomic system and associated economic implications (Culbreath et al. 2003).

Weed management for mitigating tomato spotted wilt appears to have a secondary effect compared to the previous practices. In fact, although several studies inspected TSWV weed hosts, no strong association has been found. The only two weeds which are considered a serious source of inoculum that require grower attention are volunteer peanut and *Verbesnia enceloides* plants. On the other hand, their real impact in a single field is still unknown (Culbreath et al. 2003).

Among cited integrated management practices, the presence of TSWV resistance in cultivated peanut plants is the most important factor (Culbreath et al. 2003).

Several sources of moderate resistance have already been identified and have led to the release of runner-type cultivars such as Georgia Browne (Branch 1994), Georgia Green (Branch 1996), UF MDR 98 (Culbreath et al. 1997), Tamrun 96 (Smith et al. 1998), and ViruGard (Shelton 2000), with levels of resistance similar to 'Southern Runner' (Gorbet et al. 1987). Moreover, 'C-99R' was found to have higher resistance than Southern Runner, in particular in situations where TSWV epidemics were severe (Gorbet and Shokes 2002; Culbreath et al. 2003). C-99R was developed at the University of Florida Agricultural Experiment Station and released in 1999. It is a jumbo-runner market-type peanut with LLS, white mold (caused by *Sclerotium rolfsii* Sacc.), and TSWV resistances. It has greater pod yields, larger seed size, better grades, and better quality than Southern Runner (Gorbet and Shokes 2002).

On the other hand, most of the peanut cultivars listed above can be traced back to a single source of resistance, PI 203396 (Isleib et al. 2001), and in case of strong TSWV

infections they may be still severely damaged. Studies indicate that the moderate resistance of these cultivars is not due to thrips feeding, thrips reproduction, or the reduced attractiveness to thrips vectors (Culbreath et al. 2003).

C 11-2-39 and C 11-186 breeding lines were also derived from PI 203396. They have been identified as more resistant than Georgia Green and characterized by higher yields (Culbreath et al. 2005). Their resistance levels were almost comparable to another breeding line whose resistance source was recognized as *A. hypogaea* spp. *hypogaea* var. *hirsuta* Köhler parental (PI 576638). C11-2-39 was later released as 'Georganic' (Holbrook and Culbreath, 2008) for use in organic peanut production systems.

From PI 576638, a cultivar named Florida EP™ "113" was derived and released in 2012 (Tillman and Gorbet 2012; Tillman and Mckinney 2018). In 2017, Mckinney and Tillman stated that in Florida it is the most resistant cultivar to spotted wilt available.

High Oleic:Linoleic Acid Trait in Peanut

Peanut is the fourth largest oilseed crop in the world (www.fao.org). It is characterized by a variable oil composition both in terms of quantity and relative proportion of fatty acids. Around 50% of peanut dry weight is oil and up to 12 fatty acids have been described in peanut, of which palmitic, oleic and linoleic account for around 90% (Moore and Knauft 1989; Isleib et al. 2004). By themselves, oleic and linoleic fatty acids comprise almost 80% of the fatty acid in peanut seeds and usually the ratio in standard genotypes can range from 1.0 to 4.0 (Norden et al. 1987). During the previous decades, efforts have been made to improve this ratio reaching values as high as 40 (Norden et al. 1987). Oleic acid has ten times higher oxidative stability compared to linoleic acid and to polyunsaturated fatty acids in general (O'Keefe et al. 1993). For this reason oleate oil rich plants are considered superior by the food industries (Jung et al. 2000). High oleic peanut is associated with several benefits such as longer storage life, reduction of low-density lipoprotein (LDL) levels during post-menopause for hypercholesterolemic women, repression of tumorigenesis and inflammatory diseases (O'Byrne et al. 1997; Yamaki et al. 2005; Mesa et al. 2006; Chu et al. 2009; Holbrook et al. 2016). Conversely, polyunsaturated fatty acyl residues are known to be disposed to oxidation with consequences such as potential atherogenic effects, unpleasant odors and tastes, and rancidity (Jung et al. 2000). The process of conversion from oleic to linoleic acids is mediated by oleoyl-PC desaturase which adds the second double bond to the oleate (Schwartzbeck et al. 2001; Chu et al. 2007b). In order to attain high oleic:linoleic acid ratio in peanut, the repression of *ah*FAD2A (*Ol*$_1$) and *ah*FAD2B (*Ol*$_2$) homeologous genes (A and B subgenomes respectively) which regulate the oleoyl-PC desaturase

activity are necessary (Chu et al. 2011). Nucleotide sequence homology between the two genes is 99% and both wild-type forms encode a functional desaturase (Chu et al. 2007b; Bruner et al. 2001). Chu et al. (2007b) showed how loss-of-function 448G>A substitution mutation that deactivates oleoyl-PC desaturase was found in the *ah*FAD2A gene in some peanut genotypes with no flowering on the main stem (ssp. *hypogaea*) but rare in those with flowering on the mainstem (ssp. *fastigiata*). The U.S. peanut market type classification indicates Runner and Virginia as ssp. *hypogaea*, while Spanish and Valencia are ssp. *fastigiata* (Stalker and Simpson 1995). Therefore, less segregation of *ah*FAD2A alleles is expected in Runner market type peanuts (Chu et al. 2007b; Chu et al. 2011). As regards the *ah*FAD2B recessive mutations, 441_442insA and 665_insMITE control high oleic:linoleic acid ratio. The cleaved amplified polymorphic sequence (CAPS) marker 1101/1048 targeting the 441_442insA mutation (Chu et al. 2009) was converted to a gel-free SNP marker by Chu et al. (2011) for HybProbe assay application. HybProbe assay is based on fluorescence resonance energy transfer (FRET) detection principle; during the PCR reaction annealing stage, two sequence-specific oligonucleotide probes (donor and acceptor) labeled with two different dyes hybridize to a target DNA sequence, thus bringing into proximity the two fluorescent molecules. Hence, a blue LED light is employed to excite the donor probe, which in return emits energy. The emitted energy is transferred and sensed by the acceptor probe, which then produces fluorescent light at different wavelength (Roche Applied Science 2008) detectable by LightCycler lab tool (Roche, Basel, Switzerland). After the annealing phase, the fluorescent light is continuously monitored by the LightCycler and temperature is gradually increased leading to the elongation and melting of probes from the DNA

complementary strand. Consequently, melting curves are produced. A single base change in the target single-stranded DNA results in probe melting temperature alterations and so melting curve peaks allow efficient genotypic analysis (De Silva et al. 1998; Roche Applied Science 2004; Chu et al. 2011).

In comparison to CAPS markers, the HybProbe assay reduces assay time, number of scoring errors and reagent costs during genotyping (Chu et al. 2011).

Background of Cultivated Genotypes Selected for Study

IAC 322

IAC 322 is a progeny from the initial cross of Runner IAC 886, a common cultivar in Brazil, and an ICRISAT germplasm line (ICGV 86687: CS 16 – B2 – B2) that showed high levels of resistance to LLS (ICRISAT, 1986) and low yield. The CS 16 – B2 – B2 was a derivative line from an interspecific hybrid with *A. cardenasii* as the wild parent (Company et al. 1982). This line was developed in North Carolina initially for ELS resistance and later distributed to India and Brazil to further select for LLS resistance (Stalker 2017). In 2017a, Clevenger et al. detected three major introgressed regions on the top and bottom part of A02 and bottom part of A03 chromosomes in IAC 322 that were hypothesized to contribute to LLS resistance (physical positions are based on the *A. duranensis* v1 pseudomolecules, peanutbase.org).

TifNV-High O/L

TifNV-High O/L was released by the USDA-ARS and the Georgia Agricultural Experiment Station in 2014 (Holbrook et al. 2017). It combines root-knot nematode and TSWV resistances with high oleic:linoleic acid ratio trait. Breeding populations were developed by hybridizing the nematode resistant cultivar Tifguard (Holbrook et al. 2008) with the high oleic:linoleic acid ratio cultivar Florida-07 (Gorbet and Tillman 2009). Marker-assisted selection was used to select homozygous plants for nematode resistance, defined by the *Rma*-linked dominant marker S197 (Chu et al. 2007a). Dominant cleaved amplified polymorphic sequence (CAPS) marker 1169/1170 and codominant simple sequence repeat (SSR) marker GM565 were employed to identify resistant/susceptible

and heterozygous genotypes, respectively. Whereas, the CAPS marker 1101/1048 targeting the 441_442insA mutation of the *Ah*FAD2B recessive gene was used for high oleic:linoleic trait genotyping. The TifNV-High O/L exhibits yields that are similar to other currently grown peanut cultivars such as Georgia-06G (Branch 2007) without nematode pressure, while significantly higher yields are obtained under nematode pressure. Seed size and size distribution are similar to other large-seeded runner-type cultivars (Holbrook et al. 2017).

Runner IAC 886

Runner IAC 886 is a common peanut cultivar in Brazil. It was derived from the American multiline Florunner cultivar (Prado et al. 2011). Its origin is from IAC, Sao Paulo, Brazil (Macedo et al. 2012).

Florida-07

Florida-07 was developed by the University of Florida, Florida Agricultural Experiment Station, North Florida Research and Education Center near Marianna, FL, and was released in 2006 (Gorbet and Tillman 2009). It is characterized by prostrate growth habit, high oleic/linoleic fatty acid ratio, TSWV resistance, moderate resistance to white mold, excellent pod yield potential and competitive kernel grade. Florida-07 originated from line 89xOL14-11-1-1-1-b2-B x C-99R cross. 89xOL14-11-1-1-1-b2-B is a breeding line characterized by early-maturity and the high-oleic trait, while C-99R is a late-maturing, LLS, white mold, and tomato spotted wilt resistant cultivar (Gorbet and Shokes 2002). Under irrigation conditions, Florida-07 has a medium-late maturity (about 140 d).

Georgia-06G

Georgia-06G was developed at the University of Georgia, Coastal Plain Experiment Station, Tifton, GA and released in 2006. It is a runner-type cultivar characterized by an intermediate or decumbent growth habit, darker green foliage compared to Georgia Green, medium maturity, tan testa color, large seeds, high yield and TSWV resistance. Georgia-06G originated from Georgia Green × C-99R (Branch 1996; Gorbet and Shokes 2002). Georgia Green has dark green foliage, decumbent growth habit, TSWV resistance and high yield. C-99R is a late-maturing, large seeded, TSWV, LLS and white mold resistant cultivar released in 1999 by the University of Florida. Georgia-06G has high performance stability and adaptability in all the major peanut production areas of the U.S. Compared to other runner cultivars at the time of its release, Georgia-06G had the highest TSWV and total disease resistance, the highest pod yield (4822 kg ha−1), total sound mature kernel (TSMK) grade (75%), and economic return per hectare ($1930 ha^{-1}), throughout 24 multilocation tests. It is very similar to its female parent in terms of blanchability, protein content, oil content, and roasted flavor. However, it is slightly higher (2.4 vs. 1.9) and slightly lower (90 vs. 94), in terms of oleic:linoleic acid ratio and iodine values, respectively. It is not considered a high-oleic cultivar (Branch 2007).

Georgia-13M

'Georgia-13M' was developed at the University of Georgia, Coastal Plain Experiment Station, Tifton, GA and released by the Georgia Agricultural Experiment Station in 2013 (Branch 2014). It is a runner cultivar, tested experimentally as GA

072716, characterized by high yield, small seeds, roasted peanut flavor of Georgia Green, high oleic:linoleic acid ratio, and TSWV resistance. Georgia-13M originated from 'Georgia-02C' × 'Georgia-09B' (Branch 2003; Branch 2010). Georgia-02C is a runner cultivar with TSWV and Cylindrocladium black rot (CBR) [caused by *Cylindrocladium parasiticum* Crous, Wingfield, & Alfenas syn. *Cylindrocladium crotalariae* (Loos) Bell & Sobers] resistances and medium seeds. Whereas, Georgia-09B originated from Georgia Green × GA 942004 (Branch 1996), GA 942004 is characterized by high oleic:linoleic acid ratio trait and TSWV resistance, while Georgia Green has similar roasted flavors of Florunner, considered a standard runner cultivar for this attribute from the peanut industry (Norden 1969; Branch 2014).

TUFRunner 511

'TUFRunner 511' was developed by the University of Florida, Florida Agricultural Experiment Station, North Florida Research and Education Center near Marianna, FL, and was released in 2013 (Tillman and Gorbet 2017)**.** It is a runner-type cultivar characterized by large seeds (79.65 g 100 seed^{-1} TUFRunner 511 vs. 77.2 g 100 seed^{-1} Georgia-06G, $P > t = 0.0516$), high yield (6430 kg ha^{-1} TUFRunner 511 vs. 6416 kg ha^{-1} of Georgia-06G, in a 7-yr period over 3 locations), tan testa color, medium green leaves and vines, prostrate growth habit and high oleic acid oil content (76% TUFRunner '511' vs. 60% Georgia-06G, $P > t < 0.001$). TUFRunner 511 has a total sound mature kernel grade percentage slightly lower than that of Georgia-06G (78.6% vs. 79.3%, $P > t = 0.064$). TUFRunner 511 originated from C-99R x 88x1B-OLBC1-6-1-1-1 (Gorbet and Shokes 2002). In terms of disease resistance, TUFRunner 511 proved to be more

susceptible and resistant than several cultivars such as Georgia-06G to late leaf spot and white mold, respectively (Tillman and Gorbet 2017).

DP-1

DP-1 was developed by the University of Florida, Florida Agricultural Experiment Station and released in 2002. It is a runner cultivar characterized by relatively late maturity (150 d to harvest), TSWV, LLS and white mold resistances, and competitive pod yield and grade (Gorbet et al. 2008). DP-1 was derived from the cross of Southern Runner and UF81206 (Gorbet et al. 1987; Gorbet 2003). Parents were both derived from PI 203396, which has good LLS resistance. Southern Runner was recognized as the first U.S. cultivar resistant to LLS, whereas white mold and TSWV resistances were identified later. UF81206 is an unreleased breeding line with exceptional resistance to LLS and good white mold and TSWV resistances (Chiteka et al. 1988a). DP-1 pods are approximately 26 mm long and 13 mm wide, while seeds are plump, rounded or elongated with a tan testa (similar to Southern Runner) and around 15 mm long by 9 mm wide (similar to Florunner) (Norden 1969). Average 100-seed weight of DP-1 is approximately 62 g (Gorbet 2003). Gorbet et al. (2008) described how DP-1 was not different to C-99R in terms of pod yield (3569 vs. 3424 kg ha^{-1}, ns) but more resistant to LLS, in 27 non-sprayed tests. Moreover, pod yield and disease values in Georgia and Florida studies indicate the superior TSWV resistance of DP-1 compared to Georgia Green (Branch 1996). DP-1 showed higher white mold resistance than Florunner, C-99R, and Georgia Green in studies conducted near Marianna, FL (1999–2001). It has around

62% oleic and 18% linoleic fatty acids with 51% oil content. Proteins amount to 27 % and sugar to 3.5% (Gorbet et al. 2008).

GP-NC WS 16

GP-NC WS 16 (SPT 06-06) was developed in a peanut genetics program at North Carolina State University (NCSU), Raleigh, NC (Tallury et al. 2014). Introgressions from *A. cardenasii* Krapov. & W.C. Gregory are present in this tetraploid germplasm line. It has ELS, TSWV, CBR and Sclerotinia blight (SB) resistances. GP-NC WS 16 includes in its pedigree C-99R (Gorbet and Shokes 2002), DP-1 (Gorbet et al. 2008) and GP-NC WS 12 (Stalker et al. 2002b). They were combined in a three-way cross by the modified pedigree method of inbreeding in the early generation segregating populations (Stalker et al. 2002b). GP-NC WS 16 is a runner-type line with seeds ranging around 500 to 550 mg seed^{-1}, with a "waffled" testa appearance. It has a compact growth habit with short, sparse branches and dark green foliage. ELS evaluations in 10 non-sprayed field tests between 2006 and 2012 showed that the mean defoliation was significantly lower than the mean of resistant checks (2.91 vs. 4.47, $P < 0.01$), where 1 is equal to no defoliation and 9 to complete defoliation. Moreover, in 13 tests between 2006 and 2012 it had significantly lower TSWV incidence than checks except 'Bailey' (0.17, 0.17 vs. 0.20). It was not different from resistant PI 576636 check, which also had a mean incidence of 0.17 (Isleib et al. 2011; Tallury et al. 2014).

Single Nucleotide Polymorphism (SNP) Genotyping

In the past years peanut has trailed other commercial crops such as rice, maize, sunflower, soybean, oat, cotton and wheat as regards genotyping practices (Pandey et al. 2017). This is mostly due to the lack of numerous and efficient molecular markers and the cost of related array technologies. In fact, genetic and genomic studies in *Arachis* have mostly relied on simple sequence repeat (SSR) markers, which have been claimed to be expensive, limited in number and time consuming to assay (Clevenger et al. 2017b). A remarkable change occurred with the introduction of Single Nucleotide Polymorphism (SNP) markers and the first cost-effective kompetitive allele specific polymerase chain reaction (KASP) assay (Khera et al. 2013). In 2014, Zhou et al. constructed a linkage map including 1,685 marker loci, of which 1,621 were SNPs and 64 were simple sequence repeat (SSR) markers. The map distributed the markers in 20 linkage groups. However, the real breakthrough occurred in 2017 when a high-throughput SNP array 'Axiom_*Arachis*' including 58 K informative SNPs was developed (Clevenger et al. 2017b; Pandey et al. 2017). The SNPs were derived from DNA resequencing and RNA sequencing of 41 peanut and wild *Arachis* accessions. 29,983 and 28,250 of the total 58,233 SNPs come from the A and B genomes, respectively. Their distribution is highly homogeneous, 51.5 % in the A subgenome and 48.5 % in the B subgenome. Moreover, the coverage per pseudomolecule averaged 2,912 SNPs (1 SNP per kb in the whole peanut genome). Among the SNPs, 44,501 were selected from alignment of both diploid genome assemblies with tetraploid sequence and 13,732 SNPs with other diploid sequences. Of the latter, 2,195 (3.8 %) of the SNPs were identified from *A. cardenasii*. The 'Axiom_*Arachis*' array has already been successfully employed by Clevenger et al.

(2017b) to study tetrasomic recombination phenomena and investigate U.S. Runner market type peanut genetic diversity. Moreover, Pandey et al. (2017) were able to conduct in-depth phylogenetic analysis of the 'Reference Set' comprising 300 genotypes developed by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) (Pandey et al. 2017). A version 2 of the 'Axiom_*Arachis*' array is presently available (Clevenger et al. 2018b; Korani et al. 2019).

CHAPTER 3

INTERSPECIFIC INTROGRESSION CHARACTERIZATION IN *ARACHIS* FOR LATE LEAF SPOT RESISTANCE

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Abstract

Late Leaf Spot (LLS) disease caused by *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash, Videira & Crous affects peanut (*Arachis hypogaea* L.) all around the world. IAC 322 is a breeding line with alien introgressions from *A. cardenasii* Krapov. & W.C. Gregory, a wild diploid relative of peanut, on chromosomes A02 and A03. Progenies from TifNV-High O/L x IAC 322 were genotyped and selected based on the introgressed segments they retained and phenotyped for LLS resistance under both *in vitro* and field conditions. IAC 322-derived progenies were found to have varied levels of LLS resistance. The introgressed segments on the top part of A02 and bottom part of A03 chromosomes accounted for the majority of LLS resistance. Moreover, high correlations between *in vitro* and field experiments, and between late stages of LLS severity and entire LLS infection progression under both field and *in vitro* experiments, were observed.

Introduction

Leaf Spot (LS) is a foliar disease plaguing peanut (*Arachis hypogaea* L.) (2n = 4x = 40) wherever it is cultivated (Shokes and Culbreath 1997). *Cercospora arachidicola* Hori and *Nothopassalora personata* (McDonald et al. 1985; Berk. & M.A. Curtis) U. Braun, C. Nakash., Videia & Crous (syn. *Cercosporidium personatum*) are the two fungi causing early leaf spot (ELS) and late leaf spot (LLS) diseases, respectively (McDonald et al. 1985).

