STRATEGIES FOR ENHANCING LEAF SPOT (*Cercospora arachidicola* AND *Cercosporidium personatum*) TOLERANCE IN PEANUT (*Arachis hypogaea* L.)

By

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To my family and friends, who have no idea what I've been studying, but have enthusiastically supported me nonetheless

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| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| AFLP | Amplified Fragment Length Polymorphism |
| AUDPC | Area Under the Disease Progress Curve |
| BA | N6-Benzyladenine |
| DAP | Days After Planting |
| DSO | Direct Shoot Organogenesis |
| ELS | Early Leaf Spot (caused by *Cercospora arachidicola*) |
| Explant A | Explant derived from peanut cotyledon with previously attached embryo-axis |
| Explant B | Explant derived from embryo-axis-free peanut cotyledon |
| FAWN | Florida Automated Weather Network |
| GUS | β-Glucuronidase |
| ha | hectare |
| IPT | Isopentyl Transferase |
| LLS | Late Leaf Spot (caused by *Cercosporidium personatum*) |
| MS | Murashige and Skoog |
| MT | Metric Ton |
| NS | Non-Sprayed |
| PCD | Programmed Cell Death |
| PI | Plan Introduction |
| %RH | Percent Relative Humidity |
| RAPD | Random Amplification of Polymorphic DNA |
| RFLP | Restriction Fragment Length Polymophism |
| RIM | Root Induction Medium |

| | |
|---|---|
| S | Sprayed |
| SAG12 | Senescence Associated Gene 12 |
| SAGs | Senescence Associated Genes |
| SEM | Shoot Elongation Medium |
| SI% | Shoot Induction Percentage |
| SIM | Shoot Induction Medium |
| SIM10 | Shoot Induction Medium supplemented with 10 µM N6-Benzyladenine |
| SIM160 | Shoot Induction Medium supplemented with 160 µM N6-Benzyladenine |
| SIM20 | Shoot Induction Medium supplemented with 20 µM N6-Benzyladenine |
| SIM320 | Shoot Induction Medium supplemented with 320 µM N6-Benzyladenine |
| SIM40 | Shoot Induction Medium supplemented with 40 µM N6-Benzyladenine |
| SIM640 | Shoot Induction Medium supplemented with 640 µM N6-Benzyladenine |
| SIM80 | Shoot Induction Medium supplemented with 80 µM N6-Benzyladenine |
| SSR | Simple Sequence Repeat |
| TSWV | Tomato Spotted Wilt Virus |

Abstract of Thesis Presented to the Graduate School
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STRATEGIES FOR ENHANCING LEAF SPOT (*Cercospora arachidicola* AND
*Cerscosporidium personatum*) TOLERANCE IN PEANUT (*Arachis hypogaea* L.)

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*Cercospora arachidicola* S. Hori and *Cercosporidium personatum* (Berk and M. A. Curtis) Deighton are fungal pathogens that cause leaf spot, the most significant disease in peanut. Early leaf spot (*C. arachidicola*) and late leaf spot (*C. personatum*) are found in all peanut-growing regions worldwide. In Florida, if fungicides are not used, pod yields can be reduced by as much as 50% by these leaf spot diseases. The present research focused on developing novel strategies for improving leaf spot tolerance in peanut.

The first objective of this study was to confirm and characterize the source of suspected leaf spot tolerance in Florida-07. It was hypothesized that Florida-07 displayed classically defined tolerance. With regard to visual rating, lesion/leaf percentage, and lesion density, the rate of disease progression was the same in sprayed and non-sprayed York sprayed AP-3, and sprayed Florida-07. Similar disease progression was observed for non-sprayed AP-3 and non-sprayed Florida-07, but at a faster rate than the aforementioned cultivar*treatments. Lesion growth occurred at the same rate. Based on these data, it was concluded that Florida-07 and AP-3 possessed the same degree of susceptibility to late leaf spot disease. The impact of leaf spot on

pod yield of Florida-07 was similar to its impact on pod yield of AP-3 in two out of three tests, but in the third test, leaf spot impacted pod yield of Florida-07 (1084 kg ha$^{-1}$) less than it did AP-3 (1991 kg ha$^{-1}$) (P > t =0.0524).  On average, however, yield loss to leaf spot (sprayed minus non-sprayed) of AP-3 (1564 kg ha$^{-1}$) was not different than that of Florida-07 (1177 kg ha$^{-1}$). On average, Florida-07 does not appear to possess significant tolerance to leaf spot.

The second objective of this research was to optimize a peanut direct shoot organogenesis tissue culture system that had been optimized for an Indian cultivar, JL-24 (Sharma and Anjaiah, 2000) for U.S. cultivars. A difference in shoot induction was found for the cotyledon explants examined (P > t = <0.0001). Explant A had more shoot induction with a visual rating of 1.8, than explant B that had a rating of 1.6 (P > t = <0.0001).  Cultivars responded to the culture conditions differently (cultivar * BA interaction). Georgia Green on 10 µM BA produced the most shoot buds (24.56%) and had the highest visual rating (2.1), followed by VC-2 on 10 µM BA (22.1%, 1.8), Valencia-A on 640 µM BA (21.4%, 1.8), Georgia Brown on 80 µM BA (9.0%, 1.7), and Florida-07 on 40 µM BA (7.1%, 1.8). Georgia Green, VC-2, and Valencia-A appear to be the best suited for future *Agrobacterium*-mediated transformation experiments based on their shoot bud production.

The third objective of this research was to identify an *Agrobacterium* strain that was highly virulent for selected cultivars. Transient expression studies were conducted using a *CaMV35S-uidA* construct. It was hypothesized that a highly virulent

*Agrobacterium* strain could be identified by testing for *uidA* expression in cotyledon explants. It was concluded that *Agrobacterium* strain ABI was virulent and should be used for future stable transformation experiments.

CHAPTER 1
LITERATURE REVIEW

**Peanut as a Crop**

The cultivated peanut, *Arachis hypogaea* L., is a self-pollinating, indeterminate, annual herbaceous legume crop of global importance. Peanut's center of genetic diversity is believed to be in South America, specifically southern Brazil and northern Paraguay (Pattee and Young, 1982). During the sixteenth and seventeenth centuries, early Spanish and Portuguese explorers found indigenous people of Central and South America cultivating peanut. Subsequently, these explorers introduced peanut first to Europe and eventually to both African coasts, Asia, the Pacific Islands, and finally to North America. Currently, peanut is grown on six continents and in over 100 countries (Nwokolo, 1996).

The vast majority of the world grows peanut as a low input, small scale subsistence oilseed crop. Presently, it is the fifth most important oilseed crop in the world. Peanut oil is versatile and has been widely used as a bio-fuel, in cooking, and as a food constituent. However, in the U.S., peanut is used primarily as a food product for direct consumption, e.g. peanut butter, dry roasted nuts, and flour. Nutritionally, peanut is high in protein, as well as mono- and poly-unsaturated fats (e.g. linoleic and oleic acids). In many developing countries, peanut serves as a crucial dietary component for the indigenous people.

In 2007, an estimated 22,365,760 hectares (ha) of peanuts were harvested worldwide. China led the world in peanut production and value (13,079,363 metric tons (MT), Int. $6,112,785,000, respectively), followed by India (9,182,500 MT, Int. $4,205,879,000), Nigeria (estimated 3,835,600 MT, estimated Int. $1,778,082,000), and

the U.S. (1,696,728 MT, Int. $778,851,000) (FAO 2010). Although the U.S. does not lead the world in peanut production, it has ranked first in yield per land unit for over 15 years (Chenault et al. 2008). In 2009, 443,536 ha of peanuts were planted in the U.S. Georgia had the largest tract of land dedicated to peanut production (186,155 ha), followed by Alabama (68,797 ha), Texas (64,750 ha), Florida (48,562 ha), and North Carolina (30,351 ha). In 2009, the farm-gate level value of peanut production was $835,172,000, while the peanut industry, as a whole, generated approximately $4 billion for the U.S. economy. Georgia had the largest farm-gate level input toward value ($390,400,000), followed by Texas ($129,658,000), Alabama ($104,606,000), Florida ($69,552,000), and North Carolina ($66,911,000) (USDA NASS 2010). U.S. peanut production plays a major role in the overall economic prosperity of many rural production areas across the peanut growing regions.

## Peanut Morphology and Taxonomy

The peanut plant can be upright or prostrate in growth. At emergence, plants develop a main stem with many auxiliary lateral branches extending from the main stem. Leaves are alternate and compound, consisting of three to four leaflets. Botanically, peanut is unique among most other cultivated crops due to its geocarpic growth habit. Geocarpy is the production of aerial flowers but subterranean fruits. Peanut flowers are papilionaceous in appearance and contain both male and female reproductive parts (perfect flower). Natural cross-pollination of peanut is rare and breeding efforts require hand pollination. Post-pollination, flowers produce an elongated ovarian structure known as a gynophore or peg. The aerial peg grows vertically and penetrates the soil where the mature fruit (pod) develops.

*Arachis hypogaea* consists of two subspecies, *hypogaea* and *fastigiata*. The *ssp.* *hypogaea* does not flower on the main stem and, in general terms, matures later, has a high water requirement, an alternate branching pattern, and produces large seed. The *ssp. fastigiata* produces flowers on the main stem, has sequential branching, and, relative to the other subspecies, matures earlier, with a lower water requirement, and produces smaller seed. Subspecies can be further classified into six botanical varieties based on their morphology and growth habits (Krapovickas and Gregory 1994). Botanical varieties '*hypogaea'* and '*hirsuta'* belong to *ssp. hypogaea* while varieties '*fastigiata'*, '*peruviana'*, '*aequatoriana'* and '*vulgaris'* belong to ssp. *fastigiata*.

The four U.S. peanut market types fall within the botanical varieties *vulgaris*, *fastigata*, and *hypogaea*. Botanical variety *vulgaris* contains cultivars belonging to the Spanish market type, *fastigata* includes the Valencia market type cultivars, and *hypogaea* consists of Runner and Virginia market types. Market type forms a rough classification system which is primarily based on relative pod and seed size characteristics (small, medium, and large), and to a lesser extent on growth habit, growing region, and center of genetic origin (Pattee and Young 1982; Knauft et al. 1987).

Cultivars classified as Spanish market types typically have small, two seeded pods containing small seeds. The genetic origin of Spanish market types is the Guarani region of northeast Argentina, Paraguay, and southern Brazil. In the U.S., Spanish market types are generally grown in the southwestern portion of the peanut producing region (Texas, and Oklahoma), and their seeds are used primarily in candy and for oil.

Valencia market types typically have medium two- and three-seeded pods containing medium sized seed and originated in Paraguay and central Brazil. This market type is grown primarily in the southeastern producing region (Georgia, Alabama, and Florida). Valencia peanuts, especially the three-seeded type, are whole roasted and boiled as snack foods.

The center of origin for Runner and Virginia market type peanuts is unclear. The precursor to these market types originated in South America, but may have arisen, as we know them today, while being grown in Africa. Runner and Virginia type peanuts tend to have larger pods and seeds compared to Spanish and Valencia peanuts. However, Virginia type peanuts have larger pods and seeds compared to Runner type pods and seeds. Runner type peanuts are most widely grown in the southeastern growing region of the U.S. and are used for oil and peanut butter production. Virginia types are primarily grown in the northeastern peanut producing region (Virginia, and North Carolina) for use as whole roasted, "ball park" nuts.

## Peanut Genetic Diversity

Within the genus *Arachis, A. hypogaea* is the only species that has been domesticated and grown worldwide. Despite extensive morphological and physiological variation, many studies have concluded that *A. hypogaea* has low genetic diversity. These studies have used pedigree analysis (Knauft and Gorbet 1989), protein profiles (Singh et al. 1991b, 1994), isozymes (Grieshammer and Wynne 1990; Lacks and Stalker 1993; Lu and Pickersgill 1993; Stalker et al. 1994), restriction fragment length polymorphism (RFLP) (Galgaro et al. 1998; Garcia et al. 1995; Halward et al. 1991, 1993; Kochert et al. 1991, 1996; Paik-Ro et al. 1992), and random amplification of polymorphic DNA (RAPD) (Halward et al. 1992; Lanham et al. 1992; Garcia et al. 1995;

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Galgaro et al. 1998; Subramanian et al. 2000; Raina et al. 2001) but have found low

levels of polymorphism. Additional studies have identified more polymorphism using

amplified fragment length polymorphism (AFLP) (He and Prakash 1997, 2001;

Herselman 2003) and simple sequence repeat (SSR) (Hopkins et al. 1999; Raina et al.

2001; Tang et al. 2007) techniques. However, the genetic diversity that exists in

domesticated peanut remains narrow when compared to other important crops.

Because most *Arachis* species are diploid, with the exception of *Arachis monticola*

Krapov. and Rigonc., they do not readily cross with tetraploid *A. hypogaea*. The limited

genetic diversity found in cultivated peanut is most likely due to a relatively recent,

single hybridization event between wild, diploid *Arachis* species (Halward et al. 1991).

This narrow genetic base in peanut has been further compounded by the self-pollinating

nature of peanut and breeding programs using very few elite breeding lines (Herselman

2003).

As mentioned above, peanut is a tetraploid, specifically an allotetraploid ($2n = 4x =$

$40$), containing two distinct A and B genomes. Genome A has a set of chromosomes

that is significantly smaller when compared to the chromosomes of the B genome

(Husted 1936). Of the approximately 70 known *Arachis* species, only a few possess the

B genome, which limits the number of candidate parent *Arachis* species (Smartt et al.

1978; Gregory et al. 1980). Morphology, chromosome pairing, cross compatibility, and

molecular markers have been used to identify likely progenitors of cultivated peanut.

Several studies point to *Arachis cardenasii* Krapov. and W.C.Greg.*, Arachis villosa*

Benth.*, Arachis correntina* (Burkart) Krapov. and W.C. Greg., or *Arachis duranensis*

Krapov. and W.C. Greg as being likely A genome donors (Seetheram et al. 1973;

Gregory and Gregory, 1976; Smartt et al. 1978; Singh and Moss 1982; Kirti et al. 1983; Murty and Jahnavi 1986; Singh, 1988; Kochert et al. 1991, 1996; Singh et al. 1996; Raina and Mukai 1999) and *Arachis batizocoi* Krapov. and W.C. Greg or *Arachis ipaensis* Krapov. and W.C. Greg as being B genome donors (Smartt et al. 1978; Singh and Moss 1984; Singh, 1988; Klosova et al. 1983; Kochert et al. 1991, 1996; Fernandez and Krapovickas 1994). Studies conducted by Kochert et al. (1996), Seijo et al. (2004, 2007) and Favero et al. (2006) propose that *A. duranensis* and *A. ipaensis* are the likely progenitors of peanut. Currently, this theory is the most commonly accepted one. As technologies improve and whole genome sequencing becomes more efficient and affordable, additional polymorphisms (e.g. single nucleotide polymorphisms) should be identified, and that along with a better understanding of epigenetic effects should help explain the morphological and physiological diversity observed in cultivated peanut.

## Peanut Diseases

Peanut is susceptible to a variety of biotic stressors. In the U.S., several foliar and soilborne diseases/pests exist that lower yields, as well as profits for growers. Domestically, the most prevalent pathogens/pests of peanut include tomato spotted wilt virus (TSWV; Tospovirus vectored by thrips), root-knot nematode (*Meloidogyne arenaria* (Neal) Chitwood race 1), *Sclerotium rolfsii* Sacc., the casual agent of white mold, *Cylindrocladium parasiticum* Crous, Wingfield and Alfenas, the casual agent of *Cylindrocladium* Black Rot, *Sclerotinia minor* Jagger, that results in *Sclerotinia* blight, *Puccinia arachidis* Speg., that causes rust, and *Cercospora arachidicola* S. Hori and *Cercospiridium personatum* (Berk and M. A.Curtis) Deighton, that are the casual agents

of early and late leaf spot. In addition to yield, seed vigor and grade, disease/pest resistance is a primary breeding objective for peanut breeding programs throughout the U.S.