Yield losses in peanut due to LS diseases can range from 10% to 80% even in treated fields (Shokes et al. 1983; Knauft et al. 1986; Knauft et al. 1988; Shokes and Culbreath 1997). Chemicals are effective and economical for LS disease control only when widely applied in large-scale farms in developed countries. Conversely, they remain ineffective and expensive in small-scale farms in developing countries. Generally, 6-8 chemical applications are necessary to prevent LS development during the growing season, although the frequency of sprays can increase when the environmental conditions favor LS progression which in turn elevates the peanut production costs (McDonald et al. 1985; Shokes and Culbreath 1997). Therefore, developing LS resistant peanut cultivars is imperative to mitigate the damage from LS diseases and increase the profitability of peanut (McDonald et al. 1985).

A few sources of host resistance to LLS have been identified both in cultivated peanut and wild *Arachis* species such as *A. stenosperma* and *A. cardenasii* (Abdou et al. 1974; Hassan and Beute 1977; Subrahmanyam et al. 1982; Holbrook and Anderson 1995; Leal-Bertioli et al. 2009). Unfortunately, in the former case resistance levels were moderate and not strong enough to avoid chemical treatments during the season, whereas the wild

species possessed strong resistance, yet introgression of resistance is hampered by ploidy level differences between diploid wild species and tetraploid cultivated peanut (Nevill 1982).

The detached leaf method has already been effectively used to evaluate peanut plants for LLS resistance both under *in vitro* and greenhouse conditions (Melouk and Banks 1978). The original detached leaf method used fungal conidia isolated from susceptible genotypes and cultured on oatmeal-agar medium (Melouk and Banks 1978). A suspension of inoculum is prepared by flooding 15-20 d-old cultures in petri-plates with distilled water and filtering it through four layers of cheesecloth in order to remove mycelial fragments. Petioles of detached peanut leaves, reinforced by a foam plug, are immersed in Hoagland's solution (Hoagland and Arnon 1950) in test tubes, and both leaf surfaces are misted with a suspension of conidia (2×10^4 conidia/ml) by means of an atomizer. The inoculated tubes then are placed in racks in a clear polyethylene chamber in the greenhouse. Temperatures should be maintained at 26 ± 2 ºC and 31 ± 2 ºC, night and day respectively, while humidity should range from 80-90% by hanging wicks of cheesecloth with their bases immersed in water on both sides of the chamber (Melouk and Banks 1978). The detached leaf method is cheap and requires minimal amounts of inoculum compared to entire plant inoculation as well as less leaf tissue and limited working space. On the other hand, this technique to date has not been able to completely substitute for field evaluations due to sporadic occurrence of differing disease reactions of the same genotypes in the two environmental conditions (Melouk and Banks 1978). Recent applications of a modified detached leaf method have been performed on F_1 peanut interspecific hybrids and cultivated varieties for LLS phenotyping (Leal-Bertioli

et al. 2009) and hairy root induction (Guimaraes et al. 2017), respectively. In these experiments, test tubes were substituted by Petri-dishes in order to create moist chambers and peanut leaf petioles were covered with moistened cotton wool. The introduced modifications were applied in order to favor rooting of petioles and prolong leaf vitality (Leal-Bertioli et al. 2009; Guimaraes et al. 2017). In addition, in the latter case, moist chambers where placed in growth chambers at 25 ± 2 ºC with a 16:8 h light:dark photoperiod instead of the greenhouse.

Culture media and techniques are currently available to induce *N. personata* conidia production *in vitro* (Abdou and Coopers 1974), but other procedures such as direct spore collection from field-infected peanut leaves provides an ample supply of rapidly available spores for a prolonged period of time (Gill 2013).

In laboratory conditions, the detached leaf method was employed to evaluate an F_2 generation from the cross of three LLS disease resistant germplasm lines (NC Ac 17133 (RF), NC Ac 17506 and Krapovickas Strain No. 16) and two susceptible cultivars (Nevill 1982). Most of the tested F_2 progenies were similar to the two susceptible cultivars with only few exceptions. The number of lesions per unit area and defoliation traits were correlated but no evidence of genetic control was present in the first case.

Under greenhouse conditions, the detached leaf method was used to estimate components of resistance to both ELS and LLS of appoximately 60 F_2 plants derived from two crosses and reciprocals of LLS resistant (PI 350680 and FESR 5-P2-B1) and ELS resistant parentals (P1 269685 and GP-NC 343) (Anderson et al. 1986a; Anderson et al. 1986b). Only a few F_2 genotypes had greater partial resistance to LS diseases than their parents,

but the correlation data indicated that peanut cultivars resistant either to ELS or LLS can be developed.

Moreover, estimates of combining abilities of an F_1 generation from the M x N mating design of nine parentals were calculated under greenhouse conditions using this method in order to identify the best sources of resistance (Wall and Wynne 1985). Estimates of general combining ability were significant for all considered LLS resistance traits, such as lesion number, lesion area, defoliation, latent period, and sporulation, while for specific combining abilities only sporulation estimates were significant.

Positive correlations among lesion number, lesion size, defoliation and sporulation components have been detected in resistant material (Nevill 1982; Anderson et al. 1986a; Anderson et al. 1986b; Jogloy et al. 1987). These resistance components were typically negatively correlated to incubation and latent period (Wall and Wynne 1985; Dwivedi et al. 2002).

The Florida scale (1-10) is the most commonly used scale for rating LS disease in Georgia since its first introduction in 1988 due to its efficiency and expediency (Chiteka et al. 1988a; Chiteka et al. 1988b). It simultaneously considers several LS resistance components such as number of lesions, lesion positions and degree of defoliation per plot. Therefore, contrary to the detached leaf method, it does not allow identification of specific components of resistance. The scores range from 1 to 10 where, 1 = No leaf spot disease; 2 = Very few lesions on the leaves with none on upper canopy; 3 = Few lesions with very few lesions on upper canopy; 4 = Some lesions with more on upper canopy and slight defoliation (5%); 5 = Noticeable lesions even on upper canopy with noticeable defoliation (20%); 6 = Numerous lesions and very evident on upper canopy with

significant defoliation (50%); 7 = Numerous lesions on upper canopy with much defoliation (75%); 8 = Upper canopy covered with lesions with high defoliation (90%); 9 = Very few leaves remaining and covered with lesions, and some plants completely defoliated; 10 = Dead plants (Chiteka et al. 1988a; Chiteka et al. 1988b; Li et al. 2012). The Florida scale (1-10) at its first introduction was successfully employed to evaluate 105 genotypes in the field and measure variability and consistency between greenhouse and field conditions (Chiteka et al. 1988a; Chiteka et al. 1988b). Ratings were performed at 120 and 135 DAP and Southern Runner and Florunner cultivars were employed as controls. At 120 DAP, the average rating of the 8 most resistant genotypes was 2.9, while Southern Runner (Gorbet et al. 1987) and Florunner (Norden et al. 1969) were 3.5 and 9.0, respectively. At 135 DAP, the average rating of the 8 most resistant genotypes was 4.9, while Southern Runner and Florunner were 7.0 and 10.0 respectively (Chiteka et al. 1988a). Positive and moderate correlations ($r = 0.46\text{-}0.59$, $p = 0.01$) have been found between field and greenhouse LLS phenotyping experiments (Chiteka et al. 1988b). Thus, both detached leaf assay and field phenotyping using the Florida scale (1-10) were considered effective tools to determine host resistance to leaf spot (Melouk and Banks 1978; Chiteka et al. 1988a; Chiteka et al. 1988b; Leal-Bertioli et al. 2009).

IAC 322 is a progeny from the cross of Runner IAC 886, a common cultivar in Brazil derived from a component line of Florunner, and an ICRISAT germplasm line (ICGV 86687: CS 16 – B2 – B2) that showed high levels of resistance to LLS (ICRISAT, 1986) and low yield. CS 16 – B2 – B2 was a derivative line from an interspecific hybrid with *A. cardenasii* as the wild parent (Company et al. 1982). This line was developed in North Carolina initially for ELS resistance and later distributed to India and Brazil to further

select for LLS resistance (Stalker 2017). In 2017, three major introgressed regions on the top and bottom part of A02 and bottom part of A03 chromosomes in the IAC 322 breeding line were detected (physical positions are based on the *A. duranensis* v1 pseudomolecules, peanutbase.org) (Clevenger et al. 2017a), and it was presumed that the three introgressed segments from *A. cardenasii* conferred LLS resistance (Clevenger et al. 2017a).

Peanut breeding is accelerated by selecting for genetic markers shown to be associated with a trait (Chu et al. 2011; Khera et al. 2013; Ozias-Akins et al. 2017). The development of an 'Axiom_*Arachis*' SNP array version 2 provided a high throughput genotyping platform for marker development (Clevenger et al. 2017a; Clevenger et al. 2018b; Korani et al. 2019). The SNPs were derived from DNA and RNA sequencing of 41 peanut and wild *Arachis* accessions. This SNP array was used to genotype our materials to confirm the introgressions from *A. cardenasii* (Clevenger et al. 2017a).

This study focuses on determining the relevance of *A. cardenasii* introgressions to LLS resistance by genotyping and phenotyping progenies from the cross TifNV-High O/L x IAC 322. TifNV-High O/L is a peanut cultivar with high levels of resistance to root-knot nematode and TSWV, and a high oleic versus linoleic acid ratio (Holbrook et al. 2017). Nematode resistance in TifNV-High O/L was also introgressed from *A. cardenasii* and it is controlled from an alien segment in chromosome A09 (Chu et al. 2007, Nagy et al. 2010 & Nagy et al. 2012; Holbrook et al. 2017). Moreover, LS resistance for each of the single introgressed segments, along with all possible combinations will provide valuable information to determine the best breeding approach with these materials.

Materials and Methods

Plant material

The breeding population was created from the cross of TifNV-High O/L x IAC 322 at The University of Georgia, Coastal Plain Experiment Station, Tifton, GA. The F_2 population comprehensive of 392 individuals was genotyped by KASPar assays and selected for presence or absence of the three *A. cardenasii* introgressed segments on chromosomes A02 and A03. Six individuals carrying each of the three introgressed regions were advanced one generation ahead (F_3) than those with all other combinations, including no segments. Consequently, F_2 and F_3 plants were selected for vigor and seeds were scanned (Epson Expression 1640XL, Seiko Epson Corporation, Suwa, Japan) in order to record testa color. F_3 individuals were genotyped by KASPar assay for nematode resistance and with HybProbe assay for high oleic trait and phenotyped for yield and testa color traits. F_3 and F_4 generations were phenotyped for LLS resistance under *in vitro* conditions with a modified detached leaf method (Melouk and Banks 1978; Leal-Bertioli et al. 2009), whereas, F_4 and F_5 generations were grown in the field for LS disease resistance phenotyping. The tested progenies containing different introgressed segment combinations were named by combining the chromosome names where segments were present and the corresponding positions (i.e., "**A02 top**", "**A02 bottom**", "**A03 bottom**", "**A02 top A02 bottom**", "**A02 bottom A03 bottom**" and "**A02 top A03 bottom**"). The only two exceptions were progenies without any segment and presence of all the three segments which were named "**No segments**" and "**All segments**", respectively.

Genotyping

The DNeasy® Plant Mini Kit (Qiagen, Hilden, DK) was used for DNA extractions. KASPar assays were used to determine the presence or absence of the three introgressed segments on chromosomes A02 and A03 and the segment conferring nematode resistance on A09. SNP markers employed for nematode resistance genotyping were AdSNP92 and AdSNP124 targeting the top and bottom regions of chromosome A09, respectively (Nagy et al. 2012; Chu et al. 2016). Whereas, gel-free SNP marker converted from 1101/1048 for HybProbe assay applications targeted the 441_442insA mutation of the *Ah*FAD2B recessive gene and was used for high oleic:linoleic trait genotyping (Chu et al. 2009; Chu et al. 2011).

Thermocycling was performed on a Roche LC480 (Roche Applied Science, Indianapolis, IN). Endpoint genotyping analysis was used for *A. cardenasii* introgressed segments and nematode resistance markers. Each 5 µl of PCR reaction contained 2.5 µl of KASP Genotyping Mix, 0.07 µl of primer assay mix, 1.93 µl of water and 0.5 µl of genomic DNA template. The following thermal cycling procedure was employed: first cycle at 95 ºC for 15 min, followed by 9 cycles of 94 ºC for 20 sec and 61 ºC for 60 sec, the annealing temperature step size reduction was equal to 0.6 ºC per cycle, followed by 32 cycles at 94 ºC for 10 sec and 55 ºC for 60 sec and 6 cycles of 94 ºC for 20 sec and 57 ºC for 60 sec. The last cycle was at 30 ºC for 1 sec and cooling at 25 ºC during plate reading. Three additional cycles of 94 ºC for 20 sec and 57 ºC for 60 sec were performed if signals did not separate appropriately. The two 5'-labeled hydrolysis probes with FAM and HEX fluorophores and the automated scatterplot analysis allowed to discriminate genotypes (Chu et al. 2016b).

A melting curve genotyping analysis method was employed for the high oleic:linoleic acid trait. Each 2.95 µl of PCR reaction contained 0.6 µl of Roche Genotyping Master Mix, 0.18 µl of $MgCl_2$, 0.12 µl of ahFAD2B_Hyb_601as primer, 0.03 µl of ahFAD2B_Hyb_436as primer, 0.03 µl of each Hybprobe, 1.51 µl of water and 0.45 µl of diluted DNA extracts. The following thermal cycling procedure was employed: a preincubation cycle of 10 min at 95°C was followed by 55 cycles of 95°C for 10 sec, 57°C for 10 sec, and 72°C for 10 sec. The melting curve cycle was completed at 95°C for 1 min, 40°C for 2 min, and a gradual increase in temperature to 95°C at 0.11°C sec^{-1} followed by a cooling to 40°C for 30 sec. A derivative formula –(d/dT) was employed to produce the melting peaks after the fluorescence signal was plotted in real time against temperature. Similar melting curves were automatically grouped by the software giving genotyping calls based on known standards (Chu et al. 2011).

To confirm the selection of introgressed segments, DNAs were submitted to Affymetrix (Thermo Fisher Scientific, Waltham, United States) for genotyping by the *Arachis* SNP array version 2. Genotyping data were analyzed with the Axiom analysis suit (Thermo Fisher Scientific).

***In vitro* phenotyping**

Phenotyping of selected F_3 and F_4 lines was performed using a modified *in vitro* detached leaf method (Melouk and Banks 1978; Leal-Bertioli et al. 2009; Guimaraes et al. 2017).

Samples of Georgia-13M leaves infected by *N. personata* were collected at The University of Georgia Coastal Plain Experiment Station Lang-Rigdon Farm, Tifton, GA in summer 2017. Conidia were harvested only from lesions on the abaxial surfaces of

leaves employing a Mini Cyclone Spore Collector (Tallgrass Solutions Inc., Kansas, United States) attached to a Chemical Duty Pump, model WP6111560 (115 V/60 Hz) (EMD Millipore Corporation, Massachusetts, United States). The collected spores were stored either in 20 ml glass vials or in gelatin capsules at 4°C, inside a box filled with Drierite to maintain low humidity.

The second youngest fully expanded quadrifoliate leaves both from main stem and laterals of F_3 and F_4 individuals were harvested from at least 6-week-old greenhouse-grown plants. Petioles were cut 5 cm below the first leaflet's attachment point. The collected leaves were washed in a Tween 20 solution (0.005%).

A thin cotton layer was placed inside each 100 mm x 15 mm Petri-dish (Thermo Fisher Scientific) and covered with a filter paper in order to create moist chambers (Guimaraes et al. 2017). Subsequently, moist chambers were humidified and a glass slide was placed on top of the filter paper to keep the leaf surfaces dry (Guimaraes et al. 2017). Each leaf was placed with the adaxial surface up with the petiole covered with moist cotton (Guimaraes et al. 2017). Leaves in the moist chambers were acclimated overnight in a growth chamber (28°C and 16 h of photoperiod).

Complete randomized block design was applied in this study with three replications. The first and second blocks were inoculated with LLS conidial suspensions, while the third block was mock inoculated for control purposes. Runner IAC 886, Florida-07, and TifNV-High O/L cultivars and IAC 322 breeding and GP-NC WS 16 germplasm lines were included as controls.

The inoculation of the spores was accomplished by brushing LLS conidia at a concentration of 4.8×10^6 spores/ml onto both sides of the leaves with an interval of 90

minutes to allow leaves to dry. After inoculation, each leaf was placed again onto a microscope slide in its Petri-dish with the adaxial surface facing up. The Petri-dishes were placed again in the growth chamber (28°C and 16 h of photoperiod) where they were maintained until the end of the experiment. Viability of spores was tested on a 0.8% water-agar plate, and germinated spores were counted 6 d after inoculation (DAI).

Data of the total number of LLS lesions, both non-sporulating and sporulating, and the number of sporulating LLS lesions per day were periodically collected for each leaf until 20 DAI. This practice allowed determination of incubation and latent periods for each genotype. The incubation period was defined as the period of time required from the inoculation to the appearance of the first LLS lesion, while the latent period as the period of time required from the inoculation to the appearance of the first sporulating LLS lesion (Chiteka et al. 1988a; Chiteka et al. 1988b). Moreover, leaflet defoliation from the petiole of each leaf was recorded until 56 DAI.

At 22 and 23 DAI, the first and second blocks of inoculated leaves were scanned, respectively, and the digital images were analyzed with ASSESS 2.0 image analysis software (The American Phytopathological Society, St. Paul, U.S.) in order determine the lesion area percentage of each leaf.

Field phenotyping

F_4 and F_5 progenies with different combinations of the three introgressed regions, IAC 322, GP-NC WS-16, DP-1 resistant controls, TifNV-High O/L, Florida-07 and Georgia-06G moderately susceptible controls, and Runner IAC 886, Georgia-13M and TUFrunner-511 (Tillman and Gorbet 2017) highly susceptible controls were planted in

Gibbs and Lang-Rigdon farms at The University of Georgia Coastal Plain Experiment Station, Tifton, GA.

A randomized complete block design with three blocks and one replication was followed. In the Gibbs farm, 40 seeds per plot were planted in two-row plots separated by 1.5 m-long alleys. Plot rows were 3.0 m-long and plants were spaced 15 cm within a row and 50 cm between rows. At the Lang-Rigdon farm, 40 seeds per plot were planted in two-row plots separated by 1.5 m-long alleys. Plot rows were 1.5 m-long and plants were spaced 7.5 cm within a row and 50 cm between rows.

Common cultural practices were adopted during the growing season apart from fungicide applications. TSWV severity was evaluated calculating the infected plot percentage at 88 and 83 DAP at Gibbs and Lang-Rigdon farms, respectively.

LS severity was assessed with the Florida scale (1-10) at 76, 88, 99, 110, 123 and 132 DAP at the Gibbs farm (Chiteka et al. 1988a), whereas it was measured at 71, 83, 94, 105, 118 and 127 DAP at the Lang-Rigdon farm.

Data on total pod weight per plot, 100-pod weight, total seed from 100 pods, shelling percentage and 100-seed weight were collected from peanut plots at the Gibbs farm.

Data Analysis

AUDPC was calculated to analyze LLS lesions, sporulating LLS lesions and leaflet defoliation traits of the *in vitro* phenotyping experiment. Furthermore, it was used to investigate the Florida scale (1-10) ratings for LS disease severity in the field experiment. LLS lesions and sporulating LLS lesions measured the last day of data collection (20 DAI) on detached leaves were also analyzed singularly. Field LS and TSWV severity traits were analyzed separately for Gibbs and Lang-Rigdon farms.