## Peanut Leaf Spots

Early leaf spot (ELS) (teleomorph *Mycosphaerella arachidi* Deighton) and late leaf spot (LLS) (teleomorph *Mycosphaerella berkeleyi* Jenk.] diseases are the most widespread foliar diseases of peanut. Both *C. arachidicola* and *C. personatum* can be found wherever peanut is grown, making them the most significant of all peanut pathogens (Zhang et al. 2001). If fungicides are not used, pod yields can be reduced by 50% or more in diseased plants (Knauft et al. 1986, Pixley et al. 1990ab, Shokes et al. 1983, Damicone et al. 1994, Smith and Littrell 1980, Zhang et al. 2001).

### Identification and Classification

During the early production years of peanut, leaf spots were regarded as a common and natural feature of the peanut plant (Backman et al. 1977). The first documented description of an organism causing peanut leaf spot was by Berkley (1875). Berkley identified a single fungal species and proposed the name *Cladosporium personatum* as being the source of leaf spot disease. Studies following the work of Berkley led to a highly variable nomenclature and classification system for leaf spot disease. Comparison of specimens and earlier reports by Woodruff (1933) led to the determination that the casual agent of leaf spot disease was actually due to two distinct fungal organisms. The two pathogens were identified and then named, *Cercospora arachidicola* Hori and *Cercospora personata* (Berk. and Curt.) Ellis and Everhart.

The sexual stages for each pathogen were later identified by Jenkins (1938) and named *Mycosphaerella arachidicola* (ELS) and *Mycoshaerella berkeleyii* (LLS). *Cercospora personata* was later re-classified by Deighton (1967) as belonging to the genus, *Cercosporidium*. Deighton re-named the pathogen to *Cercosporidium personatum* (LLS).

**Symptoms and Signs**

ELS and LLS diseases are characterized by necrotic flecks that enlarge to necrotic lesions that reduce light interception and photosynthesis (Boote et al. 1983). Lesions caused by either disease can occur on pegs, stems, or petioles, but are most commonly found on leaves (Hemingway, 1954; Gibbons, 1966). Lesion appearance on leaves infected by *C. arachidicola* and *C. personatum* can differ slightly. ELS disease produces tan to reddish-brown to black foliar lesions that are typically, but not always, surrounded by a distinct yellow halo (frog-eye). Because the yellow halo is not always indicative of ELS, conclusive identification can only be made by microscopically examining conidiophores/conidia. In ELS, conidiophores form on the upper leaf surface within the lesion covered area and conidia are often sparsely present or not present at all. LLS disease, on the other hand, produces brown to black lesions with no halo ever being present. However, similar to ELS, conclusive identification can only be made by microscopic examination of conidiophores/conidia. The formation of *C. personatum* conidiophores/conidia is far more prolific than *C. arachidicola*. Conidiophores of *C. personatum* tend to be densely packed into lesions with numerous conidia being present.

Regardless of lesion appearance, lesions caused by the presence of either *C. arachidicola* or *C. personatum* have the same effect of reducing photosynthetic activity

in leaf tissue, as mentioned above. The reduction of photosynthetic leaf area is the primary factor associated with loss of yield in peanut. Pre-mature defoliation (due to early onset of senescence mechanisms), another symptom associated with both leaf spot pathogens, of course, further compounds the reduction of active photosynthetic area.

**Disease Cycle**

*C. arachidicola* and *C. personatum* are very similar in respect to their life cycles. Both produce conidia and mycelia that are capable of overwintering in crop residue. They are necrophilic, thriving on the dead cells and tissues of the host. Conidial-spores and mycelia overwintering in crop residue provide the inoculum source for the following season's initial infection.

Infection begins when conidial-spores germinate and form germ tubes that penetrate open stomata or lateral faces of epidermal cells. Following penetration, germ tubes form into networks of mycelia. These mycelia produce cellulolytic and pectolyic enzymes, i.e., dothistromin (Stoessl, 1984) and/or cercosporin, which diffuse and degrade host cell wall and middle lamellae constitutients. Intercelluar hyphae of *C. arachidicola* have been shown to kill host cells in advance of hyphal penetration (Alabi and Naqvi, 1977; Stoessl et al. 1990; Daub et al. 2000). Conversely, *C. personatum* does not kill prior to penetration, but instead develops into haustoria. As mycelia spread into host tissues and enzymatic degradation occurs, cells collapse and produce necrotic lesions (Abdou et al. 1974; Jenkins, 1938). In addition to their degradative properties, enzymes produced by these pathogenic fungi have also been shown to promote ethylene production, enhancing the rate of leaf abscission (Bourgeois et al. 1991).

Sporulation of these organisms is characterized by the formation of long, thin multicellular conidia on short, darkly pigmented conidiophores (Agrios, 2005). Conidia and conidiophores for both organisms are very similar in appearance. Conidia are easily detached and can be dispersed by wind, water, or any other mechanical movement. *C. arachidicola* and *C. personatum* favor warm temperatures and are most destructive during the summer months in warmer climates, such as those found in the southeastern peanut growing states (e.g., Alabama, Florida, Georgia, Mississippi, and South Carolina) (Culbreath et al. 2009). Development and dispersal of conidia of both pathogens are most prevalent in temperatures ranging from 16°C - 30°C and relative humidity exceeding 90%. High temperatures and leaf wetness, either due to humidity or rainfall, are necessary for the rapid growth and widespread dispersal of leaf spot disease (Jensen and Boyle, 1965; Alderman and Beute, 1986; Shew, 1988; Jacobi et al. 1995a, b).

**Management Strategies**

Current management strategies for controlling leaf spot epidemics rely heavily on foliar fungicide application, crop rotation, tillage, planting date, and cultivar selection (Wright et al. 2009; Cantonwine et al. 2006, 2007a; Zhang et al. 2001).

**Foliar fungicide application**. Numerous reports are available describing the successful control of leaf spot diseases using fungicides. Without the use of fungicides, commercial peanut cultivation would not be practical. Disease control for the 2010 growing season has been estimated to be approximately $216/ha, with a large portion of that amount going toward fungicides for leaf spot control (Smith and Smith, 2009). Annually, purchasing and applying fungicides is one of the most expensive investments for a grower. As previously mentioned, without fungicides, peanut yields may be

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reduced by more than 50%, which is unacceptable if one is trying to make a profit.

Foliar fungicide products commonly used on peanuts include sulfur, tebuconazole, propiconazole, chlorothalonil, trifloxystrobin, pyraclostrobin, and azoxystrobin. Less commonly used fungicides include copper, maneb, mancozeb, thiophanate, boscalid, iprodione, fluazinam, prothioconazole, and phoshite (Mossler and Aerts, 2007). Current recommendations call for fungicides to be applied every 10 - 14 days beginning 30 - 35 days after planting (DAP) (Wright et al. 2009). As a result, typically seven or more applications are made during a growing season. Additionally, it is recommended that multiple fungicides with different modes of activity be used throughout the growing season, to avoid the development of fungicide-specific, resistant strains. With the use of fungicides, leaf spot control may approach 100%, but on average, growers can expect 60 - 70% protection from recommended fungicide applications (Culbreath et al. 2009).

**Crop rotation**. Rotation has long been recognized as one of the most effective means of controlling disease in any crop. Crop rotation provides a time period for degradation of crop debris, which in turn deprives any surviving inoculum of host tissues. After foliar fungicide applications, crop rotation is the next most important management practice for reducing leaf spot pressure (Culbreath et al. 2009).

Unfortunately, in the southeastern U.S., low value crops are generally the only alternative for rotating with high cash value crops like peanut (Wright et al. 2009). Due to the discrepancy in crop-value, many growers have opted to continually grow peanuts in the same fields. Current extension recommendations suggest rotation with non-leguminous crops such as cotton, corn, sorghum, or bahiagrass. Rotating these crops with peanut will reduce disease pressure and thereby result in higher yields (Culbreath

et al. 2009; Wright et al. 2009). In fact, peanut yields were 19% higher after two years of

corn and 41% higher after two years of bahiagrass (Wright et al. 2009). Mossler and

Aerts (2007) reported that a rotation interval of three to four years will further reduce

disease pressure and increase yields.

**Tillage**. Because *C. arachidicola* and *C. personatum* are necrophilic and survive

from season to season on crop debris, tillage will create a soil layer (physical barrier)

preventing fungal inoculum from coming into contact with new growth. Conventional

tillage of peanut involves turning the soil in an entire field. Recently, the increased cost

of fuel has led to the investigation of conservation tillage methods. A particularly

effective conservation method is strip tillage, which differs from conventional tillage in

that the entire field is not turned. Rather, a narrow strip of planting area (8 - 12" wide) is

sub-soiled (inversion of top soil) (Wright et al. 2009). Although the exact mechanism is

unclear, leaf spot appearance is delayed and late-season pressure is less severe in

strip-tilled peanut fields (Cantonwine et al. 2007b; Culbreath et al. 2009). Because of

the reduced time investment, cost, and incidence of disease, strip tillage has been

regionally adopted in the southeastern states by some peanut producers.

**Planting date**. Peanuts planted in early- to mid-April generally have less leaf spot

pressure than those planted later in mid-May to early-June. Peanuts planted during the

earlier months have less exposure time to hot, humid conditions which are most

conducive for pathogen development. Fungicide applications in early planted fields

(mid-April) can be delayed to 60 DAP (Mossler and Aerts, 2007). However, this

advantage is overcome in early planted peanuts because they are more susceptible to

outbreaks of white mold and TSWV (Culbreath et al. 2009). Although first identified in

the early 1980s in the southeastern U.S. growing region, during the mid-1990s, TSWV severity became more prevalent. To avoid significant TSWV damage, planting dates were shifted later in the season and this increased leaf spot pressure. Current recommendations call for the use of environmental modeling systems to determine planting dates.  Ideally, planting will occur late enough in a season to avoid TSWV damage, but early enough to avoid the most conducive leaf spot environment.

**Cultivar selection**. In a typical growing season in the southeastern U.S. peanut growing region, it can be expected that leaf spot will be the most severe disease encountered. Breeding programs have invested a great deal of effort in developing leaf spot resistant cultivars. Breeding for leaf spot resistance has led to the release of several cultivars with negligible lesion coverage, reduced defoliation, and high yield potential. Some cultivars possess enough resistance to reduce fungicide spray regimes. Resistant cultivars provide financial protection to growers because less investment is required for chemical fungicides/applications and final yield potential is protected.

Several peanut cultivars have been released that are classified as "resistant" to ELS and/or LLS disease(s). These cultivars include Georgia-01R (Branch, 2002), Tifrunner (Holbrook et al. 2007), Georgia-02C (Branch, 2003), Georganic (Holbrook et al. 2008), Georgia-07W (Branch et al. 2008), Southern Runner (Gorbet et al. 1987), York, DP-1 (Gorbet and Tillman, 2008), C99-R (Gorbet, 2002a), Hull (Gorbet, 2007b), and Florida MDR-98 (Gorbet, 2002b). Although classified as resistant, the degree of protection in many of these cultivars is incomplete and still allows for significant damage under severe disease pressure.  Additionally, several of these cultivars are associated with characteristics that have hindered their wide-spread acceptance among growers,

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such as poor germination, late maturity, and large seed size. For example, when

multiplied by commercial seed producers, York, DP-1, C-99R, Hull, and MDR-98 often

exhibit poor field emergence.  Poor field emergence results in unacceptable field stands

that in turn affect final yield (Morton, 2007). Additionally, the development of leaf spot

resistant, Runner-type cultivars have typically been limited to cultivars with late maturity

(maturity reached 14 - 21 days after other Runner-types), and these cultivars tend to

have larger seed size which presents problems to shelling facilities and has further

contributed to the limited acceptance of such cultivars. The unfavorable characteristics

associated with many leaf spot resistant cultivars may be due to a common parent in

their lineage, plant introduction (PI) 203396, which is the primary source for superior

leaf spot resistance. PI 203396 is one of only a few peanuts that consistently results in

progeny with high leaf spot resistance, consequently the genetic diversity available for

leaf spot resistance is narrow.

## Breeding for Leaf Spot Resistance

Peanut breeding in the U.S. began in Florida during the 1920s (Tillman and

Stalker, 2009). Since that time, breeding efforts have led to drastic improvements in

peanut performance. The University of Florida has led breeding efforts over the past 30

years to develop leaf spot resistant cultivars. Breeding methods in peanut are similar to

that of other self-pollinating crops. Pedigree selection, single seed descent, and mass

selection are all common strategies for improvement. In terms of breeding for leaf spot

resistance, the major hurdle encountered is the lack of genetic diversity available, as

previously mentioned. Southern Runner was the first cultivar to be released with

resistance to leaf spot. Cultivars with a genetic background similar to Southern Runner

have been recently released: York, DP-1, C99-R, Hull, and Florida MDR-98. Along with

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leaf spot protection, these genetically similar cultivars also inherited many agronomically unfavorable characteristics that were described above.

In an effort to increase genetic diversity and incorporate favorable traits, alternative breeding methods and new genetic technologies (e.g., hybrid introgression, embryo rescue, and genetic transformation) have been used in peanut breeding programs.  Wild *Arachis* germplasm has been collected with nearly complete resistance to both leaf spot pathogens. However, the production of fertile *A. hypogaea* x *Arachis sp.* progeny are complicated by differences in ploidy levels of the parents.  However, *A.villosa, A. correntina, A. diogoi* Hoehne*, A. stenosperma, A. cardenasii* Krapov. and W.C. Greg.*, A. duranensis*, and  *A. batizocoi* have all been successfully crossed with *A. hypogaea* (Singh, 1986; Stalker and Simpson 1995).

Simpson and Starr (2001) released the first commercial peanut cultivar, COAN, which possessed an identifiable gene derived from a wild *Arachis* species that provided resistance to root-knot nematode. Although not bred for the purposes of leaf spot resistance, the development of COAN proved that hybrid introgression was a viable method for improving genetic diversity and bringing biotic resistance factors into cultivated peanut. Recently, germplasm lines have been released with very high levels of leaf spot resistance derived from *A. cardenasii* (Stalker et al. 2002). PI 261942 was crossed with *A. cardenasii* to produce triploid hybrids. First generation hybrids were collected and colchicine-treated to restore fertility. Fertile plants were self-pollinated, and offspring were field screened for disease resistance. Germplasm possessing leaf spot resistance was further screened for ploidy level. Lines that were tetraploid were

selected as breeding stock (Stalker et al. 2002). Isleib et al. (2006) used these stocks to develop a germplasm line resistant to ELS, N96076L.

## Peanut Transformation

Recently, interest has increased in transgenic approaches to complement traditional breeding for improved agronomic performance in peanuts. Transgenic cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), and corn (*Zea mays* L.) have been widely accepted and very successful in streamlining cultivation practices and improving yields.

Numerous studies have focused on transforming peanuts using particle bombardment as well as *Agrobacterium*-mediated transformation systems (see Table B-1 for details of these studies). Presently, the most successful attempts at producing transgenic peanuts have used particle bombardment to introduce constructs into peanut somatic embryos. Although an effective means for generating transgenics, bombardment protocols have several disadvantages: complex rearrangements and integration patterns, gene silencing, high cost, difficulty of use/accessibility, and limited end product utility (Altpeter et al. 2005). Among these disadvantages, perhaps the most unfavorable issue associated with bombardment protocols, is the length of time required to generate mature plants. Most biolistic protocols require 8-16 months to produce mature, transgenic lines capable of producing seed. These lengthy tissue culture requirements allow for an increased likelihood of somaclonal variation. In addition, these lengthy protocols often require extensive subsequent sub-culturing, which is highly labor intensive and increases the chances for putative transgenics to be lost to contamination.