A Shapiro-Wilk test was performed to test data normality, while Levene's median test was used to assess the homogeneity of variances. Significant p-values implied non-normal distribution of the data and absence of homogeneity of variances, respectively. A Kruskal-Wallis non-parametric test was employed to determine the presence or absence of significant differences among genotypes. Grouping of genotypes was performed using the criterion of Fisher's least significance difference (LSD) ($\alpha = 0.05$). Pearson's r correlation coefficients were computed among Gibbs and Lang-Rigdon farms LS disease severities, among *in vitro* phenotyping experiment LLS resistance traits and between the two as well as between yield traits, and LS and TSWV disease severities for the Gibbs farm. All statistical analyses and data plotting were performed with RStudio (RStudio, Boston, United States) software. The employed RStudio packages were "car", "agricolae" and "ggplot2".

Results

Testa color trait was segregating among breeding lines during the F_2 generation with different combinations of introgressed segments (**Fig. 1**). 17 of the F_2 peanut lines had seeds testa characterized by pink/tan color, typical of the TifNV-High O/L parent. Four lines (71, 85, 132 and 383) had red seed testa color, typical of the IAC 322 parent. Two F_2 lines (91 & 129) had both pink/tan and red seed testa. Segregation in terms of testa color was recorded also on F_4 seeds derived from F_3 individuals with all three segments present (**Table 1 & Fig. 1**). F_3 individuals had also variation in terms of yield traits (**Table 1**).

Genotyping

KASPar assays effectively genotyped the F_2 generation for presence or absence of the three introgressed segments (**Table 2**) and allowed selection of 15 lines with all possible combinations of the three introgressed segments. Furthermore, KASPar and HybProbe assays were used to screen the selected F_3 plants for nematode resistance markers and high oleic:linoleic acid mutants, respectively (**Table 1)**.

The Axiom_*Arachis* SNP array genotyping confirmed presence and positions of the three introgressed segments detected by the KASPar assays and was able to better define the segment sizes of introgressions on the top and bottom of chromosome A02, which were 5,095,872 bp and 86,013,395 bp, respectively (**Table 2**). 210 markers were related with the introgressed segment in the top part of chromosome A02, while 117 with the introgressed segment in the bottom part of the chromosome A02 (**Table 3**). Introgressed segment position on the bottom part of chromosome A03 was confirmed by the SNP

array. On the other hand, it was not able to define the introgressed A03 segment size due to lack of informative markers in the bottom part of the chromosome A03.

***In vitro* phenotyping**

LLS spores had a germination rate of 41% and effectively infected leaves of tested genotypes (**Fig. 2**). The mock inoculated block did not show any LLS contamination and the leaves were healthy throughout the experiment.

LLS lesions started to be visible on inoculated peanut leaves around 9 DAI (**Fig. 3A**) and the number of lesions continued to increase until the end of the experiment. A slight reduction in number of lesions has been found for some genotypes from around 17 to 20 DAI due to coalescence of lesions. Sporulating LLS lesions started to be noticeable around 13 DAI and increased in number throughout the duration of the experiment (**Fig. 3B**).

The incubation and latent periods were identified as 9-10 DAI and 13-14 DAI, respectively, for most tested genotypes (**Fig. 3A & B**). It is noticeable that individuals with delays in terms of incubation periods were also showing a reduced number of LLS lesions at 20 DAI suggesting a negative correlation between the two. In fact, this was confirmed by the correlation analysis (**Table 4**). Runner IAC 886 and IAC 322 were the genotypes that showed the highest and lowest numbers of LLS lesions and sporulating LLS lesions, respectively. Furthermore, IAC 322, **All segments** and **A02 top A03 bottom** were the only individuals that on average showed less than 100 total LLS lesions per inoculated leaf at 20 DAI and were among tested genotypes that on average showed less than 25 sporulating LLS lesions per leaf at 20 DAI (**Fig. 3A & B**).

IAC 322, **All segments**, and **A02 top A03 bottom** incubation and latent periods were 10 and 20, 10 and 20, and 9 and 14 DAI, respectively (**Fig. 3A & B**). IAC 322 showed only one sporulating lesion during the whole *in vitro* phenotyping experiment ending at 20 DAI (**Fig. 3B**).

In vitro experiment p-values of the Shapiro-Wilk test were significant for all traits (**Table 5**). Whereas, for Levene's test, they were significant for all traits except LLS lesions at 20 DAI and sporulating LLS lesion at 20 DAI.

Detached leaf trait p-values of the Kruskal-Wallis test were significant for all traits analyzed indicating presence of significant differences among genotypes (**Table 5**). Runner IAC 886 was significantly more susceptible than progenies (except **No segments** and **A02 bottom)** for all tested LLS resistance traits (**Fig. 4-9**). Among progenies, the line with no introgressed segment and the line with A02 bottom segment showed the highest values in terms of AUDPC of LLS lesions, LLS lesions at 20 DAI, AUDPC of leaflet defoliation and lesion area percentage traits. Whereas, **No segments** and **A03 bottom** were the most susceptible in terms of AUDPC of sporulating LLS lesions and sporulating LLS lesions at 20 DAI traits. IAC 322 was the most resistant genotype to LLS for all detached leaf traits, and **A02 top A03 bottom** was the only genotype that was always grouped with the resistant parent for all tested traits.

For AUDPC of LLS lesions and LLS lesions at 20 DAI, Fisher's LSD test grouped IAC 322, **All segments** and **A02 top A3 bottom** together. On the other hand, only IAC 322 and **A02 top A03 bottom** were significantly different from all the other progenies and controls (**Fig. 4 & 5**).

IAC 322, **All segments**, **A02 top A03 bottom** and **A02 top** were grouped together for AUDPC of sporulating LLS lesion and sporulating LLS lesions at 20 DAI and significant differences were identified only with controls, **No segments** and **A03 bottom** (**Fig. 6 & 7**). For the AUDPC of leaflet defoliation trait, only **A02 top A03 bottom** was grouped with IAC 322, but it was not significantly different from most of the other progenies (**Fig. 8**). Lastly, for lesion area percentage, IAC 322, **All segments** and **A02 top A03 bottom** were grouped together and were significantly different from controls and progenies except **A02 bottom A03 bottom** (**Fig. 9**).

Pearson's r correlation coefficients among detached leaf disease traits were all significant, and equal or higher than 0.71 except for incubation period vs AUDPC of sporulating LLS lesions (**Table 4**). High levels of correlation ($r = 0.99$ & 0.98, $p < 0.001$) were found for LLS lesions at 20 DAI vs AUDPC of LLS lesions and sporulating LLS lesions at 20 DAI vs AUDPC of sporulating LLS lesions, respectively.

Field phenotyping

Peanut plots in both locations were infected by LS and TSWV diseases (**Fig. 10**). Field experiment p-values of the Shapiro-Wilk test were significant at both locations in case of LS severity at the last day of data collection and TSWV severity traits. While, they were not significant for AUDPC of LS severity (**Table 6**). On the other hand, field experiment p-values of the Levene's test were not significant for all traits at both locations.

P-values of the Kruskal-Wallis test were significant for LS severity at the last day of data collection and AUDPC of LS severity traits for both locations implying significant

differences among genotypes (**Table 6**). Conversely, there was no significant difference for genotype and TSWV severity.

The Lang-Rigdon farm was characterized by higher LS disease pressure and lower TSWV severity than the Gibbs farm. In fact, all tested genotypes had higher AUDPC values, higher Florida scale (1-10) ratings and lower TSWV ratings at the Lang-Rigdon farm (**Figs. 11-16)**.

Generally, progenies with at least one introgressed segment, IAC 322 and GP-NC WS 16 lines were less susceptible to LS diseases than **No segments** progeny and the other controls (**Fig. 11-14**). The IAC 322 breeding line was the most resistant genotype conforming to the findings of the detached leaf experiment.

For the AUDPC of LS severity trait at the Gibbs farm, GP-NC WS 16 and **A02 top A03 bottom** were grouped with IAC 322 (**Fig. 11**). IAC 322 and GP-NC WS 16 were significantly more resistant than other controls and progenies, whereas **A02 top A03 bottom** was also clustered with most of the progenies and moderately resistant checks TifNV-High O/L and Georgia-06G. All progenies (except **No segments**), Georgia-06G, DP-1 and TifNV-High O/L were significantly more resistant than Georgia-13M, Runner IAC 886 and **No segments** progeny. At the Lang-Rigdon farm, GP-NC WS 16, **A02 top A03 bottom** and **All segments** were grouped with IAC 322, but they were also clustered with **A02 top** progeny (**Fig. 12**). These five genotypes, DP-1, **A02 bottom** and **A02 top A02 bottom** were significantly more resistant than Georgia-06G, Georgia-13M, TUFrunner-511, Runner IAC 886, Florida-07 and **No segments**.

For LS severity at the last day of data collection at the Gibbs farm (132 DAP), GP-NC WS 16, **A02 top**, **A02 bottom**, **A02 top A02 bottom**, **A02 top A03 bottom** and **All**

segments were grouped with IAC 322 (**Fig. 13**). These genotypes were significantly more resistant than Georgia-13M, TUFrunner-511, Runner IAC 886, Florida-07, TifNV-High O/L and **no segments**. At the Lang-Rigdon farm (127 DAP), only **All segments** and **A02 top A03 bottom** were grouped with IAC 322 (**Fig. 14**). **A02 top A03 bottom** and IAC 322 were significantly more resistant than all tested genotypes except **All segments** and GP-NC WS 16.

As predicted by the Kruskal-Wallace test, separation of genotypes was not possible for TSWV severity for both locations (**Fig. 15 & 16**).

Pearson's r correlation coefficients between AUDPC of LS severity at the Gibbs farm and detached leaf LLS resistance traits were significant only for AUDPC of LS severity vs incubation period (-0.77, $p < 0.01$), AUDPC of LS severity vs LLS lesions at 20 DAI (0.62, $p < 0.05$) and AUDPC of LS severity vs sporulating LLS lesions at 20 DAI (0.58, $p < 0.05$) (**Table 4**). On the other hand, they were all significant between AUDPC of LS severity at the Lang-Rigdon farm and detached leaf LLS resistance traits except for AUDPC of LS severity vs leaflet defoliation. All significant Pearson's r correlation coefficients values were equal to or higher than 0.59.

Pearson's r correlation coefficients among the field phenotyping experiment LS resistance traits were all significant at the 0.001 level and equal to or higher than 0.75 (**Table 7**). High values ($r = 0.81$ & 0.93, $p < 0.001$) were observed for AUDPC of LS severity at the Gibbs farm vs LS severity at 132 DAP at the Gibbs farm and AUDPC of LS severity at the Lang-Rigdon farm vs LS severity at 127 DAP at the Lang-Rigdon farm, respectively.

P-values of both the Shapiro-Wilk and Levene tests were not significant for all collected yield and pod traits at the Gibbs farm location (**Table 8**).

P-values of the Kruskal-Wallis test for Gibbs farm yield and pod traits were all significant indicating differences among genotypes (**Table 8**). DP-1, Florida-07, TifNV-High O/L, **A02 top** and **A02 top A03 bottom** were the genotypes with highest values in terms of total pod weight and they were significantly different from Georgia-13M, TUFrunner-511, Runner IAC 886, IAC 322, **A02 bottom** and **A03 bottom** (**Fig. 17**). Georgia-06G, TUFrunner-511, DP-1, Florida-07 and TifNV-High O/L checks were the genotypes with the highest values in terms of 100 pod weight, total number of seeds from 100 pods and 100 seed weight traits (**Fig. 18-20**). Moreover, Florida-07 and TifNV-High O/L were always significantly different from Georgia-13M, Runner IAC 886, IAC 322, GP-NC WS 16 and all tested progenies.

Georgia-06G and Georgia-13M were the best genotypes in terms of shelling percentage and they were significantly different from all controls and tested progenies except TUFrunner-511, DP-1 and Runner IAC 886 (**Fig. 21**).

Pearson's r correlation coefficients between yield traits (except shelling percentage) were all significant and equal to or higher than 0.72 (**Table 9**). Pearson's r correlation coefficients of AUDPC of LS severity at the Gibbs farm vs yield traits were not significant except for shelling percentage ($r = 0.60$, $p < 0.05$). Correlation coefficients of TSWV severity at the Gibbs farm vs yield traits were all negative and not significant.

Discussion

Testa color was still segregating in the F_2 and F_3 generations of tested peanut lines as expected given the early stages of population advancement. Seed testa colors of the F_2 and F_3 plants were pink/tan or red as parental (TifNV-High O/L and IAC 322 respectively) colors. Conclusions on the actual gene actions involved in the tested population cannot be made given the employed experimental layout and previous selection of peanut lines.

KASPar markers (Smith and Maughan 2015) designed to target three chromosomal segments introgressed from *A. cardenasii* into peanut were demonstrated to be cheap, flexible and effective tools to select for segments in progenies. Their positions were within Mb-size chromosomal regions characterized by both the IntroMap diagnostic tool (Clevenger et al. 2017a) and Axiom_*Arachis* SNP array analyses.

The KASPar assay technology and marker-assisted selection (MAS) was applied to rapidly screen 392 genotypes of the F_2 population derived from the TifNV-High O/L x IAC 322 cross and reduce the total population size to only 15 selected lines with all possible combinations of three introgressed segments in just one breeding generation. This MAS approach, based on previous IntroMap analysis outcomes, involved more risk than classical methods such as the development of recombinant inbred line (RIL) populations which allow the creation of a massive and consistent database of phenotypic information. In the case of inaccurate IntroMap analysis or lack of association between introgressed segments and LLS resistance, this method would have resulted in a waste of time and resources. On the other hand, MAS, as in the present study, saved several breeding generations and field space which significantly reduced breeding program costs.

Therefore, its employment is recommended in cases where reliable phenotypic data are already available, along with genotypic associations.

Furthermore, in the present study, when comparisons were possible, boundaries of the detected introgressed segments by IntroMap analysis entirely fit within those of the *Arachis* SNP array confirming the IntroMap bioinformatic tool as valuable for genotypic studies. For the segment in the top part of chromosome A02, IntroMap detected a region that occupied a central position compared to the one detected by the Axiom_*Arachis* SNP array. Whereas, for the segment in the bottom part of chromosome A02, IntroMap detected a region that was proximal to the inferior border of the one detected by the Axiom_*Arachis* SNP array.

The Axiom_*Arachis* SNP array version 2 accurately defined introgressed segments boundaries and sizes on the top and bottom part of chromosome A02, which were larger (more than twelve times in the case of the segment in the bottom part of chromosome A02) than what was detected by IntoMap analysis. Unfortunately, this was not possible with the last introgressed segment due to a lack of informative markers in the bottom part of the chromosome A03. This fact implies that further research would be necessary to identify which regions are closely linked to LLS resistance and which ones are not within a single introgressed segment and if it would be possible to avoid the introgression of parts of them during breeding programs.

IAC 322 and **A02 top A03 bottom** were the most resistant genotypes in each field trial location and condition. Their levels of resistance were superior to any check, even those that are nowadays considered resistant or moderately resistant cultivars such as DP-1 and TifNV-High O/L, respectively (Gorbet et al. 2008; Holbrook et al. 2017). Furthermore,

other derived progenies with at least one introgressed segment also had generally higher LLS resistance than most checks, and statistically significant separation of introgression lines with at least one check genotype was always possible for each resistance trait in both detached leaf and field experiments (the only exception was **A02 bottom** progeny for detached leaf). Thus, these results confirmed that the wild peanut relative *A. cardenasii* is a strong source of LLS disease resistance (Abdou et al. 1974; Stalker 2017). Although, **all segment** and **A02 top A03 bottom** progenies were both really similar to IAC 322 in terms of LS disease resistance, **A02 top A03 bottom** progeny was the only genotype among all tested progenies that was always grouped with IAC 322 both in detached leaf and field experiments at multiple locations. Consequently, these findings suggest dispensability of the introgressed segment in the bottom part of chromosome A02. Avoiding introgression of this segment limits potential linkage drag where deleterious genes may cause fitness reduction during introgression from wild *Arachis* species into cultivated peanut varieties (Bertioli et al. 2011; Pandey et al. 2012). Future breeding programs that may introgress LLS resistance from the low-yielding breeding line IAC into a modern, high-yielding cultivar should consider the potential for linkage drag associated with the bottom part of chromosome A02 that could make the difference between low or high yield classification. This is further emphasized by the fact that A02 bottom progeny had the lowest yield both in terms of total pod weight and shelling percentage at the Gibbs farm. Conversely, **A02 top A03 bottom** was the highest yielding line among IAC 322 progenies in terms of total pod weight.

Overall, the detached leaf method was demonstrated to be an effective tool for LLS studies under *in vitro* conditions. It allowed the screening of a large number of genotypes

for disease resistance requiring limited time and effort (Melouk and Banks 1978; Leal-Bertioli et al. 2009; Guimaraes et al. 2017). Data collection was the most time consuming and laborious phase of the *in vitro* experiment since periodic leaf inspection and lesion counting were necessary to record the disease progression on infected peanut leaves. Results showed extremely high Pearson's r correlation values of LLS lesions at 20 DAI vs AUDPC of LLS lesions ($r = 0.99$, $p < 0.001$) and, sporulating LLS lesions at 20 DAI vs AUDPC of sporulating LLS lesions ($r = 0.98$, $p < 0.001$) suggesting that late stages of LS infection are representative of the entire infection progress. Therefore, in future lab experiments that may use the detached leaf method for LLS resistance screening, it would be possible to save time and increase overall detached leaf experiment efficiency by deferring data collection to the last stages of LS disease infection (about 20 DAI).

This recommendation is further confirmed by the field experiment where high correlations ($r = 0.81$ and 0.93, $p < 0.001$) were found at both locations (Gibbs & Lang-Rigdon farms, respectively) between LS severity at the last day of data collection (132 & 127 DAP, respectively) and AUDPC of LS severity. The r values of the field experiment were slightly lower compared to the lab experiment, perhaps due to the fact that field infected peanut plants were exposed to variable climatic conditions and biotic and abiotic stresses which may have somehow altered the LS infection progression.

High and significant Pearson's r correlation values also were found among the other traits of the detached leaf experiment with the only exception of incubation period vs AUDPC of sporulating LLS lesions. Detached leaf disease traits were all positively correlated except for the incubation period and in complete agreement with previously published

work (Nevill 1982; Wall and Wynne 1985; Anderson et al. 1986a; Anderson et al. 1986b; Jogloy et al. 1987).

The Florida scale (1-10) (Chiteka et al. 1988a; Chiteka et al. 1988b) effectively described LS severity in both locations during the field experiment. The Lang-Rigdon farm was characterized by higher LS pressure compared to the Gibbs farm. Thus, genotype distinction in terms of LS resistance was more marked in the previous location. Strong correlations ($r = 0.75\text{-}0.93$, $p < 0.001$) were detected between LS severities at two different locations implying a certain level of environmental stability of tested genotypes and LS infection progression.

Higher correlation values were detected than those previously recorded between lab and field (Nevill 1982) and greenhouse and field (Chiteka et al. 1988b) experiments. Incubation period, LLS lesions and sporulating LLS lesions at late stages of LLS infection for the detached leaf experiment seem to be the ones that better predict genotype LLS resistance under open field conditions. On the contrary, leaflet defoliation for detached leaves was the only trait that was not significant versus field AUDPC of LS severity for both field locations. This may be due to the controlled environment and the absence of wind disturbance *in vitro*, both of which would affect timing of defoliation in the field and lead to expected contrasts between lab and field experiments. Hence, detached leaf leaflet defoliation does not associate with host resistance under field condition. These outcomes confirm what was previously suggested from studies on fifteen peanut interspecific derivatives under greenhouse conditions (Dwivedi et al. 2002).

Significant separation of tested genotypes for TSWV resistance was not possible. However, the Gibbs farm was characterized by higher levels of spotted wilt infection compared to the Lang-Rigdon farm. This may be due to the difference in plot sizes between the two locations. In fact, for the same number of seed per plot (40), the Gibbs farm had rows two-times longer than the Lang-Rigdon farm which could have increased the percentage of infected plants (Culbreath et al. 2003).

Pearson's r correlation values were positive and high ($r = 0.72 – 0.99$, $p < 0.01$-0.001) among Gibbs farm yield traits, except when calculated versus the shelling percentage. On the other hand, they were not significant when calculated between yield traits and AUDPC of LS severity at the Gibbs farm, except for shelling percentage vs AUDPC of LS severity which was positive and equal to 0.60 ($p < 0.05$).

The positive, significant correlation between the foliar disease and shelling percentage is in agreement with what was previously reported by other authors, which suggested a genetic linkage between pod filling or pod thickness and LLS susceptibility (Anderson et al. 1993; Shoba et al. 2012a).

Conversely, the other correlations and significant differences were not anticipated and not in agreement with previous literature i.e., Chu et al. (2019) reported significant negative correlations ($p < 0.001$) between yield and LLS disease in a four-year test of a recombinant inbred line population from Florida-07 x GP-NC WS 16. The lowest negative value was -0.54, while several others were lower than -0.40.

An explanation to these results may be given by three facts that characterized the field phenotyping experiment at the Gibbs farm. First, plot sizes were 3.0 m-long x 1.5 m-large and only 40 plants per plot. Therefore, plot sizes and number of plants in the present

study were not representative enough to record significant differences in yield reductions among the different progenies. Secondly, the Gibbs farm location, between the two tested, had lower LS disease pressure, hence less severe yield reductions are conceivable. Lastly, the South Georgia area in 2018 was characterized by unusually wet weather conditions. At the Tifton Coastal Plain Experiment Station, from planting to harvest of Gibbs farm peanut plants, precipitation reached 0.51 m with rain 55 out of 90 days (www.georgiaweather.net). Thus, yield may have been influenced more by the uncommon weather conditions than the stress caused by LS diseases.

In brief, this study was able to characterize for LS resistance three *A. cardenasii* segments introgressed in the top and bottom parts of chromosome A02 and bottom part of A03 of IAC 322 breeding line and to identify the segments on the top part of chromosome A02 and bottom part of chromosome A03 as responsible for the resistance, while the segment in the bottom part of chromosome A02 as dispensable.

Table 1: Yield, testa color and, high oleic:linoleic and nematode resistance trait data of the F_4 seeds with all the three *A. cardenasii* introgressed segments. Mutants carry the high oleic:linoleic trait. For both for AdSNP92 and AdSNP124, X is the allele associated with nematode resistance while Y is associated with susceptibility. Negative output identifies a failed reaction.

Line	F_3 Plant	Tot. Pods Wt. (g)	50 Pods Wt. (g)	50 Pods Tot. Seed Wt. (g)	50 Seed Wt. (g)	Testa Color	High Oleic:linoleic	AdSNP92 Allele	AdSNP124 Allele
07	**4**	128	73	52	33	red	mutant	Y	Y
	5	116	66	47	30	red	mutant	Y	Y
	7	130	71	53	31	red	mutant	Y	Y
	8	78	49	30	25	red	mutant	Y	Y
	11	47	45	30	26	red	mutant	Y	Y
	13	57	55	40	28	red	mutant	Y	Y
34	**6**	129	100	77	46	pink	wildtype	Y	Y
	7	133	88	70	36	tan	heterozygote	Y	Y
259	**1**	87	46	34	24	red	heterozygote	X	Y
	12	99	47	34	25	tan	heterozygote	both	Y
	15	83	49	37	26	tan	heterozygote	X	Y
	16	72	57	41	30	pink	wildtype	X	Y
	18	131	66	46	34	pink	wildtype	X	Y
	22	95	46	69	28	pink	wildtype	both	Y
	24	49	42	29	25	pink	heterozygote	X	Y
	25	148	52	44	27	pink	mutant	Y	Y
	27	64	55	41	26	red	heterozygote	both	Y
	34	53	53	39	27	tan	heterozygote	both	Y
	45	51	45	30	24	pink	heterozygote	both	Y
293	**2**	143	88	66	43	tan	wildtype	Y	Y

	3	90	77	53	38	red	negative	negative	negative
	6	57	57	38	31	light red	mutant	X	X
	7	78	65	39	34	tan	heterozygote	both	both
	9	72	67	48	36	pink	mutant	both	both
	12	151	64	39	31	pink	heterozygote	X	both
	13	102	60	45	30	pink & red	mutant	both	both
	15	70	64	47	38	pink	mutant	both	both
	16	77	77	59	38	pink	heterozygote	both	both
	18	103	48	63	30	pink	mutant	X	X
332	**38**	81	66	49	34	tan	heterozygote	both	both

Table 2: Genotyped F_2 and F_3 peanut lines for presence of *A. cardenasii* introgressed segments subdivided by genotyping method. Markers IDs (when present), positions and detected introgressed region sizes of each segment are listed for IntroMap diagnostic tool (yellow), KASPar assay (green) and Axiom_*Arachis* SNP array version 2 (red). Marker positions are based on the physical position of the *A. duranensis* v1 pseudomolecules (peanutbase.org).

		IntroMap		KASPar assay			Axiom_*Arachis* SNP array version 2		
Line	**Introgression**	**Segment Size**	**Marker Position**	**Marker ID**	**Marker Position**	**Segment Size**	**Markers ID**	**Marker Position**	**Segment Size**
64	No segments	---	---	---	---	---	---	---	---
24, 117, 159	A02 top	4,316,163	122,410	Aradu.A02-910314-CT	910,314	1,708,562	AX-147212106	77,630	5,095,872
			4,438,573	Aradu.A02-2618876-TA	2,618,876		AX-176805847	5,173,502	
36, 85	A02 bottom	6,699,001	79,295,214	Aradu.A02-80149907-GA	80,149,907	5,334,974	AX-176793652	5,891,273	86,013,395
				Aradu.A02-83909015-GA	83,909,015				
			85,994,215	Aradu.A02-	79,414,342		AX-17681678	91,904,668	

				79414342-GA			5		
				Aradu. A02-85484881-TA	85,484,881				
71, 91	A03 bottom	4,675,189	130,144,882	Aradu. A03-134516425-CG	134,516,425	Not applied	Not detected	Not detected	Not detected
			134,820,071						

Table 3: Marker IDs and positions of SNPs from the *Arachis* SNP array version 2 detecting *A. cardenasii* introgressions on the top and bottom parts of chromosome A02. Marker positions are based on the physical positions in the *A. duranensis* v1 pseudomolecules (peanutbase.org). In yellow, marker IDs and positions detecting introgressed segments boundaries by KASPar assay.

A02 top		A02 bottom	
ID	**Marker Position**	**ID**	**Marker Position**
AX-147212106	77,630	AX-176793652	5,891,273
AX-147239800	107,019	AX-176801214	8,512,661
AX-176823530	107,155	AX-176810300	8,655,513
AX-147212116	111,029	AX-176808768	9,330,548
AX-147239816	177,906	AX-176797993	11,812,181
AX-147239817	178,738	AX-176814042	12,426,083
AX-147212144	179,738	AX-176814043	12,554,447
AX-176820789	195,147	AX-147213418	12,945,134
AX-147212154	203,548	AX-176800811	13,453,066
AX-147212155	216,264	AX-147240197	13,636,966
AX-147212160	222,582	AX-176804271	13,654,065
AX-176823679	234,648	AX-147239900	13,667,945
AX-147239847	243,157	AX-176794678	14,455,514
AX-176821246	333,157	AX-176812657	16,465,249
AX-176820323	351,242	AX-176798403	16,559,066
AX-176820745	355,418	AX-176801359	17,000,881
AX-147239883	356,772	AX-176819930	17,000,951
AX-176820614	393,799	AX-176823893	17,734,759
AX-147212194	436,437	AX-147241087	19,126,364
AX-176821997	453,682	AX-176802985	19,747,199
AX-176821113	454,252	AX-176800523	19,769,229
AX-147239922	463,789	AX-176816512	19,823,812
AX-176820849	499,765	AX-176813196	19,997,755
AX-147212226	540,156	AX-176795971	20,805,112
AX-176823510	611,723	AX-176803467	20,926,718
AX-176823777	657,805	AX-176801028	22,275,589
AX-177638989	657,840	AX-176800735	22,977,030

AX-176823397	658,586	AX-176816605	23,446,003
AX-177639854	673,606	AX-176796979	23,576,890
AX-177639848	693,441	AX-176794914	25,627,463
AX-177640297	704,231	AX-176810983	26,715,495
AX-147260407	708,283	AX-176818723	26,754,226
AX-147260409	708,810	AX-147241193	27,622,130
AX-177637526	740,844	AX-176810398	29,637,549
AX-147212284	743,786	AX-176793269	30,314,583
AX-177639558	747,915	AX-147213671	36,422,818
AX-177640317	753,709	AX-176798955	39,688,642
AX-147212293	761,768	AX-176812735	40,783,435
AX-177637178	771,251	AX-176793482	41,025,424
AX-176813692	778,771	AX-176821039	42,620,368
AX-147260354	803,879	AX-176800599	43,648,746
AX-176791531	812,440	AX-147213744	45,214,031
AX-147260344	813,213	AX-176810110	45,839,098
AX-147212325	824,732	AX-176814097	47,263,763
AX-177638108	854,168	AX-176806618	50,280,943
AX-177639129	854,798	AX-176809952	52,028,053
AX-177638312	855,353	AX-176806824	54,442,550
AX-177639790	855,369	AX-176814102	56,249,350
AX-177638898	884,867	AX-176802925	56,409,237
AX-147212350	890,382	AX-176806354	59,637,046
AX-176820966	891,147	AX-176823747	60,167,477
AX-177637989	902,657	AX-176815478	60,192,626
AX-177639692	904,278	AX-176805915	61,353,315
AX-147212353	906,663	AX-147213898	61,624,453
AX-176822910	906,704	AX-176809280	62,714,854
AX-177639163	907,368	AX-176814399	63,240,797
AX-176822663	907,369	AX-176814664	63,510,625
Aradu.A02-910314-CT	910,314	AX-176817747	63,685,196
AX-177640564	932,009	AX-176814122	63,972,712
AX-177642451	939,788	AX-176805709	64,584,077
AX-176821437	950,154	AX-176814126	65,495,153
AX-177638799	953,042	AX-176795760	66,882,916
AX-177637822	975,144	AX-176812322	67,200,424
AX-177638087	1,050,291	AX-176813025	67,227,460

AX-177640520	1,093,279	AX-176792619	68,682,708
AX-177640089	1,093,863	AX-176814137	69,842,933
AX-177637614	1,102,848	AX-176810508	71,077,800
AX-177639007	1,104,093	AX-176816683	71,078,802
AX-177638758	1,116,431	AX-147214086	71,088,041
AX-176820144	1,131,265	AX-176800337	71,665,832
AX-147212393	1,145,405	AX-176807553	74,898,009
AX-177638648	1,154,079	AX-147214202	76,430,768
AX-176822938	1,154,240	AX-176804158	76,599,341
AX-177640008	1,158,322	AX-176799240	76,821,168
AX-177637680	1,182,185	AX-176804268	77,274,711
AX-177638558	1,198,594	AX-147241860	79,291,480
AX-177639640	1,199,195	AX-147214321	79,292,428
AX-177637225	1,199,205	AX-147214380	79,831,803
AX-177637392	1,199,530	AX-147214384	79,896,625
AX-177639821	1,199,581	AX-177644005	80,088,078
AX-177639180	1,200,775	AX-147214394	80,148,486
AX-176819339	1,200,821	AX-176805646	80,149,872
AX-176821848	1,200,950	Aradu.A02-80149907-GA	80,149,907
AX-177637730	1,201,188	AX-177642621	80,285,186
AX-176822998	1,201,365	AX-147214409	80,390,386
AX-177639061	1,201,805	AX-176798289	80,605,276
AX-177639242	1,201,941	AX-147214457	80,848,807
AX-176823219	1,202,285	AX-147241950	80,932,123
AX-176820851	1,202,370	AX-177643702	80,987,078
AX-176821242	1,202,409	AX-147214463	81,019,095
AX-176820056	1,202,960	AX-147214476	81,196,567
AX-177639533	1,203,083	AX-147241982	81,272,556
AX-176823914	1,203,231	AX-177644217	81,500,078
AX-176820663	1,204,122	AX-147214532	81,732,113
AX-147212416	1,211,208	AX-147214533	81,741,200
AX-177638302	1,212,360	AX-147214543	81,781,523
AX-177637982	1,222,049	AX-147214572	82,312,369
AX-177638300	1,222,184	AX-147242070	82,576,633
AX-147260275	1,260,292	AX-147242083	82,716,655
AX-177638297	1,262,930	AX-176803329	83,209,731
AX-177639764	1,263,023	AX-147214673	83,713,878

AX-176823791	1,274,287	AX-147214723	84,310,707
AX-177638233	1,278,914	AX-176814935	84,413,575
AX-177638294	1,278,946	AX-176809183	84,510,265
AX-147260266	1,279,992	AX-147214781	84,907,950
AX-177638997	1,283,531	AX-176823918	85,053,216
AX-177639466	1,300,068	AX-176821217	85,054,196
AX-177639686	1,303,096	AX-176823430	85,070,716
AX-177639270	1,303,781	AX-176794070	85,484,846
AX-177638473	1,304,204	Aradu.A02-85484881-TA	85,484,881
AX-177637634	1,305,146	AX-147242237	85,488,627
AX-177639531	1,306,476	AX-147242253	85,667,004
AX-176820170	1,306,564	AX-147214832	85,680,118
AX-177639179	1,332,679	AX-147242262	85,893,596
AX-177638172	1,354,551	AX-176809058	86,895,060
AX-177638477	1,355,218	AX-176812849	87,512,155
AX-147260246	1,356,851	AX-176813961	87,922,047
AX-177637840	1,358,126	AX-176791388	88,070,993
AX-177637763	1,360,024	AX-176816785	91,904,668
AX-177638020	1,366,815		
AX-177638979	1,377,704		
AX-177639262	1,398,344		
AX-177637489	1,410,655		
AX-177637871	1,413,214		
AX-147212484	1,413,700		
AX-176821303	1,487,675		
AX-147212509	1,556,236		
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AX-176819098	1,909,222		
AX-176821886	2,016,700		
AX-147212583	2,026,021		
AX-176823468	2,028,264		
AX-147212600	2,072,464		
AX-176819312	2,458,469		

AX-147240130	2,496,690		
AX-147212654	2,529,802		
AX-176823935	2,530,491		
AX-147212659	2,557,281		
AX-147212674	2,599,755		
AX-176821041	2,599,915		
Aradu.A02-2618876-TA	2,618,876		
AX-176820694	2,616,563		
AX-147212684	2,621,300		
AX-147212686	2,625,195		
AX-147212687	2,640,779		
AX-147240010	2,645,632		
AX-147212710	2,658,859		
AX-176819118	2,672,755		
AX-147212723	2,675,266		
AX-176793345	2,929,837		
AX-176822450	2,930,016		
AX-147240249	2,930,522		
AX-147212764	2,931,058		
AX-176822566	2,932,459		
AX-147212765	2,932,724		
AX-176791970	2,997,421		
AX-176820952	2,999,752		
AX-176821123	3,001,008		
AX-176821465	3,034,367		
AX-176821938	3,035,679		
AX-176822162	3,038,442		
AX-176823130	3,040,270		
AX-176820879	3,048,471		
AX-176819393	3,049,387		
AX-176822053	3,049,400		
AX-176820632	3,049,512		
AX-176821957	3,050,278		
AX-147240296	3,097,780		
AX-147240297	3,097,954		
AX-147212795	3,098,068		
AX-176821380	3,102,439		

AX-147240314	3,265,828		
AX-147212811	3,282,178		
AX-176820019	3,310,481		
AX-176821424	3,310,728		
AX-176823437	3,321,872		
AX-147212819	3,338,207		
AX-176820410	3,377,383		
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AX-176823267	3,587,389		
AX-177637928	3,665,495		
AX-177640246	3,667,809		
AX-147240468	3,897,256		
AX-176821068	3,958,452		
AX-147212876	3,995,999		
AX-147212877	3,998,295		
AX-176822106	4,056,007		
AX-176823105	4,062,056		
AX-147212881	4,084,701		
AX-147212883	4,085,230		
AX-176820927	4,163,406		
AX-176822861	4,205,522		
AX-176821507	4,206,032		
AX-176822226	4,233,793		
AX-176823442	4,235,641		
AX-176820706	4,236,088		
AX-176824001	4,237,580		
AX-176820554	4,256,298		
AX-176819436	4,259,938		
AX-147240503	4,405,635		
AX-147240504	4,405,845		
AX-176819295	4,425,908		
AX-147212908	4,426,458		
AX-176820714	4,455,839		
AX-147240508	4,455,971		
AX-147212930	4,630,386		
AX-176805847	5,173,502		

Table 4: Pearson's r correlation coefficients among ratings for late leaf spot (LLS) components in the *in vitro* phenotyping experiment (red) and between the *in vitro* phenotyping experiment and the AUDPC of LS severity of Gibbs and Lang-Rigdon farms (yellow). * p<0.05; ** p<0.01; *** p<0.001.

	AUDPC LS Severity (Gibbs)	AUDPC LS Severity (Lang-Rigdon)	Incubation Period	AUDPC LLS Lesions	AUDPC Sporulating LLS Lesions	LLS Lesions (20 DAI)	Sporulating LLS Lesions (20 DAI)	Leaflets Defoliation	Lesion Area Percentage
Incubation Period	-0.77 **	-0.76 **	1						
AUDPC LLS Lesions	0.55	0.69 **	-0.71 **	1					
AUDPC Sporulating LLS Lesions	0.46	0.59 *	-0.50	0.72 **	1				
LLS Lesions (20 DAI)	0.62 *	0.74 **	-0.75 **	0.99 ***	0.71 **	1			
Sporulating LLS Lesions (20 DAI)	0.58 *	0.72 **	-0.62 *	0.81 ***	0.98 ***	0.80 ***	1		
Leaflets Defoliation	0.37	0.51	-0.57 *	0.80 **	0.82 ***	0.76 **	0.83 ***	1	
Lesion Area Percentage	0.49	0.59 *	-0.59 *	0.75 **	0.79 **	0.72 **	0.81 ***	0.93 ***	1

Table 5: P-values of Shapiro-Wilk, Levene and Kruskal-Wallis tests of *in vitro* phenotyping experiment traits. * p<0.05; ** p<0.01; *** p<0.001.

Trait	Shapiro-Wilk Test (p-value)	Levene's Test (p-value)	Kruskal-Wallis Test (p-value)
LLS Lesions (20 DAI)	0.01 *	0.14	2.51×10^{-5} ***
Sporulating LLS Lesions (20 DAI)	6.81×10^{-9} ***	0.07	7.76×10^{-7} ***
LLS Lesions	0.01 *	0.04 *	6.33×10^{-6} ***
Sporulating LLS Lesions	5.38×10^{-11} ***	0.01 *	2.73×10^{-7} ***
Leaflet Defoliation	1.13×10^{-4} ***	0.04 *	7.16×10^{-7} ***
Lesion Area Percentage (%)	2.37×10^{-7} ***	0.01 *	7.25×10^{-7} ***

Table 6: P-values of Shapiro-Wilk, Levene and Kruskal-Wallis tests of Gibbs (red) and Lang-Rigdon farms (green) field phenotyping experiment traits. * p<0.05; ** p<0.01; *** p<0.001.