As an alternative to lengthy bombardment methods, protocols using faster, direct organogenesis and *Agrobacterium* have been investigated. Transformation by

*Agrobacterium* is believed to be superior to bombardment because integration patterns tend to be "cleaner", meaning whole gene constructs integrate into the host genome usually with low copy number (Sharma et al. 2005). Additionally, and perhaps most favorable, tissue culture requirements tend to be far less intensive in terms of sub-culturing and time to plant maturity. This reduction in time and handling lessens the likelihood for contamination and somaclonal variation. Thus, once established, protocols are far less labor intensive and more economically sound.

Despite the many advantages of *Agrobacterium*-mediated transformation over particle bombardment, it is far from an ideal system and requires intensive optimization because highly efficient *Agrobacterium* protocols are dependent upon multiple factors: bacterial strain, specialized plasmid vectors, host genotype, explant age/type, and co-cultivation conditions (Sharma et al. 2005). Due to the biological nature of *Agrobacterium*-mediated transformation (host-"pathogen" compatibility), much effort is required to determine the best infection conditions. Unlike particle bombardment protocols, that use DNA-coated gold particles to physically deliver foreign DNA to the nucleus of target tissue, *Agrobacterium* relies on a biological virulence mechanism for nuclear transgene delivery. As in nature, the interaction of host tissue susceptibility and *Agrobacterium* virulence are highly variable.

Few genetically engineered peanut lines exist today, and none are commercially available. The limited availability of transgenic peanuts is primarily due to: 1) no single peanut transformation protocol for fast and routine production of transgenic, 2) no approved transgenic lines, and 3) grower hesitancy to plant "GM peanut" for fear of non-acceptance by consumers. Recently, grower/consumer attitudes have shifted since

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observing the success of other genetically engineered crops, e.g., cotton, corn, and soybean. Because of this shift in attitude, a renewed interest in peanut transformation has led to the development of research lines with improved agronomic performance.

**Peanut Tissue Culture**

Genetic transformation has great potential for introducing novel, beneficial genes into peanut that would not be available using conventional breeding methods. While conventional breeding will always play a highly significant role in the improvement of peanut, transformation technologies may provide a means of streamlining those improvement processes.

Although many studies have reported the successful production of transgenic peanuts, none have described very efficient production in the numbers of independent lines generated. Many of the transgenic peanut lines developed have been for "proof of concept" purposes and have used easily identifiable traits that serve no agronomic function, i.e., production of β-glucuronidase (GUS) or fluorescent reporter proteins. A common factor that impedes the efficient production of multiple independent lines is the restraints associated with the tissue culture process. Somaclonal variation due to long tissue culture requirements, explant availability, cultivar specificity, and poor regeneration into mature plants are common factors attributed to the limited success of developing highly efficient transformation protocols (Livingstone and Birch 1999; Anuradha et al. 2008).

Regardless of transformation method or target crop, a requirement for all tissue culture systems is the highly prolific, *in vitro* production of actively dividing cells. The transfer and stable integration of transgenes is dependent upon the rapid regeneration of competent cells. Highly efficient transformation protocols, in which numerous stable,

independent lines are produced, are often those with the highest incidence of regeneration. Currently, the major impediment of the routine production of transgenic peanut is the lack of prolific tissue culture systems.

Most seed and seedling tissues of peanut can be used to establish regeneration-competent tissue culture systems (Ozias-Akins and Gill, 2001). Explant source material has varied widely in previous peanut tissue culture studies. Several explant types have been used to develop both embryogenic and organogenic tissue culture protocols with moderate success. These studies have repeatedly shown that regardless of explant type or developmental system, the pathway to differentiation is primarily dependent upon genotype selection and growth regulator concentration in culture medium. Peanut cultivars tend to be regionally adapted and this has led to a large number of genotypes being tested across many regeneration protocols. Likewise, numerous growth regulators at various concentrations have been tested in tissue culture protocols. Presently, cytokinin-class hormones, i.e. N6-benzyladenine (BA), kinetin, and thidiazuron, and auxin-class hormones, i.e. 2,4-dichlorophenoxyacetic acid (2,4-D), and picloram, have been the most widely tested and successful for eliciting a regeneration response.

**Embryogenesis**

To date, the most efficient method for producing transgenic peanut is particle bombardment of somatic embryos. Somatic embryogenesis is the development of embryogenic cells lines from tissues not typically involved with embryo production. Embryos are unique from other adventitious tissues because they are bipolar, having both a shoot and root pole.

Ozias-Akins et al. (1989) and Hazra et al. (1989) were among the first to report the successful generation of somatic embryos in peanut. These studies used immature cotyledons as explants, which were placed on medium supplemented with synthetic auxin hormones. Later studies also used immature explants to develop somatic embryos. The major disadvantage of using immature tissues as explants is the limited availability of this starting material. To obtain immature explants, material must be collected from flowering plants three to four weeks following soil penetration by the peg. In the southeastern U.S., field production of peanut begins in mid- to late-April and continues through early-October, with the most prevalent flowering occurring 60 - 80 DAP (Wright et al. 2009; personal communication Y. Lopez, 2010). The process of monitoring flowering and peg formation is an extremely tedious and labor intensive activity. Furthermore, flower induction is highly dependent on environmental conditions and can deviate from the general 60 - 80 day range. Along with the same problems observed in field-grown peanuts, growing peanuts in a controlled greenhouse environment is complicated by the fact that these plants tend to produce fewer flowers. Because of the unpredictable time and rate of immature embryo development, the availability of explants is extremely limited.

To circumvent the issues associated with using immature explants, investigations focused on developing protocols that used mature explants. Mature explants (generally from seeds) can remain viable when stored at low temperature and humidity, making the production of somatic embryogenesis on a year-round basis more convenient. McKently (1991) was the first to report a successful embryogenesis protocol using mature explants cultured on Murashige and Skoog (MS) medium supplemented with

picloram. Despite the convenience of using mature explant source material, many studies showed improved somatic embryogenesis efficiencies using immature tissue as explants (Ozias-Akins et al. 1993; Singsit et al. 1997; Wang et al. 1998; Chenault et al. 2002, 2003b, 2005; Yang et al. 1998, 2003; Deng et al. 2001; Chenault and Payton 2003; Athmaram et al. 2006).

In addition to explant availability, somatic embryogenesis in peanut is disadvantageous due to the low conversion rate of embryos into mature plants (Joshi et al. 2008). Ozias-Akins et al. (1992) and Chengalrayan et al. (1995, 1997) have made attempts to increase the frequency of recovering mature plants from somatic embryos of peanut. Despite previous efforts, the time required for the production and conversion of somatic embryos has led to the investigation of other tissue culture systems for use in transformation protocols.

**Organogenesis**

An alternative to lengthy somatic embryo production is direct production of organ-specific tissues from explants, a process known as organogenesis.

Illingworth (1968) was the first to report successful *in vitro* organogenesis of peanut from de-embryonated cotyledon sections cultured on hormone-free basal medium. This study, as well as many of the other early peanut organogenesis studies, was intended to develop protocols for basic research purposes, such as germplasm storage, rapid propagation, disease eradication, and embryo rescue (Martin, 1970; Kartha, 1981; Mroginski, 1981; Bajaj, 1982; Narasimhulu, 1983; Pittman, 1983; Atreya, 1984; Bhatia, 1985). These studies tested several media formulations, various growth hormones and concentrations, and explants. Although the efforts of these investigations resulted in the development of organogenesis protocols, no single protocol was highly

efficient in regenerating adventitious tissues. As the reality of routine gene transformation became more evident, efforts to improve the organogenic response in peanut intensified.

Successful organogenesis protocols have been developed using leaf material and immature seed material. These protocols, much like embryogenesis protocols using similar starting material, are not always favorable due to low explant availability. Mature seed have been investigated as an explant source. Hypocotyls, epicotyls, and cotyledonary nodes from freshly germinated seed have been investigated as explants for organongenesis. To simplify protocols, direct organogenesis from non-germinated, mature, whole seed, embryo axes, and cotyledons has been tested.

Sharma and Anajaiah (2000) developed an efficient protocol which used de-embryonated cotyledon halves as explants. This study optimized an organogenesis system using cv. JL-24. Freshly cut cotyledon-halves placed on MS medium supplemented with 20 µM BA and 10 µM 2,4-D were efficient at producing adventitious shoot buds (> 90%). Recently, Tiwari et al. (2008) expanded upon this protocol to include other Spanish market type cultivars widely grown in India: TMV-2, TAG-24, and Dh-3-30. In addition to numerous adventitious buds forming and rapid regeneration to mature plants, Sharma and Anajaiah (2000) and Tiwari et al. (2008) reported high transformation efficiencies using this tissue culture method.

### Peanut Transformation Advancements

Peanut, like other crops, encounters many biotic and abiotic stressors throughout a growing season. Although much of the early peanut transformation work was for "proof of concept" purposes, several investigators have developed transgenic lines for improved agronomic performance.

As previously discussed, TSWV is a major pathogen in most peanut growing regions throughout the U.S. Innate resistance has been observed in peanut, but is incomplete and allows for significant yield loss. In an effort to supplement natural resistance, Brar et al. (1994), Yang et al. (1998), Magbanau et al. (2000), and Chenault et al. (2003) bombarded somatic embryos with a nucleocapsid coat protein from TSWV. Li (1997) used *Agrobacterium* transformation to integrate a similar gene into peanut. $T_0$ and progeny of transgenic plants displayed a day delay in symptom development. Using a similar approach, Higgins et al. (2004) developed transgenic lines expressing peanut stripe nucleocapsid coat protein. These lines displayed resistance to peanut stripe virus, a virus common to peanut crops in Asian and Australian growing regions.

Toxin derived from *Bacillus thuringiensis* (*Bt*-toxin) has been widely used in many crops to confer resistance to insect pests. *Bt*-expressing peanut was developed using both *Agrobacterium* and biolistic transformation. Singsit et al. (1997), using bombardment, developed transgenic peanut lines expressing *Cry1Ac* providing protection to lesser cornstalk borer. Tiwari et al. (2008) successfully integated a synthetic *Cry1EC* gene into peanut using *Agrobactrium* transformation. Complete resistance to tobacco cut worm, an insect pest common to Indian production regions, was reported for several independent lines. Ingestion of *Cry1EC*-expressing plants by tobacco cut worms in *in vitro* bio-assays led to 100% fatality.

As mentioned earlier, fungal pathogens are the most prevalent peanut pests. Rohini and Rao (2001) were the first to use *Agrobacterium* to generate peanut plants with improved fungal resistance. Using a non-tissue culture-based transformation system, Rohini and Rao (2001) developed plants expressing tobacco chitinase. This

study reported transgenic lines displaying tolerance to ELS disease in small-plot field trials. Chenault et al. (2002) used biolistics to engineer peanut lines expressing genes encoding chitinase and glucanse. Livingstone et al. (2005) engineered peanut lines to produce oxalate oxidase, an enzyme which degrades oxalic acid, a compound required for *Sclerotinia* blight infection. Detached leaflet assays showed transgene expression limited lesion size resulting from direct application of oxalic acid. Lesion size was significantly reduced in transgenic plants compared to wild type controls (65% – 89% reduction at high oxalic acid concentrations). A second assay examined lesion size after inoculation of leaflets with *S. minor* mycelia. Lesion size was reduced by 75% - 97% in transformed plants, providing evidence that oxalate oxidase can confer enhanced resistance to *Sclerotinia* blight in peanut. Most recently, Anurahda et al. (2008) generated peanut plants expressing a mustard defensin protein. *In vitro* bio-assays of leaf material indicated improved resistance to multiple fungal pathogens.

**Leaf Senescence, a Nuclear Controlled Form of Programmed Cell Death**

Plants defend themselves against pathogens by activating a complex, multi-component defense response. Induced defenses of plants against pathogens are regulated by networks of interconnecting signaling pathways involving cytosolic $Ca^{2+}$ and $H^+$ ions, reactive oxygen intermediates, salicylic acid, jasmonic acid, nitric oxide, and ethylene as the primary components (Agrios, 2005). Increased activity of these pathways during pathogen infection is believed to be controlled by gene-for-gene interaction between the host and pathogen. Interactions between these defense pathways are complex and not completely understood. However, hypersensitivity is associated with nearly all defense mechanisms. Hypersensitivity is the rapid cell death at the site of attempted pathogen ingress.

In recent years, programmed cell death (PCD) has become the focus of several studies because of its potential to explain many fundamental processes common to a species. PCD is the controlled self-destruction of cells triggered by external or internal factors (Lim et al. 2007). PCD was once viewed as an unorganized process in which cellular components were randomly degraded and were relocated to newly developing tissues. More recent studies focusing on leaf senescence, a nuclear controlled form of PCD, show that the process is very much orchestrated and coordinated by a complex biochemical network (Gan and Amasino, 1997; Brault and Maldiney, 1999). Leaf senescence is a phase of a plant's life cycle that signifies the final stage of leaf development and is controlled by an extremely regulated system. Changes occur in cell structure, metabolism, and gene expression. Senescence is characterized by reduced photosynthetic capabilities, chlorosis and subsequent necrosis. A primary purpose of this process is to relocate nutrients from old, non-functional leaves to developing portions of the plant such as young leaves, growing seeds, or storage tissues (Gan and Amasino, 1997; Jordi et al. 2000). Leaf senescence is influenced by many internal and environmental signals (Lim et al. 2007). Internal factors include age and productivity of tissues, flower and seed development, and phytohormone levels (Gan and Amasino, 1997). Environmental factors controlling leaf senescence can be biotic or abiotic in nature. Examples of these factors include temperature extremes, drought, ozone, nutrient deficiency, pathogen infection, wounding and shading (Lim et al 2007). Although the exact mechanisms that regulate leaf senescence are not yet well defined, several researchers have identified a class of control genes known as senescence associated genes (SAGs). SAGs have been identified in a number of plant species.

First identified in *Arabidopsis (Arabidopsis thaliana* (L.)) Heynh. (Lohman et al. 1994),

SAGs have also been found in asparagus (*Asparagus officinalis* L.) (King et al. 1995),

barley (*Hordeum vulgare* L.) (Becker and Apel, 1993), rapeseed (*Brassica napus* L.)

(Buchanan-Wollaston and Ainsworth, 1997), maize (*Zea mays* L.) (Smart et al. 1995),

radish (*Raphanus sativus* L.) (Azumi and Watanabe, 1991), rice (*Orzya sativa* L.) (Lee

et al. 2001), and tomato (*Solanum lycopersicum* L.) (Drake et al. 1996). Many SAGs

code for similar gene products across species lines. Products often associated with

senescence genes are degradative enzymes such as proteases, lipases, nucleases,

chlorophyllases, and other nutrient recycling proteins such as glutamine synthase

(Gepstein et al. 2003; Ori et al. 1999)

Watanabe and Imaseki (1982) were the first to observe a correlation between leaf

senescence and a change in gene expression; their study indicated significant reduction

of leaf mRNAs during the progression of senescence. Subsequent work with

*Arabidopsis* showed that expression of photosynthetic genes are markedly down

regulated during the progression of leaf senescence, whereas mRNA levels increase for

other genes (later to be classified as SAGs) (Jiang et al. 1993; Humbeck et al. 1996).