Location	Trait	Shapiro-Wilk Test (p-value)	Levene's Test (p-value)	Kruskal-Wallis Test (p-value)
Gibbs Farm	**LS Severity 132 DAP**	0.02 *	0.47	6.04×10^{-4} ***
	AUDPC LS Severity	0.24	0.94	6.58×10^{-4} ***
	TSWV Severity	5.43×10^{-5} ***	0.72	0.08
Lang-Rigdon Farm	**LS Severity 127 DAP**	4.82×10^{-5} ***	0.90	1.46×10^{-4} ***
	AUDPC LS Severity	0.17	0.74	4.13×10^{-3} **
	TSWV Severity	4.61×10^{-8} ***	0.72	0.36

Table 7: Pearson's r correlation coefficients amongst the field phenotyping experiment leaf spot (LS) resistance traits. * p<0.05; ** p<0.01; *** p<0.001.

	AUDPC LS Severity (Gibbs)	LS Severity 132 DAP (Gibbs)	AUDPC LS Severity (Lang-Rigdon)	LS Severity 127 DAP (Lang-Rigdon)
AUDPC LS Severity (Gibbs)	1			
LS Severity 132 DAP (Gibbs)	0.81 ***	1		
AUDPC LS Severity (Lang-Rigdon)	0.90 ***	0.89 ***	1	
LS Severity 127 DAP (Lang-Rigdon)	0.75 ***	0.87 ***	0.93 ***	1

Table 8: P-values of Shapiro-Wilk, Levene and Kruskal-Wallis tests of Gibbs farm phenotyping experiment yield traits. * p<0.05; ** p<0.01; *** p<0.001.

Trait	Shapiro-Wilk Test (p-value)	Levene's Test (p-value)	Kruskal-Wallis Test (p-value)
Total Pod Weight	0.65	0.74	1.43×10^{-3} **
100 Pod Weight	0.27	0.84	5.45×10^{-4} ***
Total Seed Weight from 100 Pods	0.32	0.95	3.64×10^{-4} ***
Shelling Percentage (%)	0.28	0.85	7.88×10^{-4} ***
100 Seed Weight	0.75	0.95	1.04×10^{-3} **

Table 9: Gibbs farm Pearson's r correlation coefficients amongst yield traits (red) and between yield traits and, AUDPC of LS severity and TSWV severity (yellow). * p<0.05; ** p<0.01; *** p<0.001.

	AUDPC LS Severity	TSWV Severity	Total Pod Weight	100 Pod Weight	Total Seed Weight 100 Pods	Shelling Percentage	100 Seed Weight
Total Pod Weight	-0.08	-0.48	1				
100 Pod Weight	-0.01	-0.16	0.73 ***	1			
Total Seed Weight 100 Pods	0.08	-0.17	0.72 **	0.99 ***	1		
Shelling Percentage	0.60 *	-0.14	0.22	0.28	0.43	1	
100 Seed Weight	-0.02	-0.15	0.77 ***	0.94 ***	0.94 ***	0.36	1

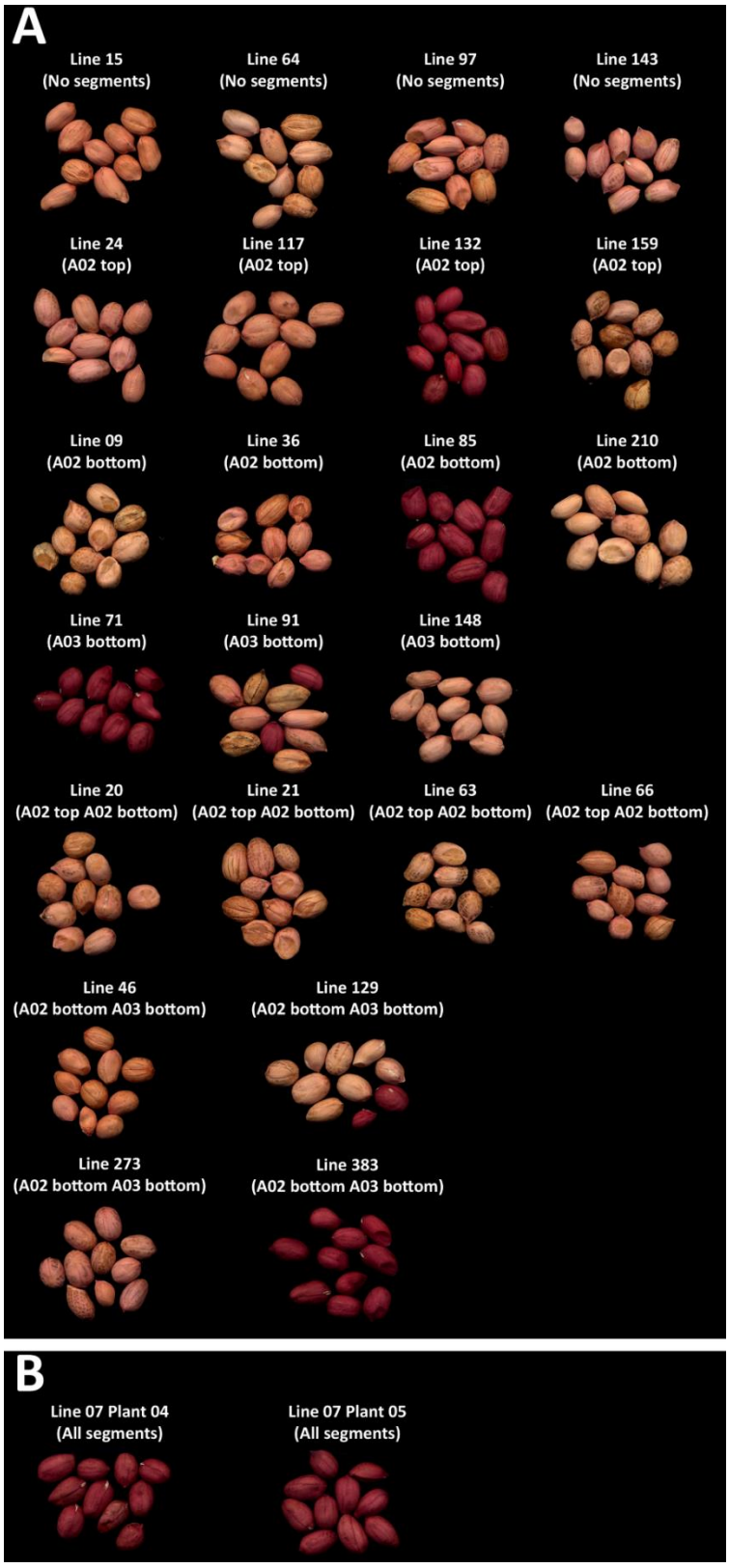
A
Line 15
(No segments)
Line 64
(No segments)
Line 97
(No segments)
Line 143
(No segments)
Line 24
(A02 top)
Line 117
(A02 top)
Line 132
(A02 top)
Line 159
(A02 top)
Line 09
(A02 bottom)
Line 36
(A02 bottom)
Line 85
(A02 bottom)
Line 210
(A02 bottom)
Line 71
(A03 bottom)
Line 91
(A03 bottom)
Line 148
(A03 bottom)
Line 20
(A02 top A02 bottom)
Line 21
(A02 top A02 bottom)
Line 63
(A02 top A02 bottom)
Line 66
(A02 top A02 bottom)
Line 46
(A02 bottom A03 bottom)
Line 129
(A02 bottom A03 bottom)
Line 273
(A02 bottom A03 bottom)
Line 383
(A02 bottom A03 bottom)
B
Line 07 Plant 04
(All segments)
Line 07 Plant 05
(All segments)

Figure 1: Testa color of F_2 seeds (**A**) with different combinations of the three *A. cardenasii* introgressed segments and F_3 seeds (**B**) with all the three introgressed segments.

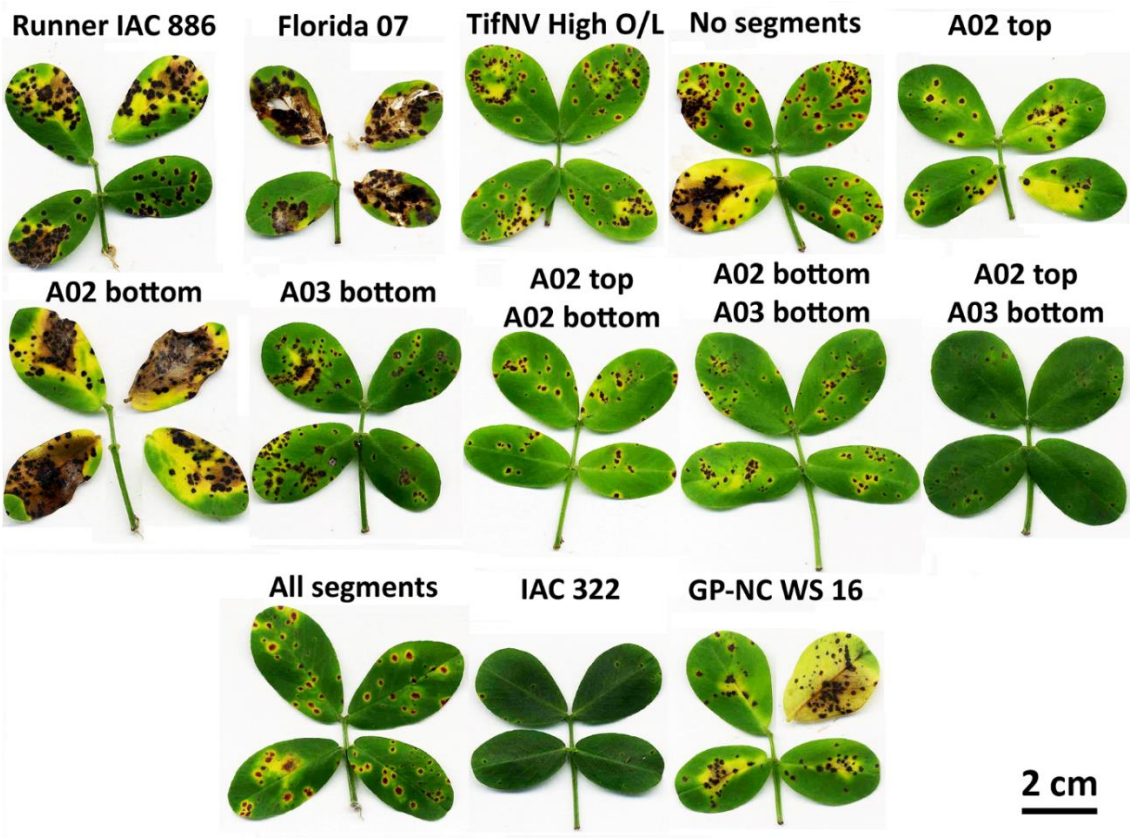

Figure 2: Detached leaves inoculated with late leaf spot spores (block 2, replication 3) and imaged at 22 DAI.

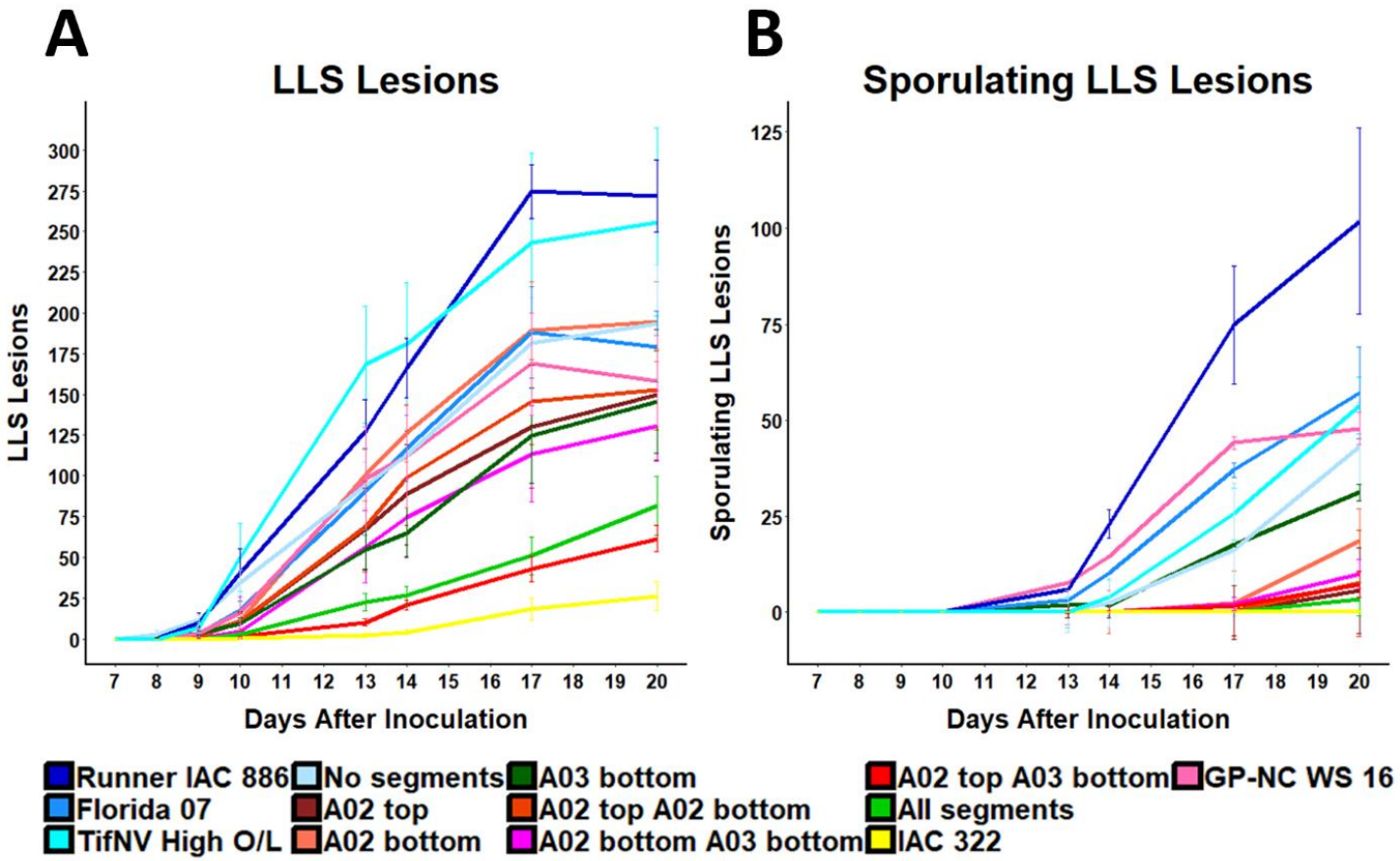

Figure 3: Late leaf spot (LLS) disease progression among detached leaf samples measured by the total number of LLS lesions (**A**) and sporulating LLS lesions (**B**).

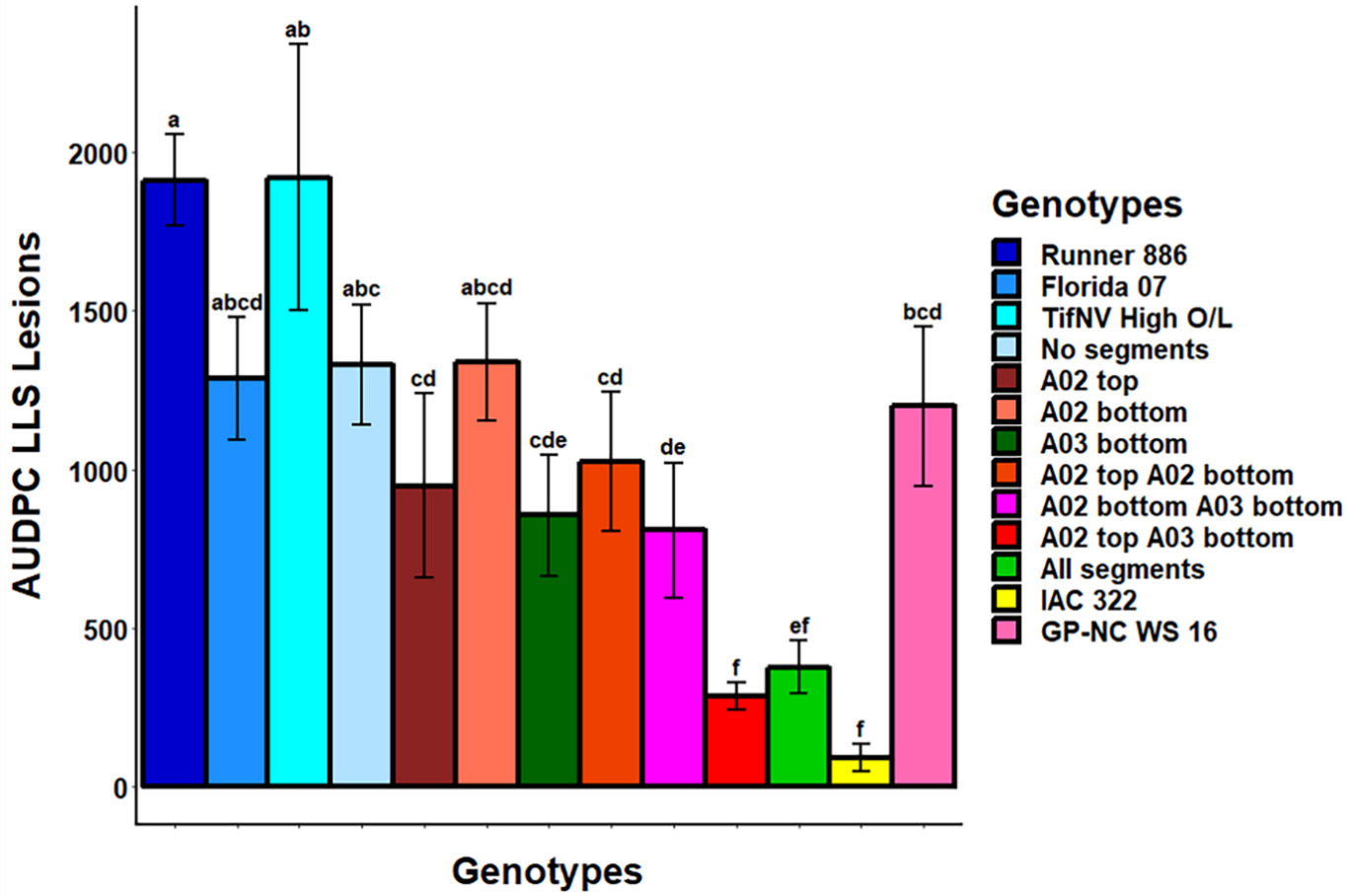

Figure 4: Genotype response measured by the AUDPC of LLS lesions for detached leaves.