Microarray analyses of *Arabidopsis* by van der Graaff et al. (2006) investigated SAGs

on a genome-wide scale. Results from this work indicated the up-regulation and down-

regulation of several hundred genes throughout the phases of senescence.

Approximately 800 SAGs of varying classes have been identified for which transcription

is initiated at various stages of leaf senescence (Gepstein et al. 2003). The large

number of SAGs expressed during leaf senescence is indicative of its tight genetic

control.

In a study focusing upon mRNA accumulating during natural senescence of *Arabidopisis* leaves, Lohman et al. (1994) observed a gene that was up-regulated throughout all phases of the process. This gene is now designated as senescence associated gene 12 (*SAG12*), and the five phases of leaf senescence are described as follows: stage one is the first visible sign of senescence, while stage five is total chlorosis. Analysis of *SAG12* expression showed that it was senescence-specific, up-regulated only slightly at stage one, and then progressed rapidly to high levels that were maintained until senescence was complete. Subsequent studies showed that *SAG12* expression was not limited to leaf tissue alone, but was also expressed in other senescing tissues such as stems, sepals, petals, and carpels (Gan and Amasino, 1997).

Gan and Amasino (1995) linked the *SAG12* promoter to a reporter gene, *uidA* which codes for β-glucuronidase, to form a *SAG12-uidA* construct. Introduction of this chimeric gene into tobacco did not alter the rate of senescence, but showed increased *uidA* expression as leaf senescence progressed. Once effectiveness of the *SAG12* promoter was confirmed, efforts then shifted toward developing an expression system that used cytokinins to delay leaf senescence.

**Cytokinins and Isopentyl Transferase**

Cytokinins are a class of plant hormones that are active in controlling several critical processes associated with the normal life cycle of a plant. Cytokinins are essential for cell division, chloroplast development, bud differentiation, shoot initiation and growth, and leaf senescence (Brault and Maldiney, 1999). Although these critical roles are widely acknowledged for cytokinins, the pathways controlling them have yet to be completely discerned.

Most of the research has focused upon the controlled expression of cytokinin biosynthetic genes (Akiyshi et al. 1984; Barry et al. 1984). These studies indicate that the gene coding for adenosine phosphate isopentyl transferase (IPT) is a key regulator of cytokinin biosynthesis in *Agrobacterium tumefaciens* (Hirose et al. 2008). This gene (*tmr*) is located on the Ti plasmid of pathogenic *A. tumefaciens* and is activated during plant infection to initiate cytokinin production and gall formation (Sakakibara et al. 2005). IPT catalyzes condensation of dimethylallylpyrophosphate and 5'-AMP to isopentenyladenosine (iPA) 5'-phosphate (Hirose et al. 2008). This reaction is generally considered the rate limiting step for cytokinin biosynthesis (Sakakibara, 2006).

One of the earliest attempts to exploit IPT activity involved linking *tmr* to a heat-shock inducible promoter, *HS6871* (Smart et al. 1991). Transgenic tobacco expressing this construct initiated IPT production under heat stress were shorter with larger side shoots, and remained green longer than wild-type controls. After several cycles of heat shock, however, plant growth and morphology became abnormal due to extremely high levels of IPT accumulating in the transgenic plants. Subsequent research tested a multitude of promoters in combination with *tmr*, with results generally similar to those reported by Smart (1991).

Gan and Amasino (1995) were the first to report transgenic tobacco plants with increased IPT levels that did not exhibit developmental abnormalities. The *tmr* gene (referred to as *IPT* in this particular study) was linked to the senescence-specific *SAG12* promoter. The *SAG12-IPT* chimeric gene resulted in an autoregulatory system that was only activated during initiation of leaf senescence. Because *IPT* expression was only activated during senescence, cytokinin levels were maintained at levels similar to wild-

type controls, thus facilitating normal development. In addition, plants transformed with

*SAG12-IPT* had delayed leaf senescence and prolonged photosynthetic activity when

compared to wild-type control plants. Subsequent research has focused on using the

autoregulatory system developed by Gan and Amasino (1995) to improve agronomic

and horticultural performance in a variety of plant species. Reports indicate successful

use of *SAG12-IPT* in lettuce (*Lactuca sativa* L.) (McCabe et al. 2001), petunia (*Petunia*

*x hybrida*) (Chang et al. 2003), tomato (*Solanum lycopersicum* L.) (Swartzberg et al.

2006), alfalfa (*Medicago sativa* L.) (Calderini et al. 2007), wheat (*Triticum aestivum* L.)

(Sykorova et al. 2008), and cassava (*Manihot esculenta* Crantz) (Zhang et al. 2010).

**Pathogen Induced Leaf Senescence**

As previously discussed, leaf senescence is the final stage of leaf development

when photosynthetic rates are reduced and nutrients are recycled to newly developing

portions of the plant. However, this process can be induced prematurely by a number of

factors, including pathogen infection, which can lead to reduced productivity and yields

(Gan and Amasino, 1995). Premature senescence in response to pathogen infection

may have evolved as a mechanism of defense (Greenberg and Yao, 2004). This

hypersensitive response would be advantageous in limiting pathogen growth and

spread. Although beneficial to the infected plant, early leaf abscission can have

negative effects in an agricultural setting. With fewer photosynthetic structures, fewer

sugars are available for developing organs, and overall yield and productivity will be

reduced. Assuming leaf senescence is induced by a lesion-producing pathogen such as

*Cercospora* spp., reduced photosynthetic capabilities can be further compounded by the

presence of lesions on the remaining, non-senesced leaves. As previously discussed,

successful efforts have been made to engineer several species of plants with the

*SAG12-IPT* chimeric gene to delay the onset of leaf senescence. Engineering plants to

retain leaves, even under pathogen attack, could potentially negate some of the

undesirable effects associated with pathogen infection. Preliminary data (M. Jones and

D. Clark, University of Florida) indicated that transgenic petunia expressing *SAG12-IPT*

had a delayed leaf senescence response (Jandrew, 2002). Transformants also

appeared to develop fewer chlorotic lesions and gained tolerance to petunia leaf spot

disease caused by *Cercospora petunia* (Jandrew 2002) (Figure 1-1). Similar results

were reported by Swartzberg et al. (2008), in which tomato plants transformed with

*SAG12-IPT* displayed suppressed symptoms (i.e. delayed leaf senescence and reduced

lesion size) of the disease caused by *Botrytis cinerea* (De Bary) Whetzel.
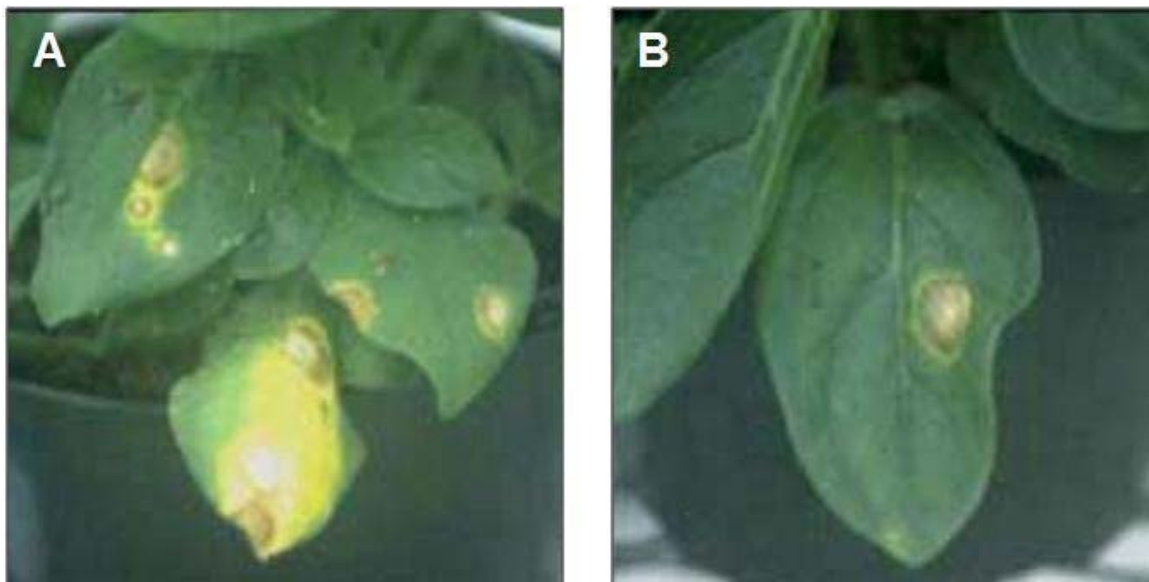


Figure 1-1.  *Petunia* Leaf Spot (*Cercospora petunia)* Infection. (A) wild type *Petunia*,
and (B) *SAG12-IPT* transgenic *Petunia* (Jandrew 2002).

# CHAPTER 2
## EVALUATING PEANUT CV. FLORIDA-07 FOR LATE LEAF SPOT TOLERANCE

**Abstract**

Florida-07, a peanut cultivar recently released by the University of Florida, displays classic symptoms of leaf spot susceptibility, having numerous lesions and heavy defoliation. However, it still produces good yields. Therefore, one hypothesis is that Florida-07 possesses tolerance to leaf spot. To test this hypothesis, Florida-07 was compared to a known leaf spot susceptible cultivar, AP-3, and a known resistant cultivar, York. Experiments were conducted in Citra, FL in 2008 and Marianna, FL in 2008 and 2009. For all years and locations, late leaf spot (*Cercosporidium personatum* (Berk and M. A. Curtis) Deighton) appeared to be the predominant pathogen. The experimental design was a randomized complete block with a split-plot treatment arrangement and three replications. Cultivars were assigned to sub-plots and fungicide treatment (full-season vs. no spray) was assigned to main plots. Data collected included area under the disease progress curve (AUDPC) for visual leaf spot rating (Florida 1-10 scale), lesion/leaf percentage, lesion density, and lesion growth rate. Following harvest, pod yield, yield loss to leaf spot, and percent yield loss to leaf spot were calculated. In regard to visual rating, lesion/leaf percentage, and lesion density, the rate of disease progression (AUDPC) was the same in sprayed and non-sprayed York, sprayed AP-3, and sprayed Florida-07. Disease progression was similar in non-sprayed AP-3 and non-sprayed Florida-07, but at a relatively faster rate compared to the aforementioned cultivar*treatment combinations. Regardless of cultivar*treatment combination, lesion growth occurred at the same rate. Based on these data, it was concluded that Florida-07 and AP-3 possessed the same degree of susceptibility to late leaf spot disease.

Because of its higher yield potential, Florida-07 appeared to overcome the impact of leaf spot disease in two out of three tests, but in the third test, leaf spot impacted pod yield of Florida-07 and AP-3 equally. In the two tests in which Florida-07's higher yield potential became evident, environmental conditions were favorable for the onset and increased severity of leaf spot disease. Therefore, it was determined that in some environments, and primarily due to its yield potential, Florida-07 may provide a degree of "protection" against late leaf spot disease that AP-3 does not possess.  However, on average, Florida-07 does not appear to possess significant tolerance to leaf spot.

## Introduction

Early leaf spot [*Cercospora arachidicola*  S. Hori (teleomorph *Mycosphaerella arachidi* Deighton)] (ELS) and late leaf spot [*Cercosporidium personatum* (Berk and M. A.Curtis) Deighton (teleomorph *Mycosphaerella berkeleyi* Jenk)] (LLS) diseases are the most widespread foliar diseases of peanut. Both ELS and LLS diseases can be found wherever peanut is grown, making them among the most significant peanut diseases (Zhang et al. 2001). ELS and LLS diseases are characterized by necrotic flecks that enlarge to necrotic lesions that reduce light interception and photosynthesis (Boote et a. 1983). The reduction in photosynthetic leaf area is the primary factor associated with loss of yield in peanut. If fungicides are not used, pod yields can be reduced by as much as 50% in diseased plants (Zhang et al. 2001). Early defoliation is also associated with both types of leaf spot infection.

Currently, management strategies for controlling leaf spot epidemics rely heavily on crop rotation or on reducing the rate of disease spread via resistant cultivars and regular applications of foliar fungicide (Zhang et al. 2001). Although leaf spot resistant cultivars are commercially available, the degree of protection in these cultivars is

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incomplete and still allows for a significant amount of damage. Previous studies have

shown that partial resistance is due to the interaction of multiple components that

additively produce varying degrees of resistance. Cultivars exist with partial resistance,

but there has been no complete or single-gene resistance to *C. arachidicola* or *C.*

*personatum* reported in cultivated peanut. Components of resistance that have been

identified include, infection frequency (dependent on density of inoculum), incubation

period (time from inoculation to appearance of symptoms), latent period (time from

inoculation to first sporulating lesion), lesion size, necrotic leaf area, spore production,

and defoliation time (Dwivedi et al. 2002; Cantonwine et al. 2008). Components of

resistance have been reported for early and/or late leaf spot for several cultivars tested

under field and greenhouse conditions (Chiteka et al. 1988a; Cook 1981; Foster et al.

1980; Green and Wynne 1986; Melouk and Banks, 1984; Ricker et al. 1985;

Subrahmanyam et al. 1982; Walls et al. 1985; Watson et al. 1998). Among the identified

resistance components, no one component has emerged as the primary mechanism for

resistance in leaf spot resistant cultivars (Cantonwine et al. 2008).

Florida-07 (released by the University of Florida in 2006) (Gorbet and Tillman,

2009) is a medium-late maturing (~140 day) Runner market-type peanut. Release of

Flordia-07 was made on the basis of its excellent pod yield potential, competitive kernel

grade, high-oleic fatty acid chemistry, and resistance to tomato spotted wilt topovirus

(TSWV) and white mold (Gorbet and Tillman, 2009). In addition to the aforementioned

characteristics, in non-sprayed preliminary field trials, under high leaf spot pressure,

Florida-07 consistently produced higher yields than other test varieties. However,

Florida-07 still displayed classic symptoms of leaf spot disease, i.e. high lesion

coverage and pre-mature defoliation. Florida-07 seemed to possess the ability to sustain the effects of leaf spot disease without dying or suffering serious injury or crop loss. Therefore, it was hypothesized that Florida-07 possessed tolerance to leaf spot disease. The purpose of this study was to confirm/characterize Florida-07 as a leaf spot tolerant cultivar and to identify a mechanism of tolerance. Currently, there are no reported formal field evaluations testing Florida-07's tolerance to ELS and LLS diseases.

AP-3 (University of Florida, 2003) is a medium-late maturing (~140 days) Runner market-type peanut. AP-3 was released because of its excellent resistance to tomato spotted wilt topovirus (TSWV) and *Sclerotium rolfsii* (white mold). The cross that produced AP-3 was made primarily to produce material to select for resistance to white mold and *Cylindrocladium* black rot (CBR - caused by *Cylindrocladium parasiticum*) (Gorbet 2007). Despite AP-3's resistance to other fungal pathogens of peanut, AP-3 is very susceptible to early and late leaf spot diseases. Without fungicide treatment, AP-3 has high lesion coverage and premature defoliation, which results in reduced yields.

York (University of Florida, 2006) is a late maturing (~150 days) runner market-type peanut. York has excellent disease resistance to TSWV, white mold, and leaf spot diseases. Under intense leaf spot pressure, lesion coverage on York is minimal and is often isolated to the uppermost portion of the canopy. Defoliation in leaf spot infected York is also minimal. Because of the observed resistance to leaf spot in York, fungicide application recommendations allow for a reduced regime when York is grown in a good crop rotation.

In this study, Florida-07 was compared to AP-3, a known leaf spot disease susceptible cultivar, and York, a known leaf spot disease resistant cultivar, in sprayed and non-sprayed field plots across multiple locations and years. Foliar leaf spot disease progression rates and yield were examined to classify Florida-07 as susceptible, tolerant, or resistant.