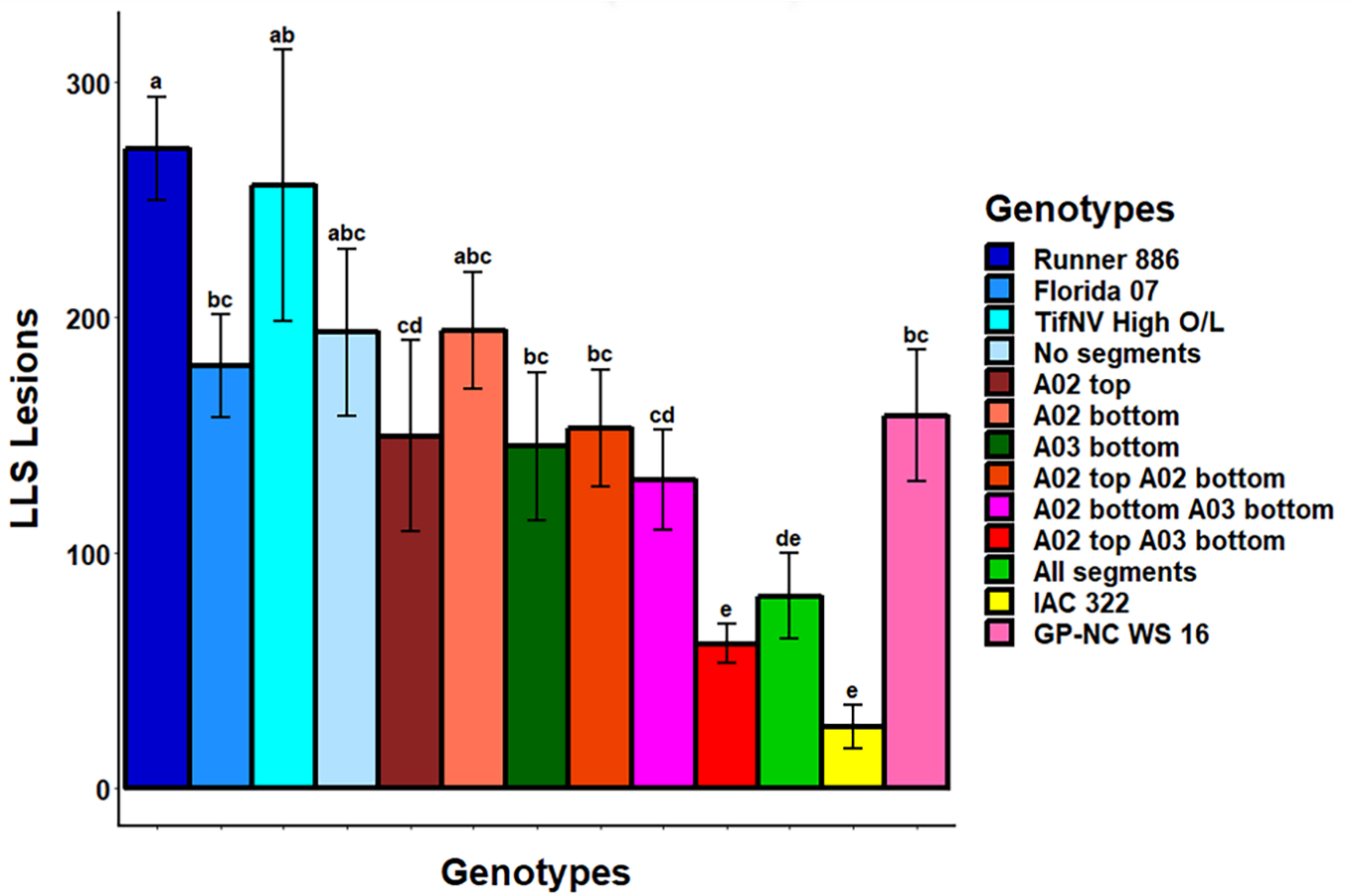

Figure 5: Genotype response measured by the number of LLS lesions at the last day of data collection (20 DAI) from detached leaves.

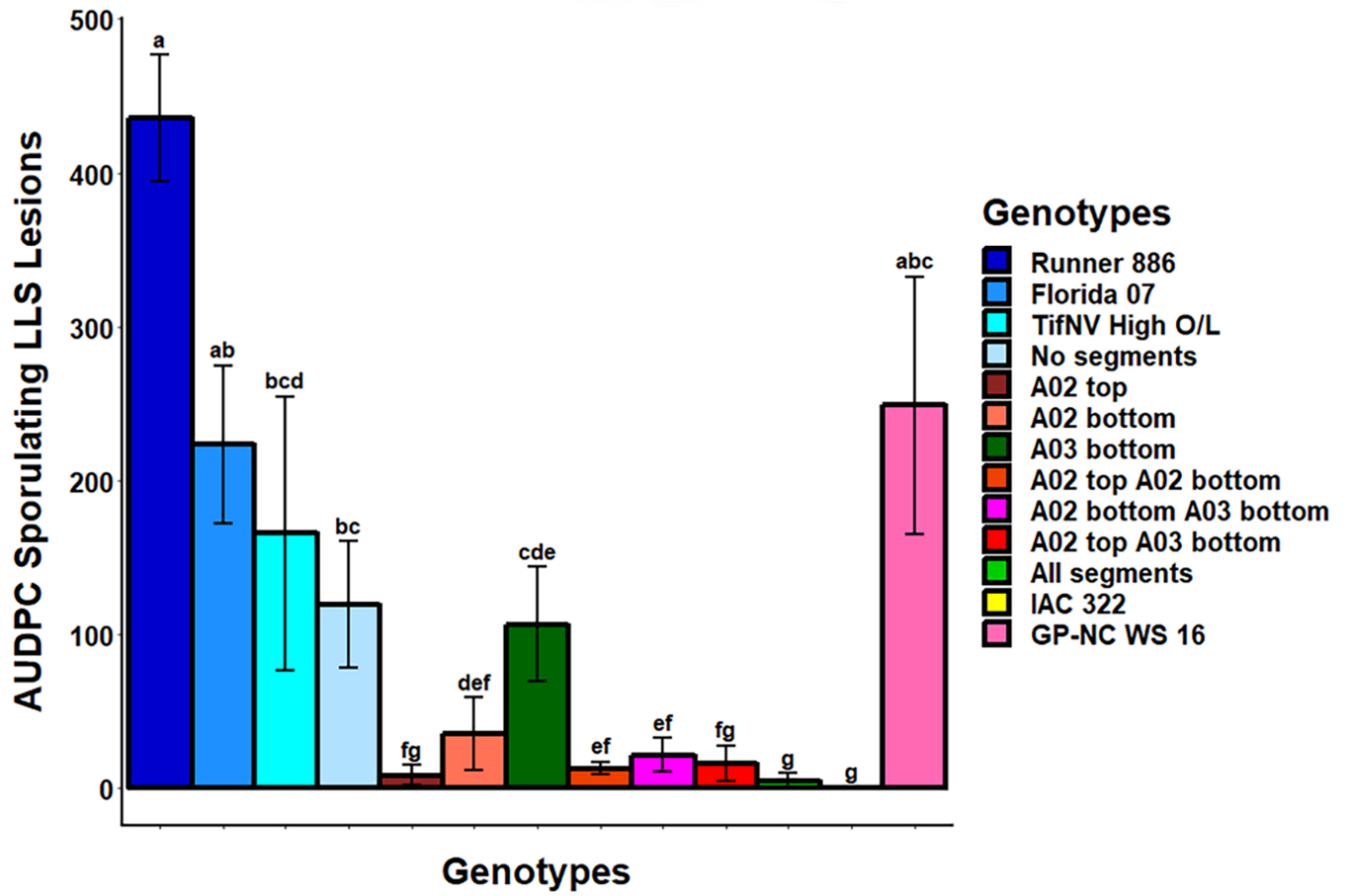

Figure 6: Genotype response measured by the AUDPC of sporulating LLS lesions for detached leaves.

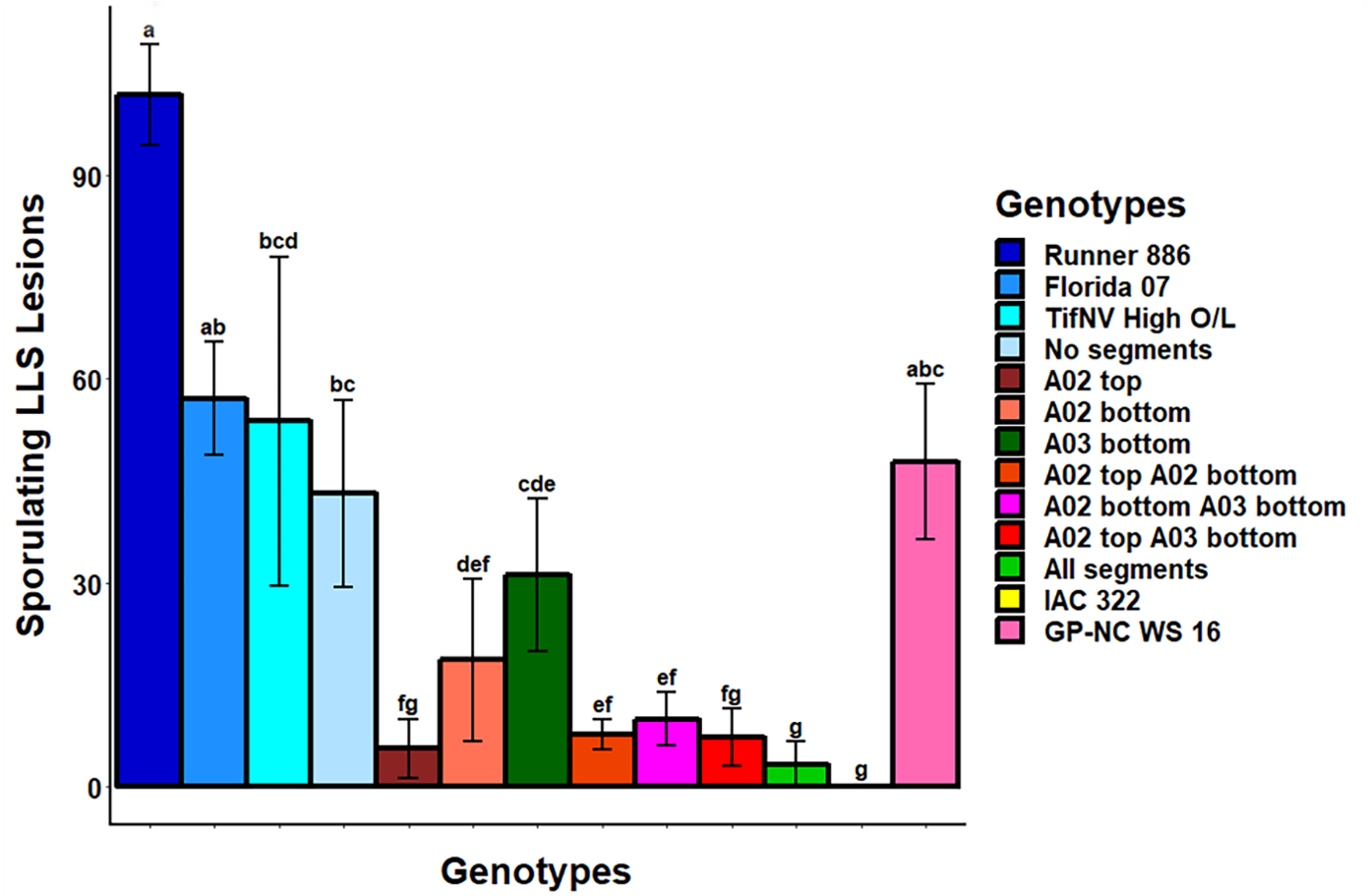

Figure 7: Genotype response measured by the number of sporulating LLS lesions the last day of data collection (20 DAI) for detached leaves.

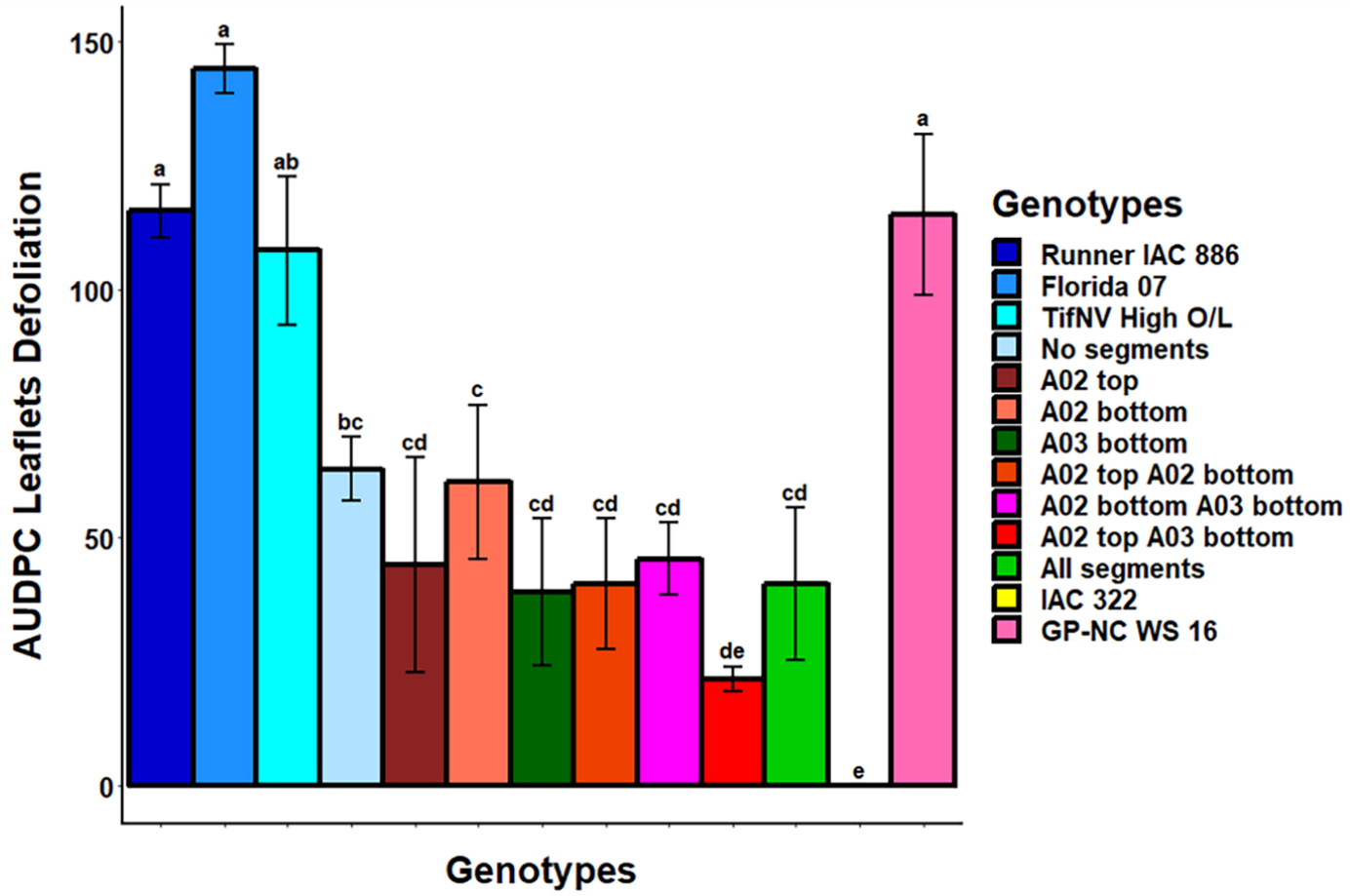

Figure 8: Genotype response measured by the AUDPC of leaflet defoliation for detached leaves.

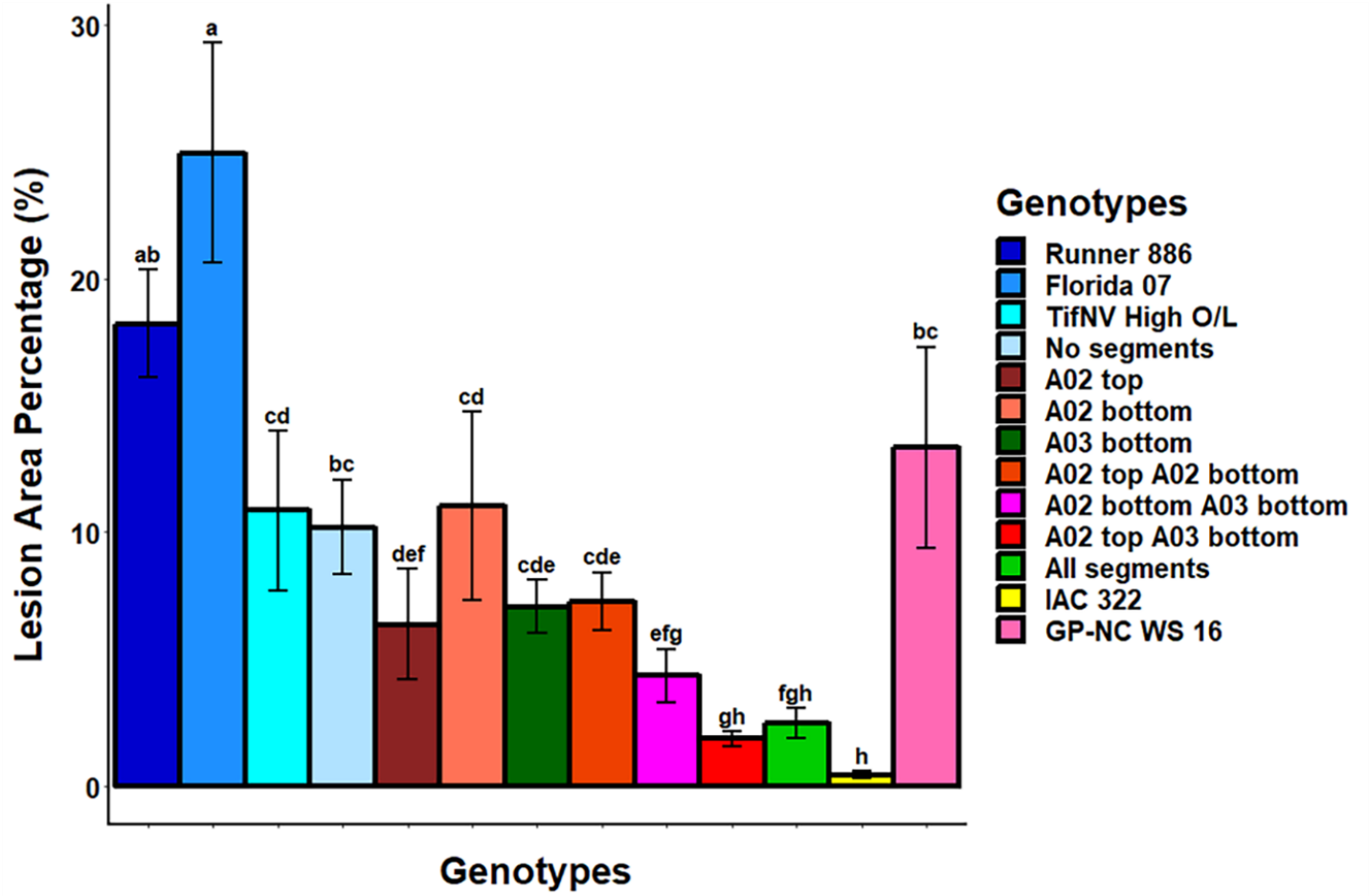

Figure 9: Genotype response measured by the leasion area percentage for detached leaves.