## Materials and Methods

**Experimental Design**

Peanut cultivars for this study included AP-3 (Gorbet, 2007a), Florida-07, and York (released by the University of Florida in 2006) (Table 2-1). The three genotypes were planted on 20 May 2008 at the Plant Science Research & Education Unit located in Citra, FL. Soil type in Citra, FL is Tavares sandy loam. The Citra, FL test site was previously planted with bahiagrass for the three years prior. A duplicate test was planted on 3 June 2008 and 20 May 2009 at the North Florida Research and Education Center located in Marianna, FL.  Soil type in Marianna, FL is Chipola sandy loam. The Marianna, FL test site was previously planted with a cotton and corn rotation. Test site locations can be seen in Figure 2-1. With the exception of fungicide applications, cultural and management practices followed the standard UF/IFAS Extension recommendations for irrigated peanut.

The experimental design was a randomized complete block with a split-plot treatment arrangement; fungicide treatment was assigned to the main plot and cultivar was assigned to the sub-plot. Plot dimensions were two rows, 4.5 m in length, with row centers set at 91 cm apart. Seed were sown at a rate of six seeds per 31 cm (90-100 seeds per row) using conventional tillage practices. Border rows of C99-R and Florida-07 were located on each side of the plots to maintain disease inoculum and to prevent

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spray-drift from affecting adjacent plots in 2008 and 2009, respectively. Plots were replicated three times at each test site and two spray regimes were used as treatments (NS = no fungicide treatment, S = standard commercial fungicide treatment). Plots receiving the standard commercial treatment were sprayed with chlorothalonil, tebuconazole, pyraclostrobin, and azoxystrobin bi-weekly beginning 30 DAP (Table 2-2).

In Citra, fungicides were applied using a $CO_2$ backpack sprayer and hand-held boom with five nozzles, spaced 51 cm apart. Boom width (swath) allowed for complete coverage of peanut plants for the entire two-row plot. The sprayer was calibrated to deliver 327 L ha$^{-1}$. In Marianna, fungicides were applied using a Hi-Boy, 12-row sprayer with flat fan nozzles. Boom width allowed for coverage of the entire treated range of test plots. The sprayer was calibrated to deliver 206 L ha$^{-1}$.

**Disease Assessment**

Disease assessment began at the first sign of leaf spot symptoms and continued weekly until harvest. Identification of the pathogen causing disease was determined in the field using a 60X-100X, handheld microscope. In this study, late leaf spot was the predominant pathogen. Disease assessment for AP-3 and Florida-07 lasted a period of four weeks and six weeks for York. For all years and locations, leaf spot symptoms first appeared in early-September.

Qualitative, visual evaluations were made in the field using the Florida 1–10 leaf spot scale as described by Chiteka et al. (1988b) (Table 2-3). Use of the Florida 1-10 rating scale allowed for the assessment of whole plot response to leaf spot pressure (lesion coverage and defoliation amount).

Lesion percentage, lesion density, and average lesion size were quantified using APS Assess 2.0 image analysis software (American Phytopathological Society). Forty compound leaves (approximately 160 leaflets) were randomly collected from each plot weekly, scanned, and imported into APS Assess 2.0 as JPEG images (Figure 2-3). Default settings were applied to determine total leaf and lesion area, lesion percentage and lesion frequency for each plot. Using the total leaf and lesion area and lesion frequency data, lesion density (lesions $cm^{-2}$) and average lesion area ($mm^2$) were calculated.

**Area Under the Disease Progress Curve (AUDPC)**

Area under the disease progress curve (AUDPC) was calculated as the total area under the graph of disease severity (Florida 1-10 rating, lesion/leaf percentage, lesion density, and lesion growth rate) against time (weekly evaluation from early-September through harvest), from the first scoring to the last:

$$AUDPC = \sum_{i=1}^{k} [\left(\frac{L_{i-1} + L_i}{2}\right)(t_i - t_{i-1})]$$

(2-1)

where, $t_i$ = days after planting (time) and $L_i$ = severity rating

**Harvest and Pod Yield**

Harvest dates were determined by maturity group and leaf spot severity. Plots with severe leaf spot pressure (high lesion coverage and high defoliation) were harvested early (plots receiving a rating ≥ 8 on the Florida 1-10 scale) to avoid substantial yield loss.  Digging was accomplished with a two-row digger/inverter.  Pod yields were determined by threshing all plants in a plot with a stationary thresher and weighing the pods after the seeds had dried to 9-10% moisture content. In addition to pod yields,

yield loss to LLS disease (S - NS), and percent yield loss to LLS disease ((S-NS) / S) were determined for each plot.

**Environmental Conditions**

Environmental conditions for each year and location were determined from various weather components (maximum (max.) temperature, minimum (min.) temperature, percent relative humidity (%RH)) obtained from the Florida Automated Weather Network (FAWN). Both test locations had FAWN stations on site. For temperatures (max/min) and %RH, daily averages were collected beginning 70 DAP and continued until harvest for each test site. Daily leaf spot hours were calculated for each year and location.  A leaf spot hour was defined as one hour with relative humidity greater than or equal to 90% and temperatures between 16°C and 30°C. Beginning 70 DAP and continuing through harvest dates, hourly average temperature and %RH data were collected. Leaf spot hours accounted for the amount of time in a given day which provided conditions that were most conducive to the rapid development and increased severity of leaf spot diseases.

**Statistical Analysis**

Analysis of variance was carried out on the means for each AUDPC and pod yield per plot using the Mixed Model procedure (PROC Mixed) in SAS software (SAS Institute, 2000). Fungicide treatment and cultivar were considered fixed effects whereas year and replication and their interactions were considered random effects. Statistical significance was determined at $P \leq 0.05$ according to Tukey's HSD mean separation test.

**Disease Response Classification**

Classification of cultivar disease response was based on descriptions reported in Agrios (2005) for resistance, tolerance, and susceptibility:

Resistance - the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor.

Tolerance - the ability of a plant to sustain the effects of a disease without dying or suffering serious injury or crop loss.

Susceptibility - the inability of a plant to resist the effect of a pathogen or other damaging factor; non-immune.

## Results and Discussion

### Citra 2008

**Disease progression.** In Citra 2008, foliar lesions were first noted during the first week of September. Unless otherwise noted, cultivar*treatment was significant for each measure of disease progression.

In terms of whole plot response (Florida 1-10 rating) and lesion percentage, Florida-07 and AP-3 were equally susceptible to LLS. AUDPC for the Florida 1-10 rating indicated that disease progression was most rapid in NS AP-3 (5.2 ± 0.3 rating*time), followed by NS Florida-07, S Florida-07 (4.5 ± 0.3 rating*time and 4.4 ± 0.3 rating*time), then S AP-3, NS York (3.9 ± 0.3 rating*time), and finally S York (2.0 ± 0.3 rating*time) (Figure 2-4A). Likewise, in respect to lesion percentage, Florida-07 and AP-3 were equally susceptible to LLS. Cultivar was the only significant main effect for necrotic lesion percentage. In this test, AUDPC means for percent lesion coverage increased at the same rate for AP-3 and Florida-07 (14 ± 1.1 %*time and 16.4 ± 1.1%*time, respectively), but more rapidly than for York (3± 1.1 %*time) (Figure 2-4B).

In terms of lesion density, LLS progression was most rapid in NS Florida-07 (4.8 ± 0.2 lesions cm$^{-1}$*time), followed by S Florida-07, NS AP-3, and S AP-3 (3.9 ± 0.2 lesions cm$^{-2}$*time, 3.6 ± 0.2 lesions cm$^{-2}$*time, and 3.4 ± 0.2 lesions cm$^{-2}$*time, respectively). Disease progression was slowest on NS York and then S York (1.8 ± 0.2 lesions cm$^{-}$

$^2$*time and 0.6 ± 0.2 lesions cm$^{-1}$*time, respectively) (Figure 2-4C). These results suggest that LLS lesions may develop more rapidly on Florida-07 than AP-3, meaning, in terms of foliar lesion density, Florida-07 may be more susceptible to LLS.

Rate of lesion growth was not affected by treatment, cultivar, or cultivar*treatment interaction. Lesion size increased at the same rate on all cultivars and treatments (Figure 2-4D).

**Yield Response.** Treatment, cultivar, and cultivar*treatment effects were significant (p>F = <0.0001, p>F = <0.0001, and p>F = 0.0007, respectively). In Citra 2008, yield response for cultivar*treatments occurred as expected under high LLS pressure. Characteristic of a leaf spot resistant cultivar, York produced the same yields under LLS pressure in the S and NS treatments (2429 kg ha$^{-1}$ and 2320 kg ha$^{-1}$, respectively) (p>t = 0.6527). AP-3, a known susceptible under LLS pressure, yielded much higher in the S treatment than the NS treatment (4452 kg ha$^{-1}$ and 2461 kg ha$^{-1}$, respectively) (p>t = <0.0001). Likewise, S Florida-07 yielded higher than NS Florida-07 under LLS pressure (4806 kg ha$^{-1}$ and 3722 kg ha$^{-1}$, respectively) (p>t = 0.0009). Despite AP-3 and Florida-07's similarity in yield response to treatments, if yields of NS AP-3 and NS Florida-07 are compared, then NS Florida-07 yielded more than NS AP-3 (P > t =0.0003). Similarly, AP-3's yield loss to LLS (S - NS) was more than that lost by Florida-07(1991 kg ha$^{-1}$ and 1084 kg ha$^{-1}$, respectively) (P > t =0.0524). However, when the yields lost to LLS are normalized to percentage values ((S – NS) / S), AP-3 and Florida-07 lost the same percent value of their yield (44.8% and 22.3%, respectively) (P > t =0.1698) (Table 2-4). However, if one compares cultivar alone, regardless of treatment, Florida-07 yielded higher than AP-3 (p<t = 0.0006). This difference in cultivar

yield in the absence of leaf spot disease may be the reason Florida-07 appeared to display tolerance to the disease in preliminary studies.

To summarize the Citra 2008 test, disease progression in whole plot evaluations and percent lesion coverage suggest that Florida-07 and AP-3 are equally susceptible o LLS. However, higher lesion frequencies developing over time indicate that Florida-07 is more susceptible to LLS. Comparison of Florida-07 and AP-3 yields show that Florida-07 has the potential to produce higher yields even under high LLS pressure. Florida-07's ability to produce high yields even under pathogen attack (i.e. high lesion density) suggests that it possesses a degree of tolerance to LLS. However, upon normalizing yield data, it becomes clear that Florida-07 did not display tolerance to LLS, but instead had a higher yield potential. Although Florida-07 did not display tolerance as defined by Agrios (2005) in this test, its higher yield potential did provide a degree of protection to final yield.

No other fungal diseases were observed in Citra 2008. However, insect pest pressure was high late in the season. An unknown species of leafhopper caused a fairly large reduction in canopy density. Reduction in canopy density might have contributed to reduced photosynthetic rates, which could have potentially impacted final yields. However, because damage occurred late in the season (occurring just prior to harvest of Florida-07 and AP-3 plots), it was determined that this reduced canopy density likely did not affect yields.

**Marianna 2008**

**Disease progression.** In Marianna 2008, foliar lesions were first noted during the second week of September. Cultivar*treatment interaction was significant main effect for each measure of disease progression unless otherwise noted.

AUDPC for the Florida 1-10 scale and lesion density indicated that LLS disease progression was more rapid in Florida-07 than for AP-3. AUDPC for Florida 1-10 ratings was most rapid on NS Florida-07, followed by NS AP-3, and then NS York (5.4 ± 0.3 rating*time, 4.4 ± 0.3  rating*time, and 2.7 ± 0.3 rating *time, respectively). S Florida-07 and S AP-3's disease progression were the same (1.8 ± 0.3 rating*time and ± 0.3 1.7 rating*time, respectively), followed by disease progression in S York (1.0 ± 0.3 rating*time) (Figure 2-5A). Likewise, the rate at which lesion density increased throughout the season was most rapid for NS Florida-07, followed by NS AP-3, and then NS York (4.0 ± 0.2 lesions cm$^{-1}$*time, 3.3 ± 0.2 lesions cm$^{-1}$*time, and 2.1 ± 0.2 lesions cm$^{-1}$*time, respectively). Disease progression as a measure of lesion density was equal in S Florida-07, S York, and S AP-3 (0.8 ± 0.2 lesions cm$^{-2}$*time, 0.7 ± 0.2 lesions cm$^{-2}$*time, 0.6 ± 0.2 lesions cm$^{-2}$*time, respectively), but occurred at a slower rate than observed in the previously mentioned cultivar*treatments (Figure 2-5C).

Percent necrotic lesion indicates that disease progression was equal in NS Florida-07 and NS AP-3, followed by NS York (15.2 ± 0.7 %*time, 14.2 ± 0.7 %*time, and 5.6 ± 0.7 %*time, respectively). S Florida-07, S AP-3, and S York were equal in rate of disease progression (2.8 ± 0.7 %*time, 2.3 ± 0.7 %*time, and 1.9 ± 0.7 %*time), but rates were slower than those observed in the aforementioned cultivar*treatments (Figure 2-5B). Under high LLS pressure, Florida-07 and AP-3 were equally susceptible.

Rate of lesion growth was not affected by treatment, cultivar, or cultivar*treatment interaction. Lesion size increased at the same rate on all cultivars and treatments (Figure 2-5D).

**Yield response.** In Marianna 2008, treatment and cultivar*treatment interaction affected pod yield ($p > F = <0.0001$ and $p > F = 0.0106$, respectively). S cultivars yielded more than their NS counterparts. NS AP-3 and NS Florida-07 produced equal yields (2790 kg ha$^{-1}$ and 2786 kg ha$^{-1}$, respectively) ($p > t = 0.9841$). AP-3 and Florida-07's yield lost to LLS were also equal (1811 and 1648, respectively) ($p > t = 0.5397$), as well as percent yield lost to LLS (39.4% and 37.2%, respectively) ($p > t = 0.7109$) (Table 2-5). Based on AP-3 and Florida-07 having equal yield under LLS pressure, yield lost to LLS, and yield percentage lost to LLS, it was determined, in terms of yield response, that Florida-07 did not display tolerance to LLS in Marianna 2008, and was equally susceptible to LLS as AP-3.

To summarize the Marianna 2008 test, yields under LLS pressure, yield lost to LLS, and percent yield lost to LLS suggest that Florida-07 and AP-3 are equally susceptible to LLS. The higher yield potential observed in Citra 2008 test was not observed in the Marianna 2008 test. Compared to Citra 2008, in Marianna 2008, Florida-07 was more susceptible to LLS than AP-3, having more rapid disease progression with respect to the Florida 1-10 rating and lesion density. The more rapid disease progression in Florida-07 may explain why a higher yield potential was not observed in this test.

In addition to LLS, the only other fungal disease observed was a small amount of rust in the 2008 Marianna test site. Signs of rust were not observed until three days prior to harvest and were found on only non-treated plots. Because of the extremely late onset and very small amount of rust found, it was determined that its presence was

negligible and had no impact on the result of the test. LLS disease was the greatest yield reducing factor in this study.

**Marianna 2009**

**Disease progression.** In Marianna 2009, foliar lesions were first noted during the second week of September. Cultivar*treatment interaction affected each measure of disease progression unless otherwise noted.