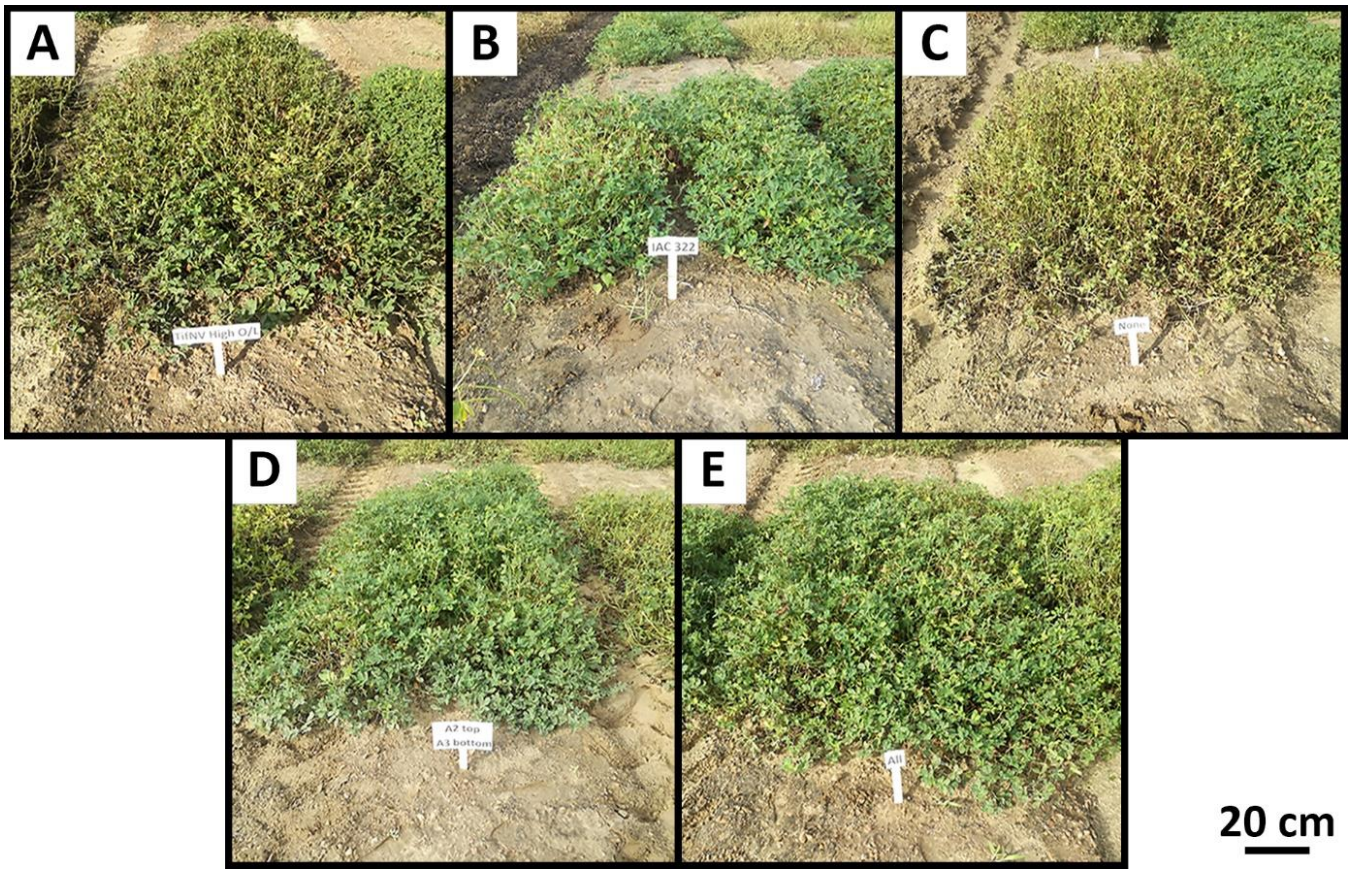

Figure 10: Gibbs farm field plots at 132 days after planting. The two population parents (TifNV-High O/L (**A**) and IAC 322 (**B**)) and the most representative progenies (No segments (**C**), A02 top A03 bottom (**D**) and All segments (**E**)).

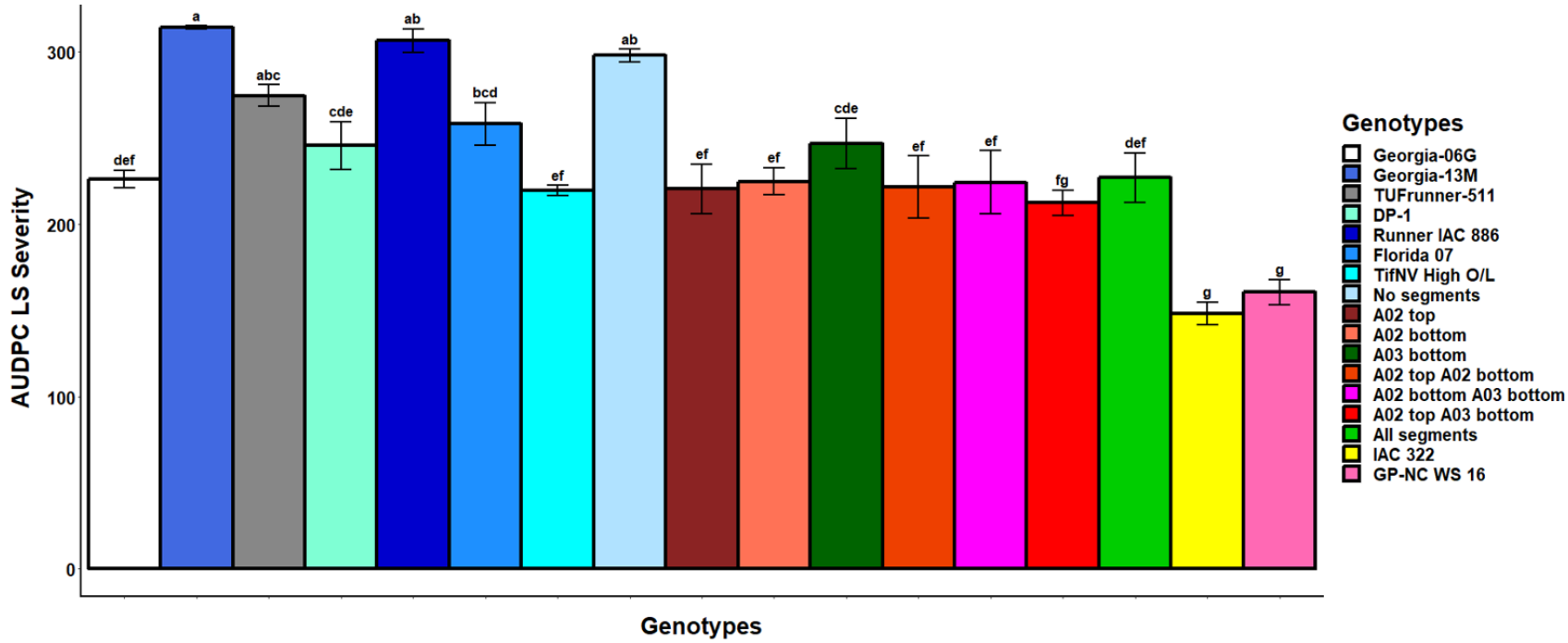

Figure 11: Genotype response measured by the AUDPC of leaf spot (LS) severity in the Gibbs farm field experiment.

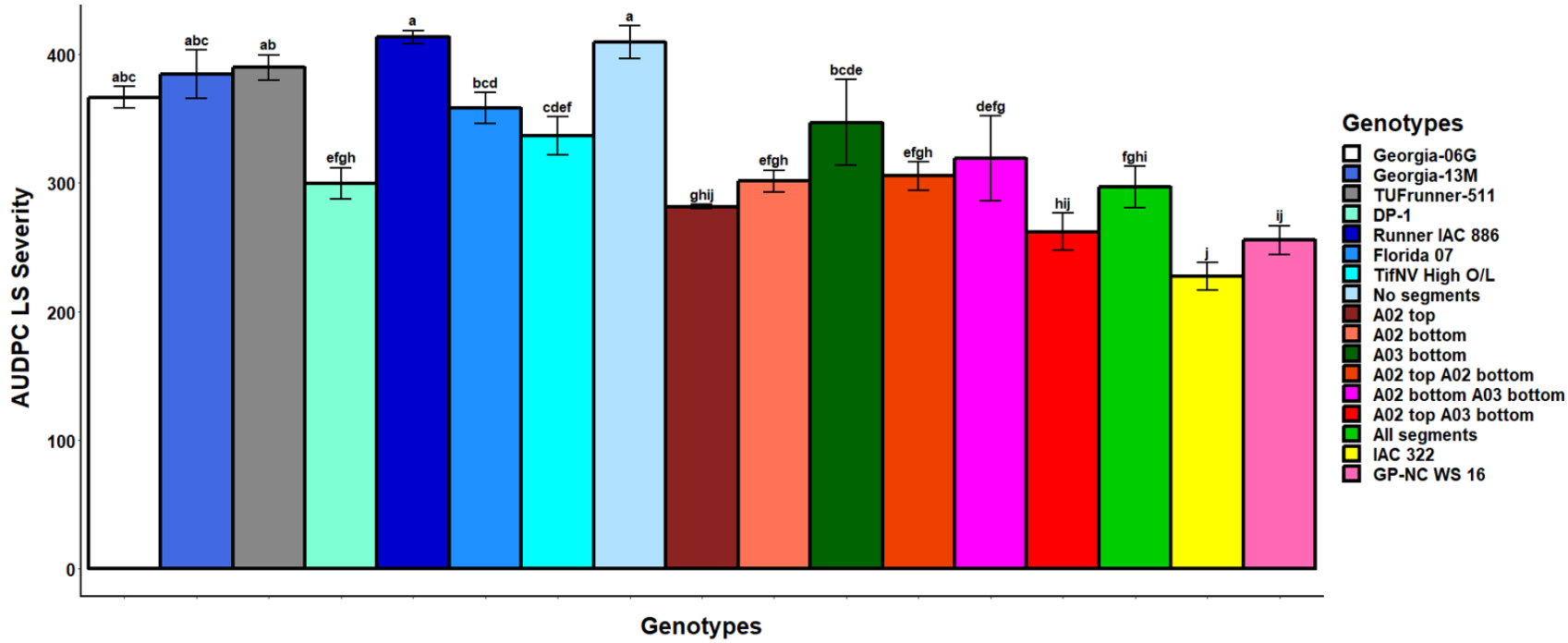

Figure 12: Genotype response measured by the AUDPC of LS severity in the Lang-Rigdon farm field experiment.

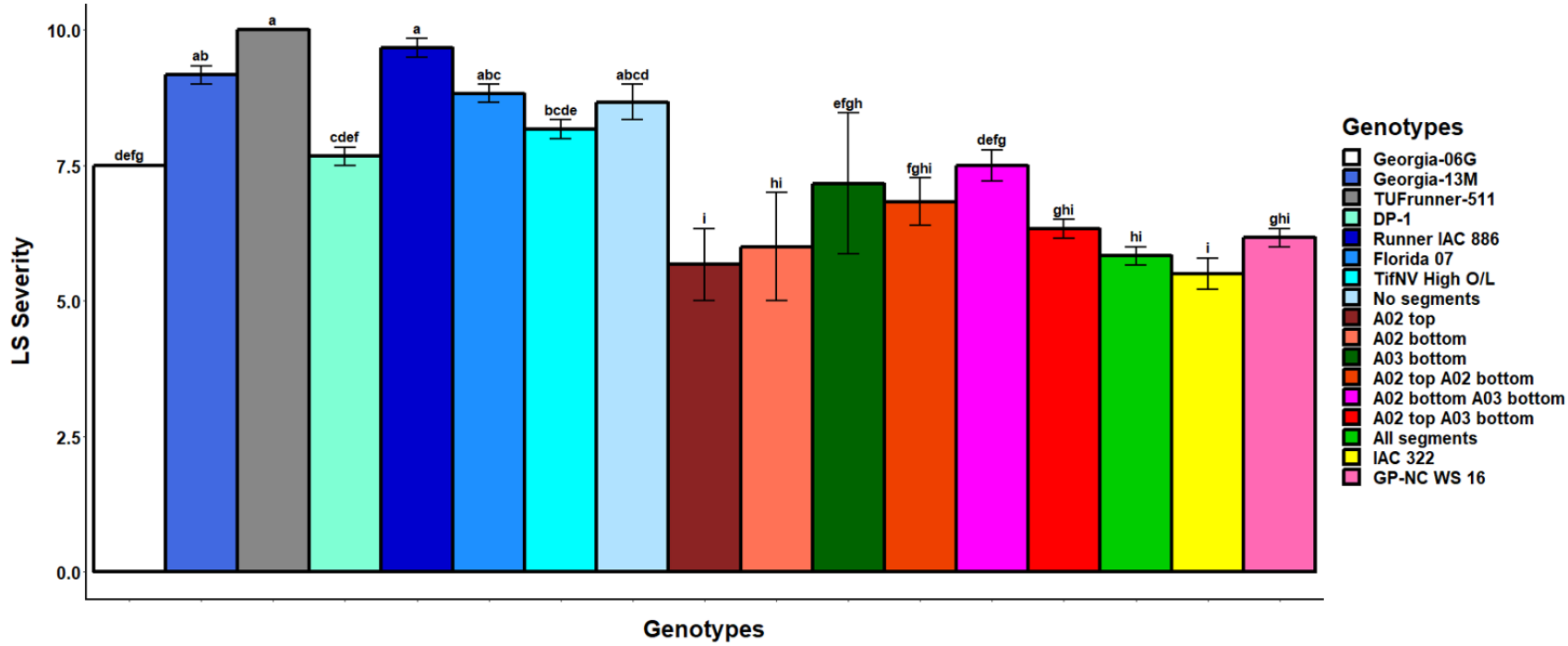

Figure 13: Genotype response measured by LS severity at the last day of data collection (132 DAP) for the Gibbs farm field experiment.

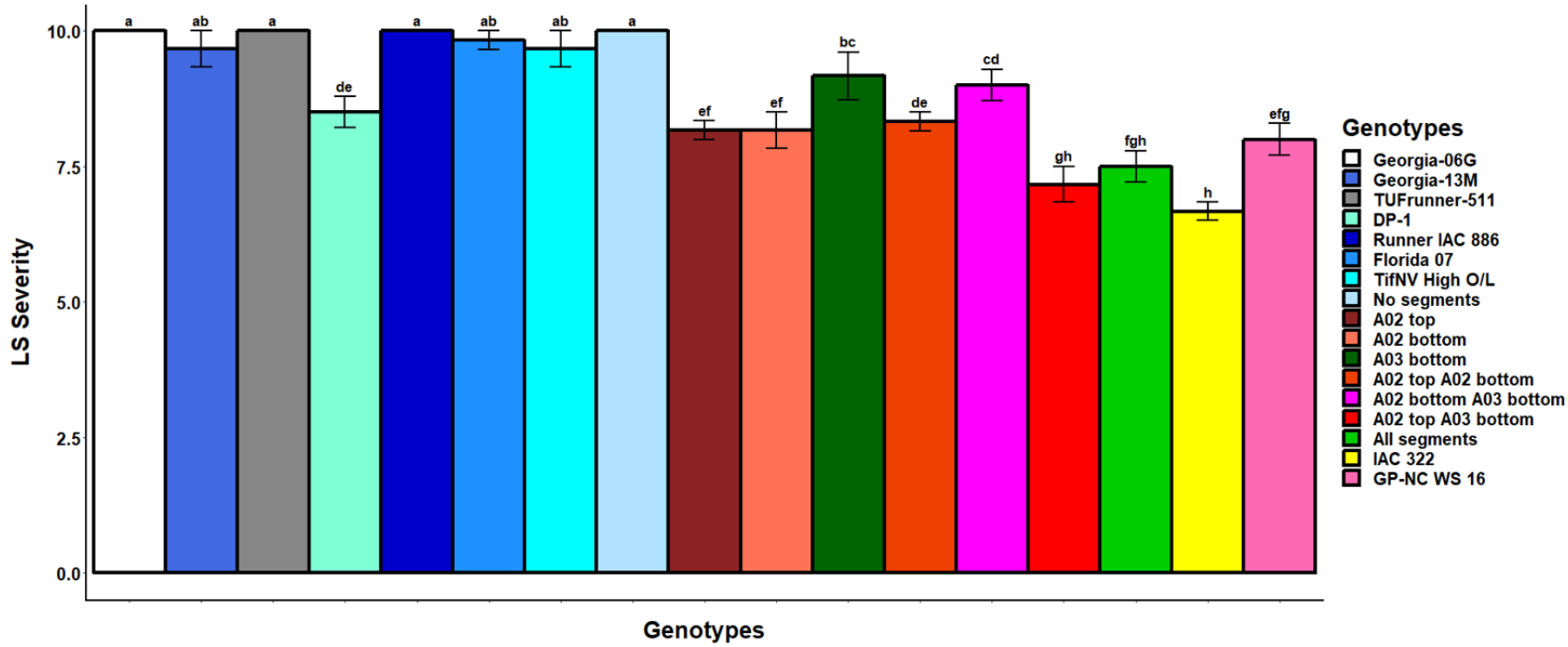

Figure 14: Genotype response measured by LS severity at the last day of data collection (127 DAP) for the Lang-Rigdon farm field experiment.

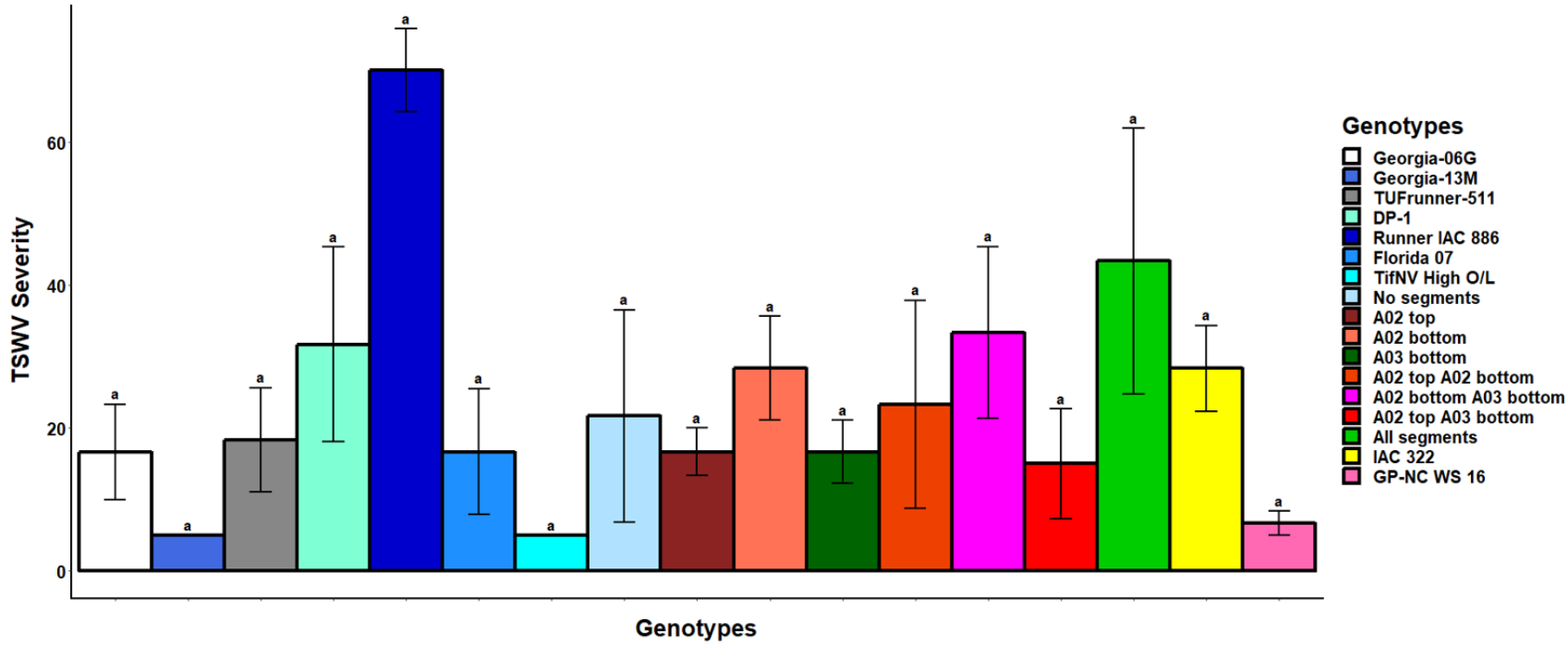

Figure 15: Genotype response measured by TSWV severity for the Gibbs farm field experiment.