Disease progression, in terms of Florida 1-10 rating, was most rapid in NS Florida-07 and NS AP-3 (3.5 ± 0.2 rating*time and 3.4 ± 0.2 rating*time, respectively). Disease progression rate was the same for NS York, S Florida-07, S AP-3, and S York (2.3 ± 0.2 rating*time, 2.1 ± 0.2 rating*time, 2.0 ± 0.2 rating*time, and 2.0 ± 0.2 rating*time, respectively), but at a slower rate than the previously mentioned cultivar*treatments (Figure 2-6A). Under high LLS pressure, in terms of the Florida 1-10 rating, Florida-07 and AP-3 were equally susceptible.

The rate of disease percent lesion coverage increased most rapidly in NS Florida-07 (25.2 ± 2.7 %*time), followed by NS AP-3 (17.6 ± 2.7 %*time). Disease progression was equal in NS York, S AP-3, S Florida-07, and S York (4.6 ± 2.7 %*time, 3.0 ± 2.7 %*time, 1.7 ± 2.7 %*time, and 1.3 ± 2.7 %*time, respectively), but was at a slower rate than the aforementioned cultivar*treatments (Figure 2-6B). Based on the results of this test, in terms of percent lesion coverage, under high LLS disease pressure, Florida-07 was more susceptible to LLS than AP-3. Disease progression, in terms of lesion density, was most rapid in NS Florida-07 (5.6 ± 0.3 lesions $cm^{-2}$*time), followed by NS AP-3 (3.9 ± 0.3 lesions $cm^{-2}$*time). Disease progression was slower in NS York, S AP-3, S Florida-07 (1.8 ± 0.3 lesions $cm^{-2}$*time, 1.1 ± 0.3 lesions $cm^{-2}$*time, and 0.8 ± 0.3 lesions $cm^{-2}$*time), followed by S York (0.6 ± 0.3 lesions $cm^{-2}$*time) (Figure 2-6C).

Based on results for percent lesion coverage and lesion density, under high disease pressure, Florida-07 was more susceptible than AP-3.

Rate of lesion growth was not affected by treatment, cultivar, or cultivar*treatment interaction. Lesion size increased at the same rate on all cultivars and treatments (Figure 2-6D).

**Yield response.** In Marianna 2009, pod yield varied by treatment and cultivar were significant main effects (P > F = 0.0166 and P > F = 0.0146, respectively). The difference in yield for S plots and NS plots was significant (3833 kg ha$^{-1}$ and 3098 kg ha$^{-1}$, respectively) (P > t = 0.0166). Florida-07 yielded (4122 kg ha$^{-1}$) higher than AP-3 (3082 kg ha$^{-1}$) (P > t = 0.0078), as well as York (3193 kg ha$^{-1}$) (P > t = 0.0142). AP-3 and York's yields were equal (P > t = 0.7311) (Table 2-6). As in the Citra 2008 test, in this test, Florida-07 produced higher pod yields than AP-3 (averaged over S and NS treatment, which indicates that Florida-07 possesses a higher genetic yield potential rather than tolerance to LLS).

To summarize the Marianna 2009 test, disease progression rate as measured by lesion percentage and lesion density showed that Florida-07 was more susceptible to LLS than AP-3. Despite foliar symptoms developing more rapidly in Florida-07 than AP-3, Florida-07's yields were higher than yields in AP-3. Florida-07 under higher disease pressure than AP-3 and possessing the ability to produce higher yields suggests that Florida-07 may have a degree of tolerance to LLS. However, this discrepancy Florida-07 and AP-3 yields is probably better explained by differences in genetic yield potential as was observed in the Citra 2008 test.

**All Years*Locations**

  **Disease progression.** Foliar lesions appeared in early September for all tests. Cultivar*treatment interaction was significant for each measure of disease progression unless otherwise noted.

  On average, means for AUDPC for the Florida 1-10 rating, lesion percentage, and lesion density indicate that Florida-07 and AP-3 are equally susceptible to LLS disease. In terms of the Florida 1-10 rating, disease progression was most rapid in NS Florida-07 and NS AP-3 (5.7 rating*time and 5.7 rating*time, respectively). Disease progression was slower and equal on S AP-3, S Florida-07, NS York, and S York (3.6 rating*time, 3.2 rating*time, 3.0 rating*time, 2.0 rating*time, respectively) (Figure 2-7A). Disease progression, as measured by lesion percentage, was most rapid for NS Florida-07 and NS AP-3 (24.6%*time and 20.3%*time, respectively). Disease progression was slower and equal on NS York, S Florida-07, S AP-3, and S York (9.5%*time, 7.4%*time, 7.3%*time, and 6.4%*time, respectively) (Figure 2-7B). Increase in lesion density throughout the season was most rapid in NS Florida-07 and NS AP-3 (6.0 lesions $cm^{-1}$*time and 4.6 lesions $cm^{-1}$*time, respectively). Disease progression occurred at a slower, but similar rate in NS York, S Florida-07, S AP-3, and S York (3.1 lesions $cm^{-2}$*time, 2.4 lesions $cm^{-2}$*time, 2.3 lesions $cm^{-2}$*time, 2.1 lesions $cm^{-2}$*time, respectively) (Figure 2-7C).

  As in all individual tests, lesion growth rate was equal in all treatments and cultivars. No main effects were significant (Figure 2-7D).

  **Yield response.** On average, in terms of yield, cultivar*treatment interaction was the only significant main effect (P > t = 0.0001). Yields under LLS pressure for S Florida-07 and S AP-3 were equal (4734 kg $ha^{-1}$ and 4092 kg $ha^{-1}$, respectively) (p>t =

0.2688). Likewise, yields under LLS pressure for NS Florida-07 and NS AP-3 were equal (3556 kg ha$^{-1}$ and 2527 kg ha$^{-1}$, respectively) (P > t = 0.0803). Yield lost to LLS were equal for Florida-07 and AP-3 (1177 kg ha$^{-1}$ and 1564 kg ha$^{-1}$, respectively) (p>t = 0.1563), as well as percent yield lost in Florida-07 and AP-3 (23.5 and 34.9, respectively) (P > t = 0.0894). S York and NS York's yields under LLS pressure were the same (2976 kg ha$^{-1}$ and 2663 kg ha$^{-1}$, respectively) (P > t = 0.2729), as is expected by a resistant cultivar (Table 2-7). York's yield lost to LLS and percent yield lost to LLS was less than those for Florida-07 and AP-3.

On average, Florida-07 displayed no tolerance to LLS. Disease progression was equal in Florida-07 and AP-3. The yield under LLS pressure for NS AP-3 and NS Florida-07 was the same. Likewise, the yield lost to LLS and the difference of percent yield loss between AP-3 and Florida-07 was the same (P > t = 0.1563 and P > t = 0.0894, respectively). However, these values approach statistical significance and it is possible that with additional testing, responses of Florida-07 and AP-3 would separate.

**Environmental Conditions**

In an effort to explain the highly variable yield response under high LLS pressure and to determine disease pressure, environmental data were collected using the Florida Automated Weather Network (FAWN). Environmental conditions required for rapid development and increased severity of LLS are warm temperatures and long periods of high humidity or leaf wetness. Differences in test site environment were determined by observing average daily leaf spot hours, percent relative humidity, and min/ max temperatures.

On average, Marianna 2009 had more daily leaf spot hours (12.1 hrs day$^{-1}$) compared to Marianna 2008 or Citra 2008 (9.2 hrs day$^{-1}$ and 10.2 hrs day$^{-1}$,

respectively). However, when analyzing individual components which comprise leaf spot hours, it appeared that Citra 2008 (80.7%, 20.9°C/32.1°C) and Marianna 2009 (83.5%, 20.4°C/31.8°C) had higher and warmer daily high/low temperatures when compared to Marianna 2008 (77.2%, 19.4°C/88.1°F) (Table 2-8). Overall, Citra 2008 and Marianna 2009 provided an environment more conducive to the rapid development and increased severity of LLS disease.

## Conclusions

Limited research has been conducted to identify and characterize tolerance as a mechanism for overcoming LLS disease (Pixley et al 1990). Previous research has primarily focused on identifying sources of resistance to leaf spot disease in peanut. Although resistant cultivars are available, many of these are derived from a similar genetic lineage and have several undesirable characteristics associated with their resistance, i.e. late maturity, large seeded, and poor germination (Morton, 2007). Tolerance provides an alternative to the limited genetic resistance available in cultivated peanut.

Based on the rate at which foliar disease symptoms progressed over time, it was concluded, that under high LLS pressure, AP-3 and Florida-07 showed the same degree of susceptibility. However, in specific tests and measurements of foliar disease progression, Florida-07 did appear to be more susceptible to LLS than AP-3. In all tests, the rate of lesion growth was equal for all treatments and cultivars tested. This result is likely due to the limited rate at which hyphae of *C. personatum* can grow and penetrate new tissue.

Yield response suggests that Florida-07 has a higher genetic yield potential than either York or AP-3. In this study, York yields were low due to poor germination which

led to poor plant stands. However, research has shown that when germination in York is high, yields were competitive with Florida-07. On average, Florida-07 did not display tolerance to LLS. However, in two of the three tests, pod yield of Florida-07 was greater than that of AP-3. The higher pod yield of Florida-07 is what led to it being mistakenly classified as a possible leaf spot tolerant cultivar. In this study, because of its higher yield potential, Florida-07 appeared to overcome the impact of leaf spot disease in two out of three tests, but in the third test, leaf spot impacted pod yield of Florida-07 and AP-3 equally.

However, Citra 2008 and Marianna 2009, the two tests in which Florida-07's higher yield potential became evident, had weather conditions were more conducive for the rapid development and increased severity of LLS. In an environment where rapid growth and development of leaf spot likely occurred, Florida-07 proved to be more resilient to LLS. Although Florida-07 does not fit the definition of tolerance described by Agrios (2005), it does provide a degree of protection for a grower by producing higher yields than other cultivars.

Based on this evidence, it was concluded that Florida-07 did not display tolerance to LLS disease. Therefore, no tolerance mechanisms were identified. However, compared to other LLS susceptible cultivars, Florida-07 possesses a high yield potential which can act as an "insurance policy" to growers.
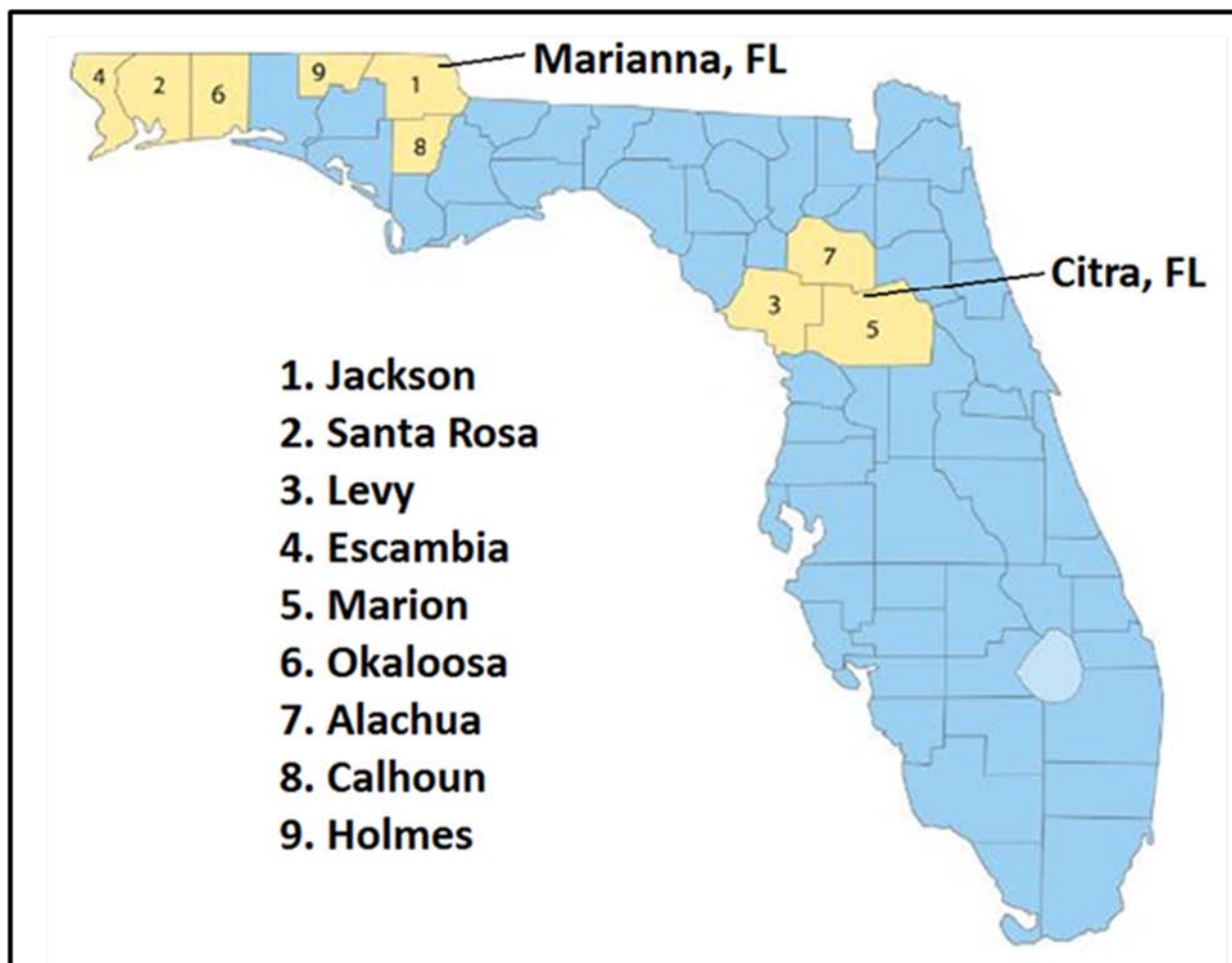
Figure 2-1.  Florida is divided into three peanut growing regions. Counties highlighted in yellow are ranked (1 – 9) by the acreage planted in peanut. Experimental locations, Marianna and Citra, are indicated on the map (modified from Mossler and Aerts, 2007).

Table 2-1.  Peanut cultivar descriptions.

| Cultivar | |
|---|---|
| York | -University of Florida, 2006<br>-89 x OL24-3-1-2-2-b2-B x C99-R<br>-Runner-type<br>-Late maturing (~150 days)<br>-High-oleic chemistry, resistance to TSWV & white mold<br>-If not sprayed to prevent LS = defoliation and lesion coverage minimal, lesions confined to upper canopy (moderate resistance) → reduced fungicide regime |
| AP-3 | -University of Florida, 2003<br>-OKFH15 x NC3033<br>-Runner-type<br>-Medium-late maturing (~140 days)<br>-High-oleic chemistry,  resistance to TSWV and white mold<br>- If not sprayed to prevent LS = high lesion coverage, premature defoliation, reduced yields |
| Florida-07 | -University of Florida, 2006<br>-89 x OL14-11-1-1-1-b2-B x C99-R<br>-Runner-type<br>-Medium-late maturing (~140 day)<br>-High-oleic chemistry, resistance to TSWV & white mold<br>- If not sprayed to prevent LS = high lesion coverage, premature defoliation,  yields higher than other susceptible cultivars |
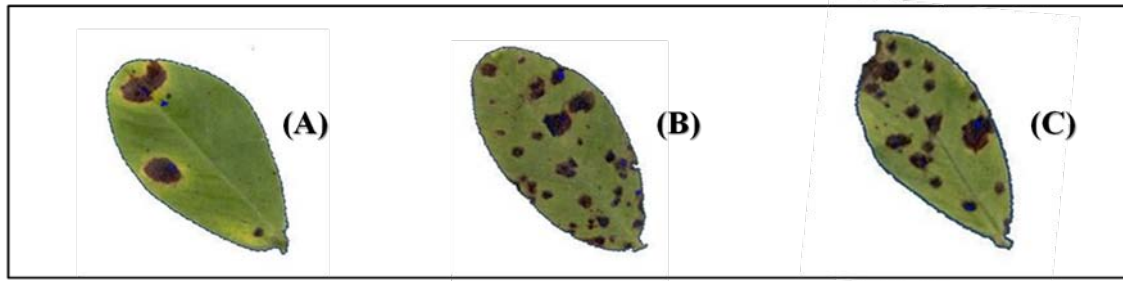
Figure 2-2.  Typical late-season, lateral-branch leaflet lesion coverage under high late
leaf spot pressure on (A) York, (B) AP-3, and (C) Florida-07 peanut cultivars
in Citra, Florida 2008 and Marianna, Florida 2008 and 2009.