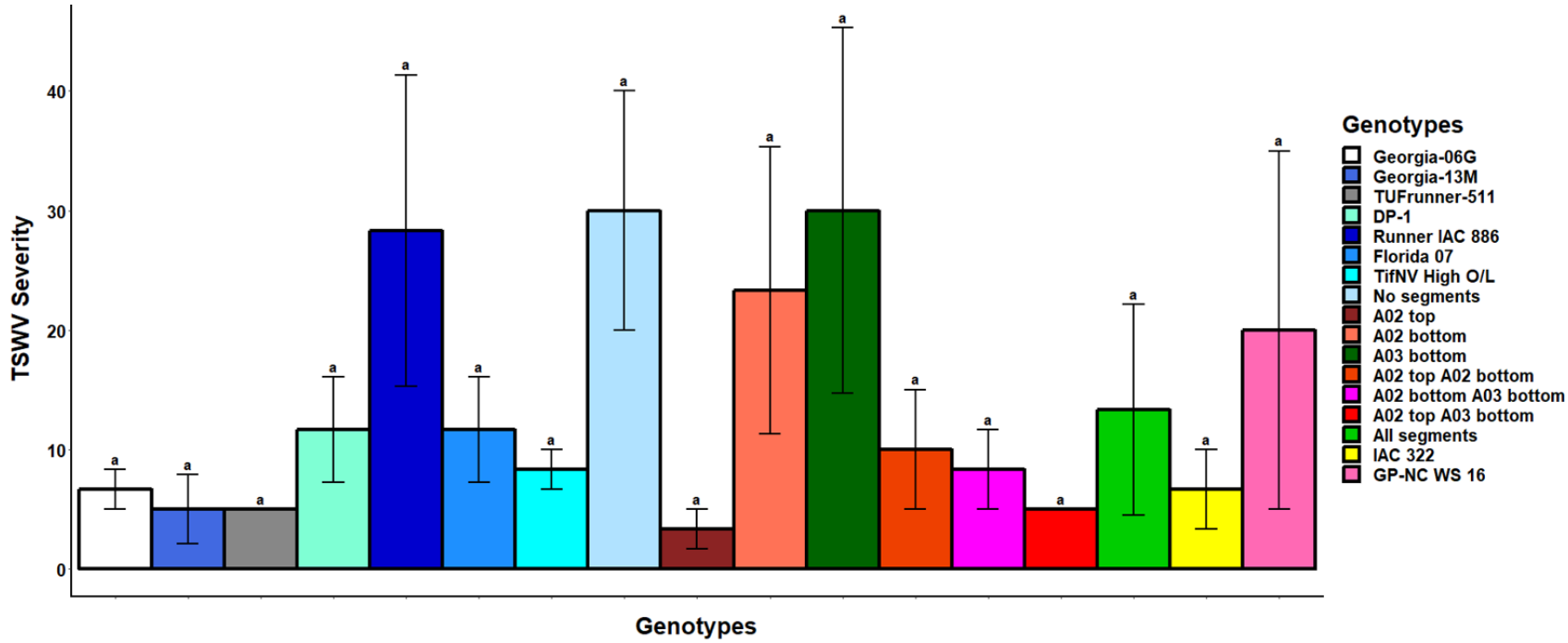

Figure 16: Genotype response measured by TSWV severity for the Lang-Rigdon farm field experiment.

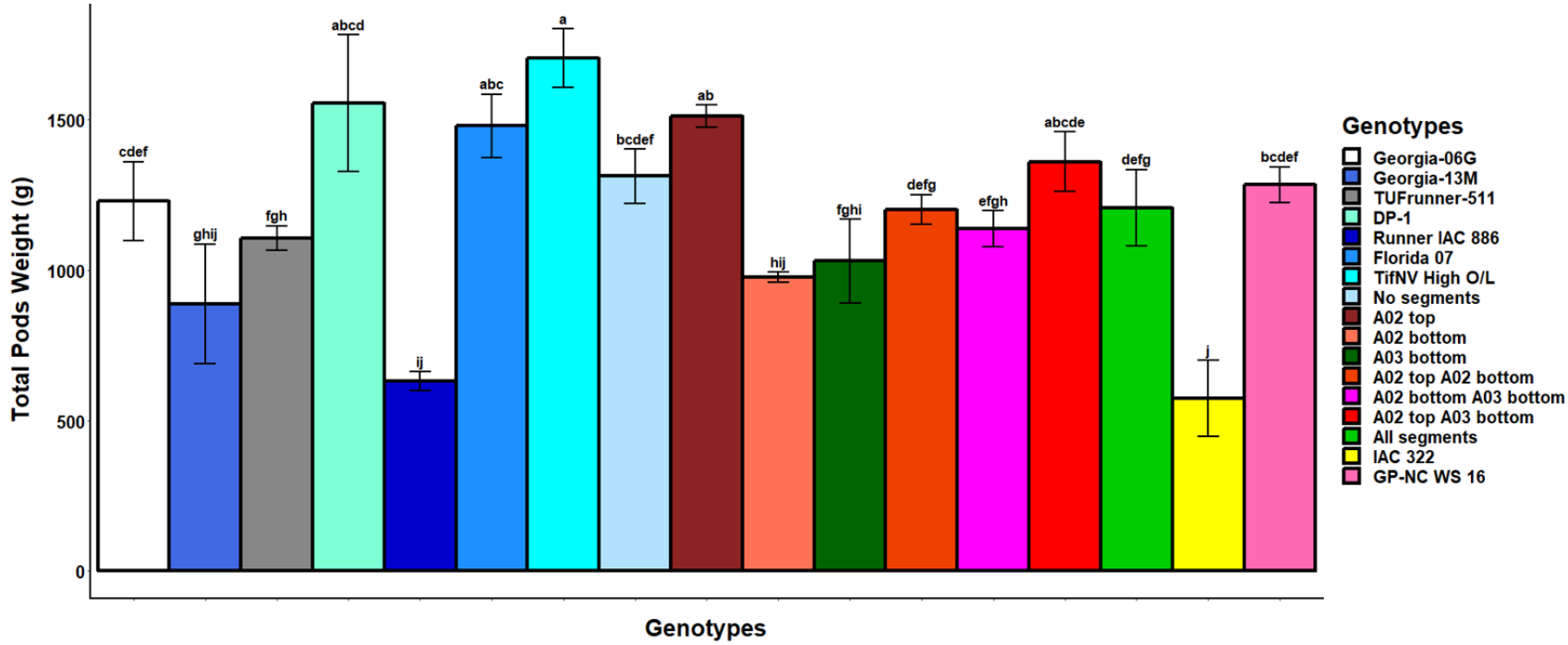

Figure 17: Genotype response measured by total pod weight in the Gibbs farm field experiment.

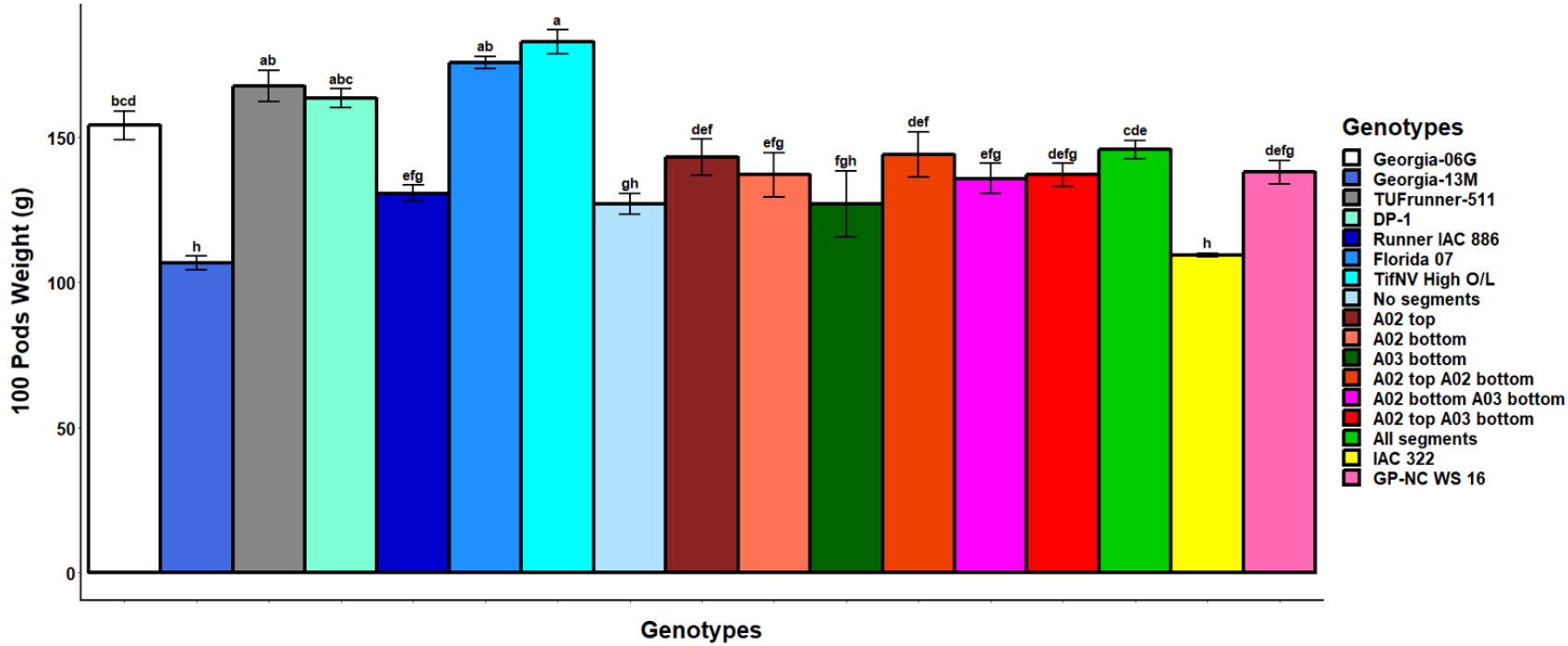

Figure 18: Genotype response measured by 100 pod weight for the Gibbs farm field experiment.

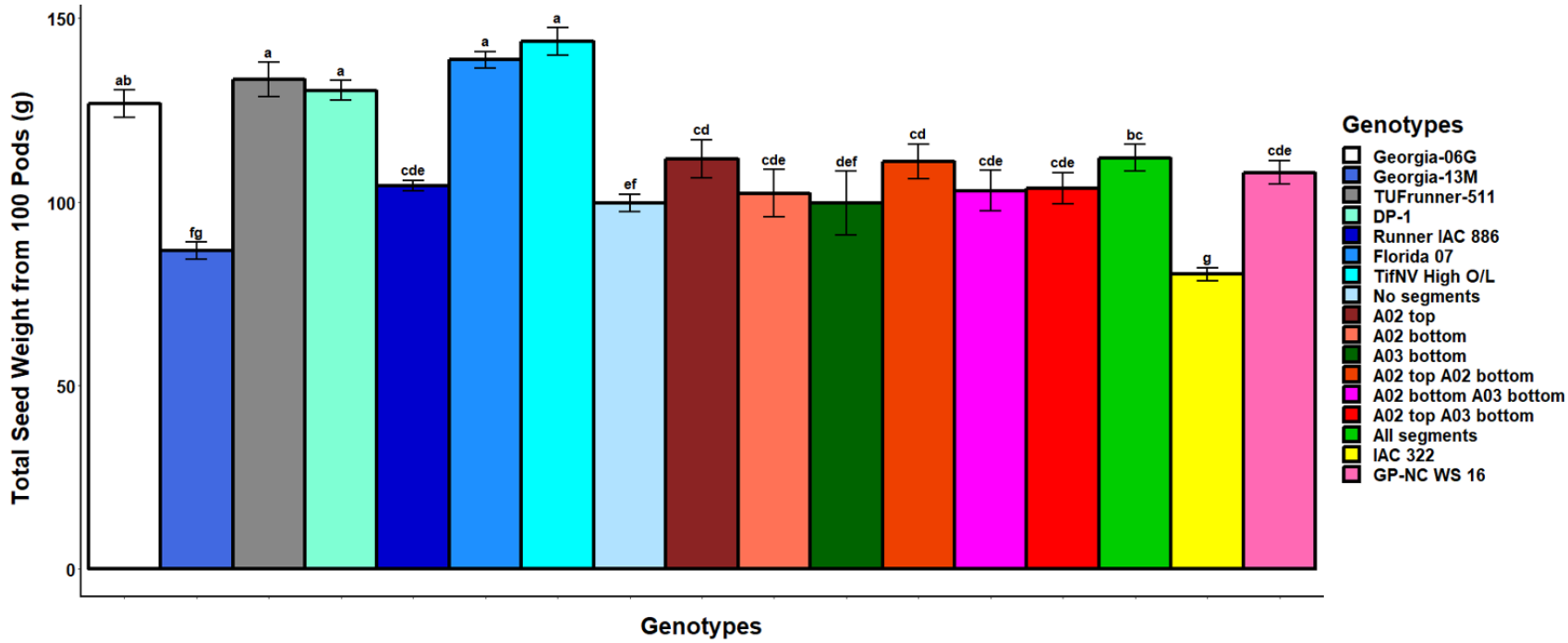

Figure 19: Genotype response measured by total number of seeds from 100 pods for the Gibbs farm field experiment.

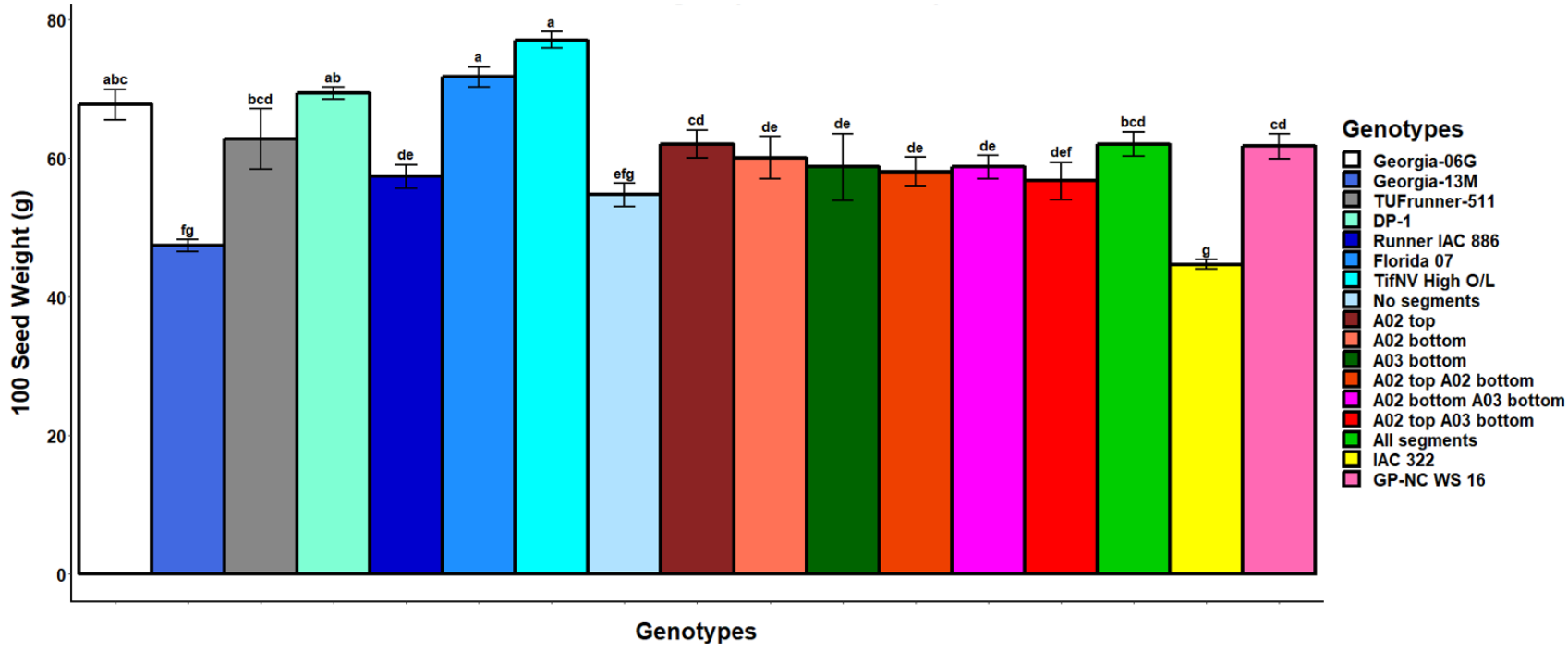

Figure 20: Genotype response measured by 100 seed weight for the Gibbs farm field experiment.

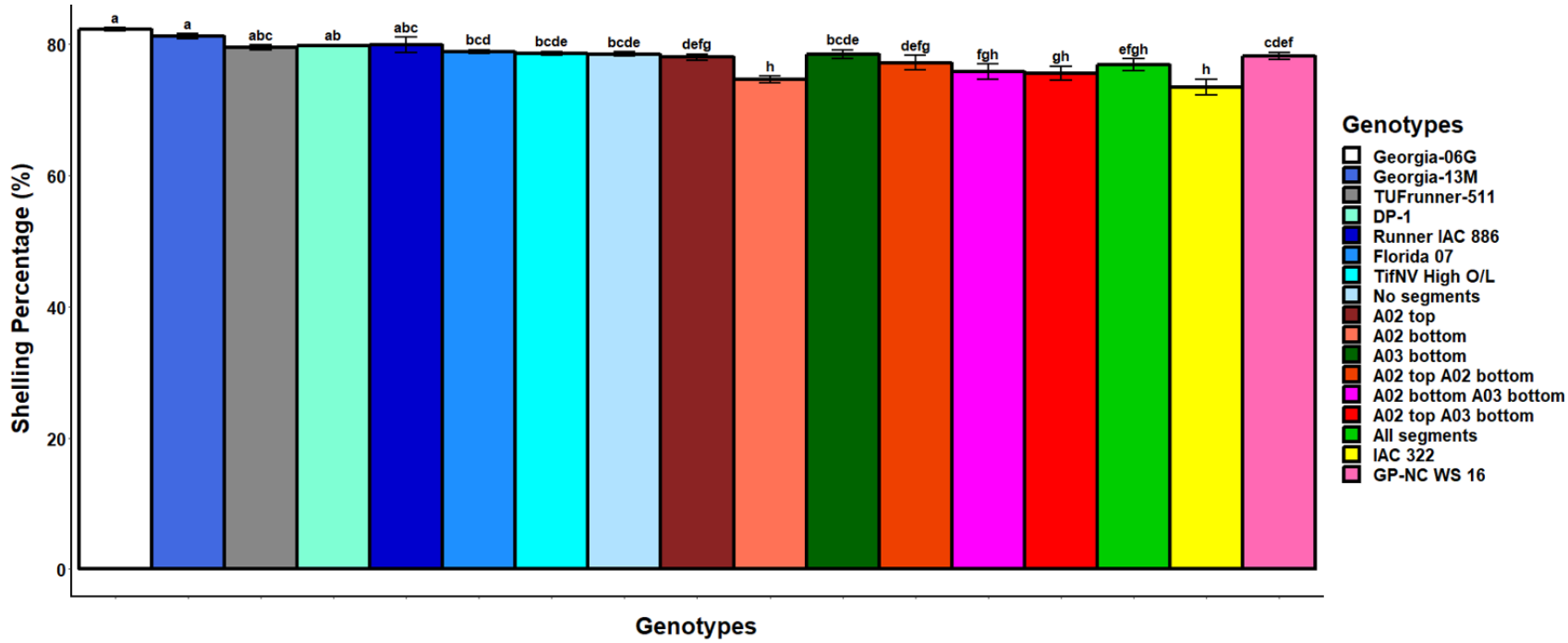

Figure 21: Genotype response measured by shelling percentage for the Gibbs farm field experiment.

CHAPTER 4

SUMMARY

The IAC 322 breeding line was characterized by high levels of LLS resistance and introgression of three *A. cardenasii* segments located in the top and bottom parts of chromosome A02 and bottom part of A03. The segments on the top part of chromosome A02 and bottom part of A03 accounted for most of the LLS resistance, while the segment in the bottom part of chromosome A02 was dispensable.

High, positive Pearson's r correlation values were detected between most LLS resistance traits in the detached leaf experiment such as lesions and sporulating lesions, defoliation and infected leaf area, although most were negatively correlated with incubation period. High, positive Pearson's r correlation values were recorded between LS infection progression at two different field locations and between detached leaf and field experiments. LLS lesions and sporulating LLS lesions at late stages of LLS infection and incubation period traits of the detached leaf experiment are the best candidate traits for predicting LLS resistance under field conditions. Furthermore, total LLS lesions and sporulating LLS lesions at late stages of LLS infection of detached leaves were representative of the entire LS infection progression.

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