Table 2-2.  Standard commercial fungicide spray treatments applied in Citra, Florida 2008 and Marianna, Florida 2008 and 2009. Treatments began approximately 30 days after planting and continued bi-weekly.

| | Commercial Name (rate ml ha-1) | |
| Treatment | Citra – 2008 | Marianna - 2008 & 2009 |
| --- | --- | --- |
| 1 | Bravo Weatherstik[1] (1753) | Bravo Weatherstik[1] (877) |
| 2 | Bravo Weatherstik[1] (1753) | Bravo Weatherstik[1] (877) |
| 3 | Headline[2] (296) | Bravo Weatherstik[1] (877) Tebustar[4] (213) |
| 4 | Abound[3] (532) | Bravo Weatherstik[1] (877) Tebustar[4] (213) |
| 5 | Bravo Weatherstik[1] (877) Folicur[4] (213) | Abound[3] (532) |
| 6 | Bravo Weatherstik[1] (877) Folicur[4] (213) | Headline[2] (296) |
| 7 | Bravo Weatherstik[1] (1753) | Bravo Weatherstik[1] (877) |
| 8 | Bravo Weatherstik[1] (1753) | --- |

Footer denotes active ingredient: [1]Chlorothalonil, [2]Pyralostrobin, [3]Azoxystrobin, [4]Tebuconazole

Table 2-3. Florida 1-10 leaf spot rating based on Chiteka et al. (1988)

| Rating | Description |
| --- | --- |
| 1 | No disease |
| 2 | Very few lesions (none on upper canopy) |
| 3 | Few lesions (very few on upper canopy) |
| 4 | Some lesions with more on upper canopy than rank for 3 and slight defoliation noticeable |
| 5 | Lesions noticeable even on upper canopy with noticeable defoliation |
| 6 | Lesions numerous on upper canopy with significant defoliation (50%+) |
| 7 | Lesions numerous on upper canopy with much defoliation (75%+) |
| 8 | Upper canopy covered with lesions with high defoliation (90%+) |
| 9 | Very few leaves remaining and those covered with lesions (some plants completely defoliated) |
| 10 | Plants dead |
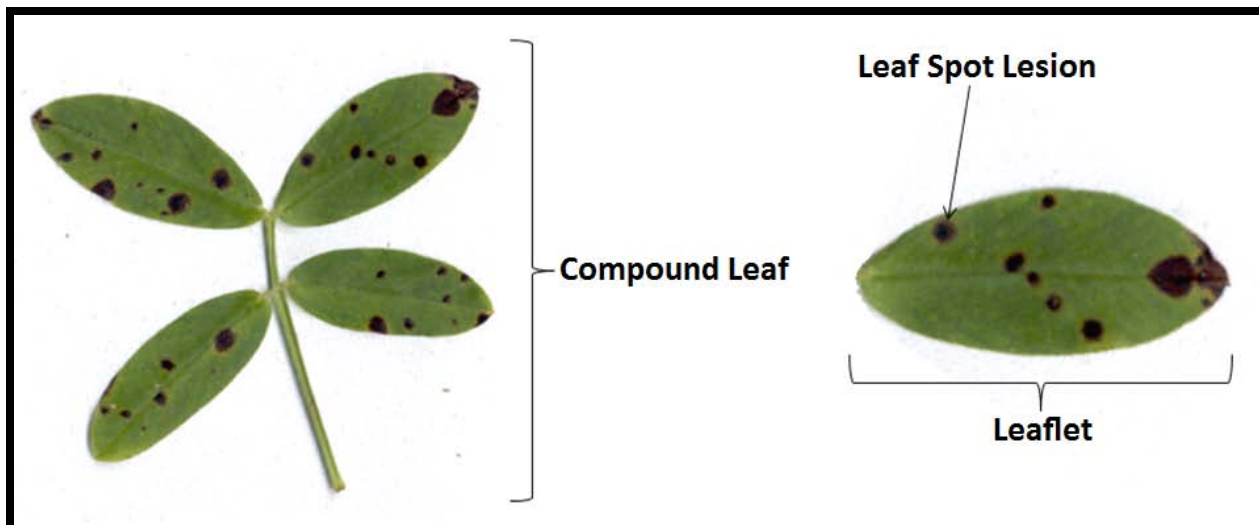
Figure 2-3.  Example of peanut leaf collection for evaluation of late leaf spot disease. Beginning at the first sign of leaf spot, compound leaves (40 compound leaves/plot = 160 leaflets) were collected weekly and scanned into APS Assess 2.0 (American Phytopathological Society) for image analysis. Tests were conducted in 2008 and 2009 in Citra, Florida and Marianna, Florida.
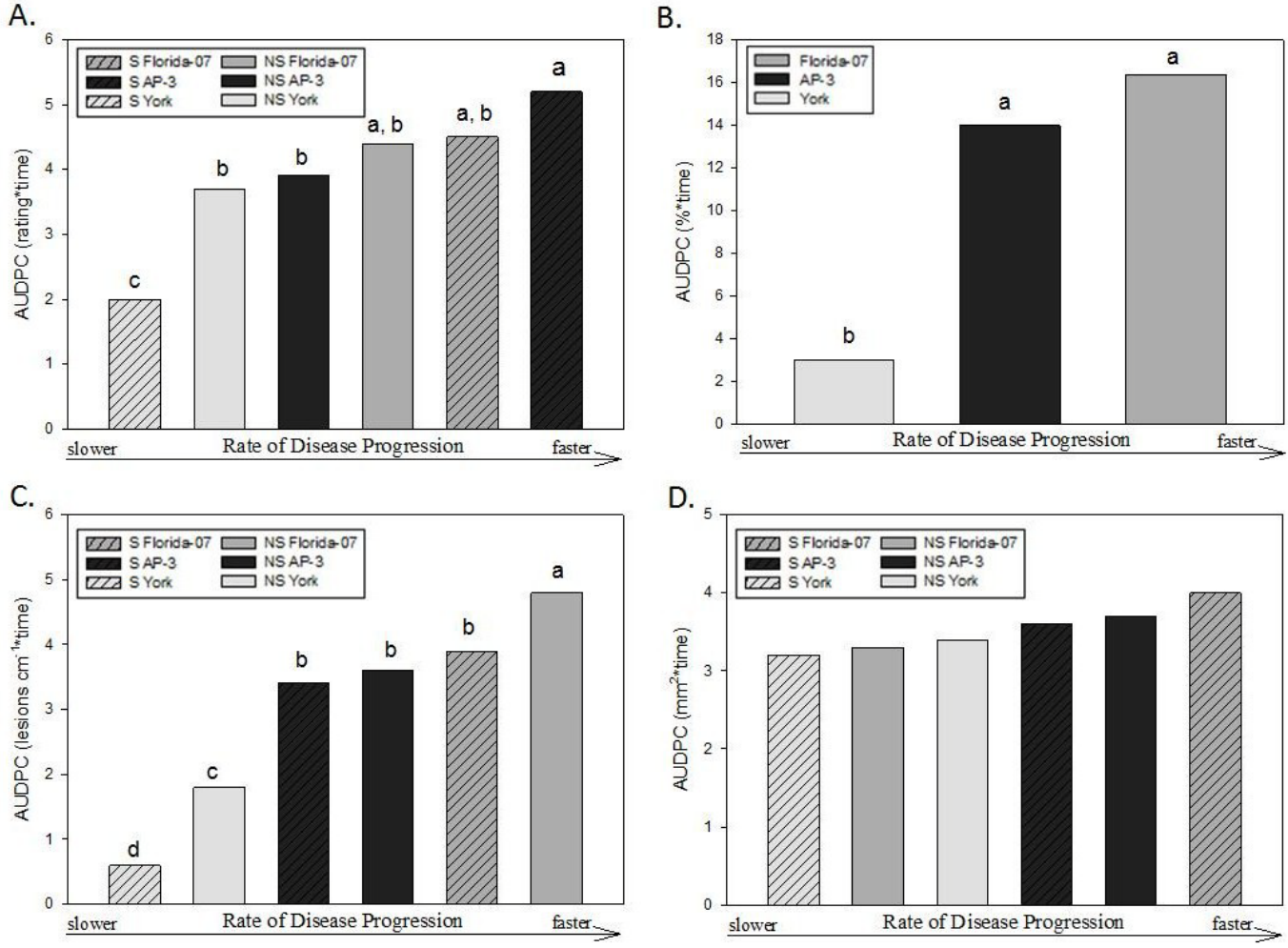
Figure 2-4.  Progression of late leaf spot disease of peanut based on AUDPC in Citra, Florida 2008 for (A) Florida 1-10 Rating, (B) lesion/leaf percentage, (C) lesion density, and (D) lesion growth (no significant main effects). Means with the same letter are not different at the P ≤ 0.05 level.
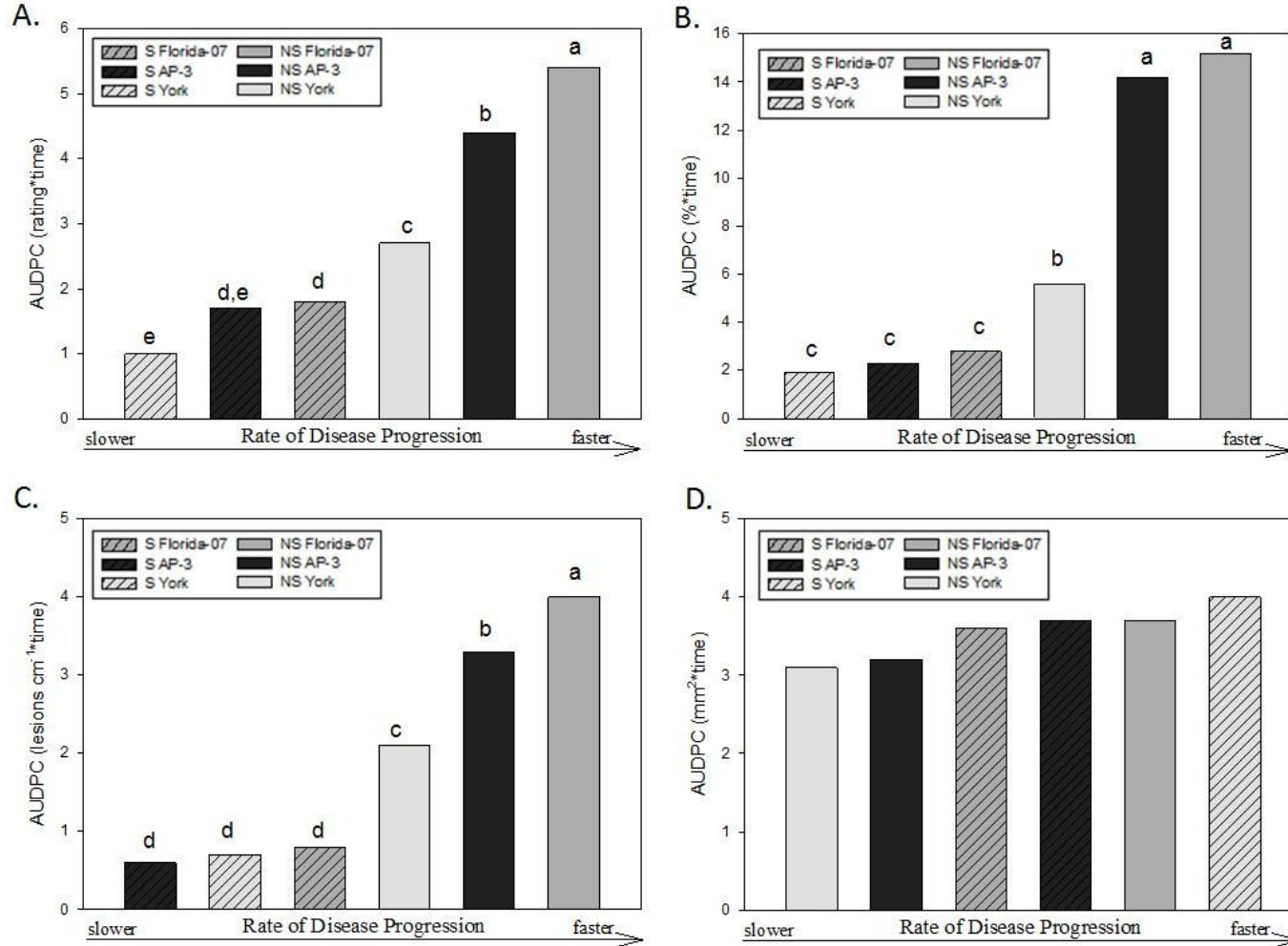
Figure 2-5. Progression of late leaf spot disease of peanut based on AUDPC in Marianna, Florida 2008 for (A) Florida 1-10 Rating, (B) lesion/leaf percentage, (C) lesion density, and (D) lesion growth (no significant main effects). Means with the same letter are not different at the P ≤ 0.05 level.
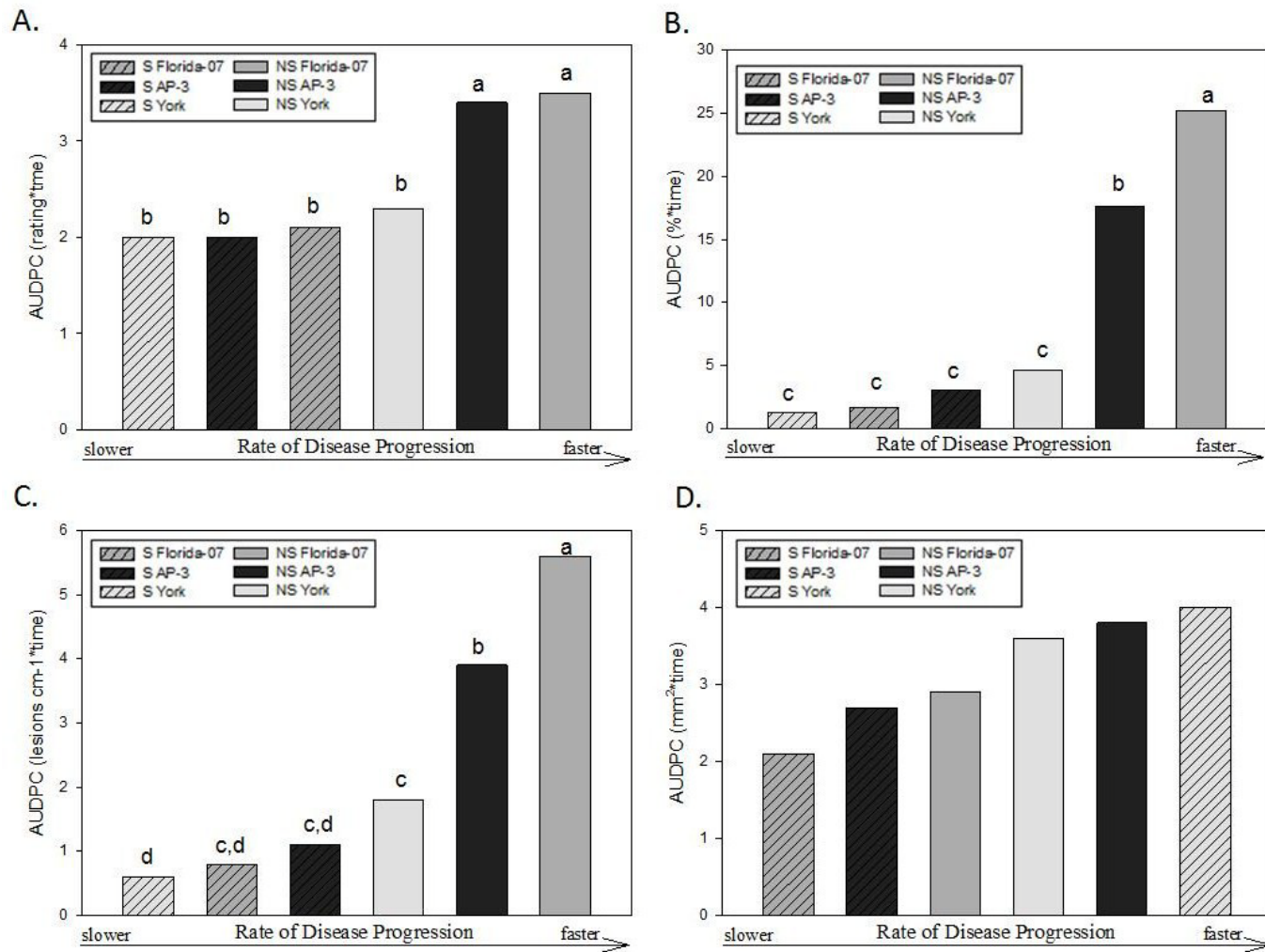
Figure 2-6. Progression of late leaf spot disease of peanut based on AUDPC in Marianna, Florida 2009 for (A) Florida 1-10 Rating, (B) lesion/leaf percentage, (C) lesion density, and (D) lesion growth (no significant main effects). Means with the same letter are not different at the P ≤ 0.05 level.
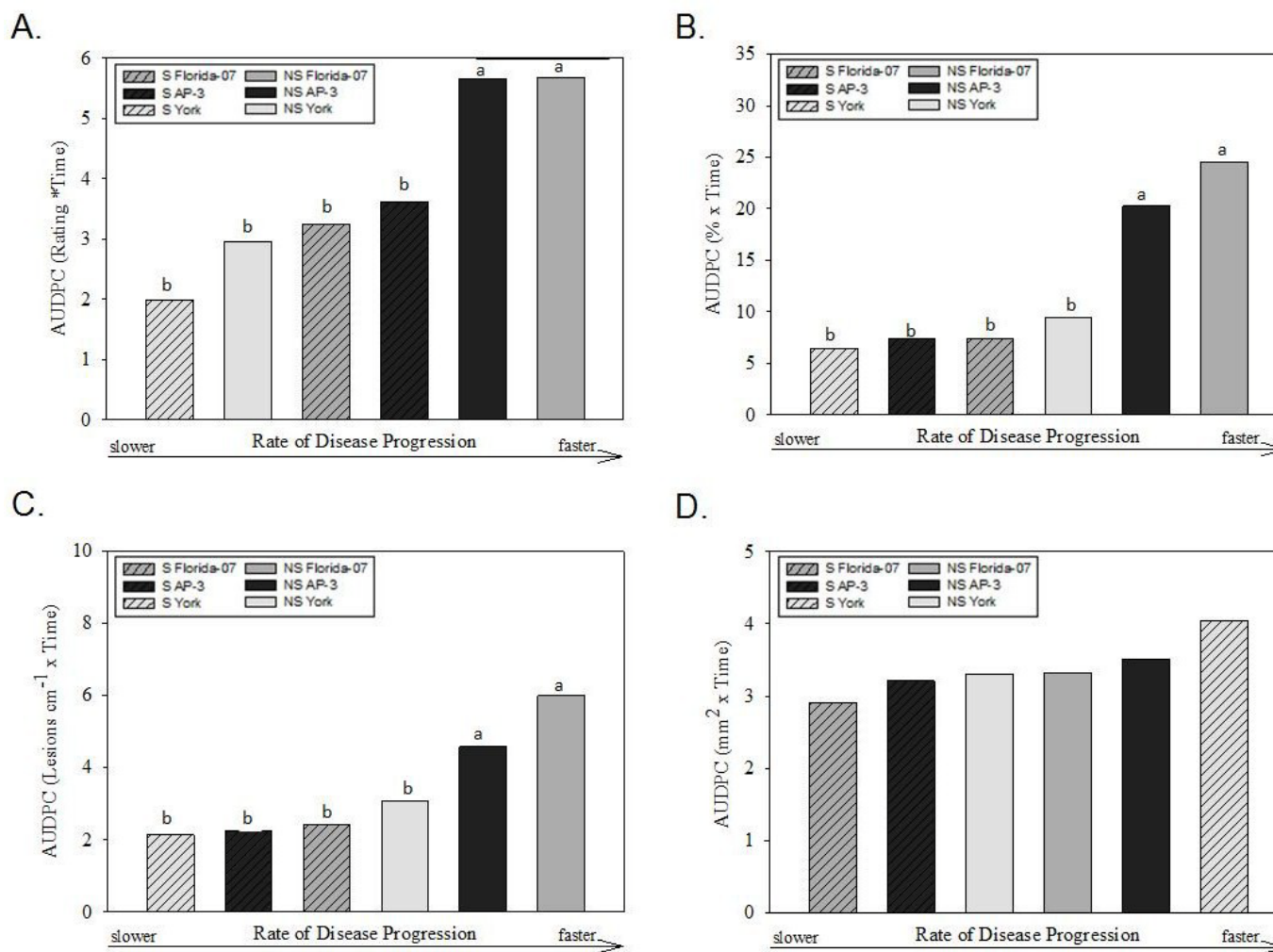
Figure 2-7. Progression of late leaf spot disease of peanut based on AUDPC in Citra, Florida 2008 and Marianna, Florida 2008 and 2009 for (A) Florida 1-10 Rating, (B) lesion/leaf percentage, (C) lesion density, and (D) lesion growth (no significant main effects). Means with the same letter are not different at the P ≤ 0.05 level.

Table 2-4.  Peanut pod yield and pod loss to late leaf spot disease in Citra, Florida in 2008.

| Cultivar | Spray | Yield Under LLS Pressure (kg ha$^{-1}$) | Yield Lost to LLS (kg ha$^{-1}$) | % Yield Lost to LLS |
|---|---|---|---|---|
| Florida-07 | S | 4806 ± 207 a | 1084 ± 207 b | 22.3 ± 9.8 a |
| | NS | 3722 ± 207 c | | |
| AP-3 | S | 4452 ± 207 b | 1991 ± 207 a | 44.8 ± 9.8 a |
| | NS | 2461 ± 207 d | | |
| York | S | 2429 ± 207 d | 109 ± 207 c | 0.9 ± 9.8 b |
| | NS | 2320 ± 207 d | | |

*Each column is a mean ± SE
**Means within columns followed by the same letter are not different at the P ≤ 0.05

Table 2-5.  Peanut pod yield and pod loss to late leaf spot disease in Marianna, Florida in 2008.

| Cultivar | Spray | Yield Under LLS Pressure (kg ha-1) | Yield Lost to LLS (kg ha-1) | % Yield Lost to LLS |
|---|---|---|---|---|
| Florida-07 | S | 4434 ± 168.29 a | 1648 ± 168 a | 37.2 ± 4.0 a,b |
| | NS | 2786 ± 168.29 c | | |
| AP-3 | S | 4601 ± 168.29 a | 1811 ± 168 a | 39.4 ± 4.0 a |
| | NS | 2790 ± 168.29 c | | |
| York | S | 3823 ± 168.29 b | 878 ± 168 b | 22.9 ± 4.0 b |
| | NS | 2946 ± 168.29 c | | |

*Each column is a mean ± SE
**Means within columns followed by the same letter are not different at the P ≤ 0.05

Table 2-6.  Peanut pod yield and pod loss to late leaf spot disease in Marianna, Florida in 2009.

| Cultivar | Spray | Yield Under LLS Pressure (kg ha-1) |
|---|---|---|
| | S | 3833 ± 183 a |
| | NS | 3098 ± 183 b |
| Florida-07 | | 4122 ± 223 a |
| AP-3 | | 3082 ± 223 b |
| York | | 3193 ± 223 b |

*Only treatment and cultivar were significant main effects.
**Each column is a mean ± SE
**Means within columns followed by the same letter are not different at the P ≤ 0.05

Table 2-7.  Peanut pod yield and pod loss to late leaf spot disease in Citra, Florida in 2008 and Marianna, Florida in 2008 and 2009.

| Cultivar | Spray | Yield Under LLS Pressure (kg ha$^{-1}$) | Yield Lost to LLS (kg ha$^{-1}$) | % Yield Lost to LLS |
|---|---|---|---|---|
| Florida-07 | S | 4734 ± 430 a | 1177 ± 430 a | 23.5 ± 8.9 a |
| | NS | 3556 ± 430 b | | |
| AP-3 | S | 4092 ± 430 a,b | 1564 ± 430 a | 34.9 ± 8.9 a |
| | NS | 2527 ± 430 c | | |
| York | S | 2976 ± 430 b,c | 314 ± 430 b | 6.3 ± 8.9 b |
| | NS | 2663 ± 430 c | | |

*Each column is a mean ± SE
**Means within columns followed by the same letter are not different at the P ≤ 0.05

Table 2--8.  Environmental conditions that impact leaf spot disease of peanut.

| Location | Year | %RH | Min. Temp. (°C) | Max. Temp. (°C) | Leaf Spot Hrs[1] (hrs/day) |
|---|---|---|---|---|---|
| Citra | 2008 | 80.7 ± 1.3 b | 20.9 ± 0.7 a | 32.1 ± 0.6 a | 10.2 ± 0.5 b |
| Marianna | 2008 | 77.2 ± 1.3 c | 19.4 ± 0.7 b | 31.2 ± 0.6 b | 9.2 ± 0.5 b |
| Marianna | 2009 | 83.5 ± 1.3 a | 20.4 ± 0.7 a, b | 31.8 ± 0.6 a, b | 12.1 ± 0.5 a |

*Mean ± SE.
**Means within individual columns followed by the same letter are not different at the P ≤ 0.05.
[1]Leaf Spot Hrs = 1 hour with percent relative humidity greater than or equal to 90% and temperatures between 16°C and 30°C.

CHAPTER 3
# A DIRECT SHOOT ORAGANOGENESIS SYSTEM FOR U.S. PEANUT CULTIVARS

**Abstract**

One of the most successful methods for producing transgenic peanut is particle bombardment of somatic embryos. A major disadvantage of this approach is the time required to produce mature plants (eight to 12 months). An alternative to lengthy bombardment and regeneration protocols is *Agrobacterium*-mediated transformation employing direct shoot organogenesis. This strategy allows for mature, transgenic plants to be obtained quickly (three to four months). Peanut cultivars, Florida-07 (Runner), Georgia Green (Runner), Georgia Brown (Spanish), Valencia-A (Valencia), and VC-2 (Virginia), were selected to represent all four market types. Two types of cotyledon explants were examined, those that previously had an attached embryo-axis upon cotyledon separation (explant A) and those that were embryo-axis-free upon separation (explant B). Explants were placed on shoot induction medium (MS salts, B5 vitamins, 3% sucrose, 0.8% agar, 10 µM 2,4-D, pH 5.8) with N6-benzyladenine (BA) concentrations ranging from 10 µM - 80 µM for Florida-07, Georgia Green, and VC2, 10 µM - 320 µM for Georgia Brown, and 10 µM - 640 µM for Valencia-A. Following a four-week culture period, explants were visually rated based on a scale of 1 to 4, where 1 indicated slight greening, but no growth; 2 indicated greening, with callus-like growth, but no adventitious bud formation; 3 indicated greening and adventitious bud formation; and 4 indicated greening, adventitious bud formation, as well as small leaflet expansion. A difference in shoot induction was observed for the cotyledon explants examined ($P > t$ = <0.0001). Explant A had greater shoot induction with a visual rating of $1.8 \pm 0.1$, while explant B had a rating of $1.6 \pm 0.1$ ($P > t$ = <0.0001). Additionally, cultivars responded

to the culture conditions differently (cultivar * BA interaction). Georgia Green on 10 µM

BA produced the most shoot buds (24.6%) and the highest visual rating (2.1), followed

by VC2 on 10 µM BA (22.1%, 1.8), Valencia-A on 640 µM BA (21.4%, 1.8), Georgia

Brown on 80 µM BA (9.0%, 1.7), and Florida-07 on 40 µM BA (7.1%, 1.8). Of the tested

varieties, Georgia Green, Valencia-A and VC2 were best suited for future transformation

experiments based on their shoot bud production.

## Introduction

Peanut production and its associated industries are important to the overall

economic prosperity of many rural areas in the southeastern U.S. The peanut industry

generates approximately $4 billion annually for the U.S. economy. Throughout the

growing season, peanut growers are faced with many biotic and abiotic threats that can

lower yields and ultimately profit. Presently, conventional breeding is the primary means

to overcome these threatening factors. Through use of conventional breeding

techniques, both cultivated and wild *Arachis* species have been used to develop

agronomically superior cultivars. However, conventional breeding is a slow and difficult

endeavor due to reproductive barriers, failure of interspecific crosses, and transfer of

undesirable traits. Recently, there has been an increased interest in using genetic

transformation to circumvent some of the problems associated with traditional breeding.

Although several studies report the successful transformation of peanut, no single

protocol has proven to be highly efficient in the number of transgenic lines recovered.

Furthermore, many of the studies used lengthy somatic embryogenesis protocols

requiring eight to 12 months to generate mature plants. This inefficient use of time and

poor *in vitro* conversion into whole, mature, seed-bearing plants, has led to the

investigation of alternative organogenesis protocols that can be successfully used in *Agrobacterium* transformation studies.

Sharma and Anjaiah (2000) reported an efficient method (> 90%) for the production of adventitious shoot buds using mature seed explants on MS medium (Murashige and Skoog, 1962) supplemented with 20 µM N6-benzyladenine (BA) and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). Combinations of BA (2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0 µM) and 2,4-D (1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 µM) were tested with six Indian cultivars belonging to the Spanish (JL-24, J-11, ICGS-11) and Virginia (Robut-3-11, ICGS-76, ICGS-44) market types. These six peanut varieties produced shoot buds with high frequencies (80.0 – 97.7%) and followed a similar pattern of growth and development on each medium formulation. Shoot proliferation appeared to be most dependent upon BA concentration. Of the six test cultivars, Sharma and Anjaiah (2000) reported that JL-24 performed the best. JL-24 is a cultivar widely grown in India, but is not readily available in the U.S. The goal of this research was to optimize direct shoot organogenesis culture conditions for use with readily available, regionally, and economically important U.S. cultivars (Georgia Green, Florida-07, Georgia Browne, VC-2, and Valencia-A). It was hypothesized that the direct shoot organogenesis protocol described by Sharma and Anajaiah (2000) could be optimized for U.S. peanut cultivars representing each market type.

## Materials and Methods

### Cultivar Selection

Peanut cultivars representing the four market types were evaluated for their potential for *in vitro* direct shoot organogenesis from cotyledon explants. Florida-07 (Gorbet and Tillman, 2009) and Georgia Green (Branch, 1996), Runner market types,

were selected because the former was a recent release by the University of Florida with many agronomically favorable traits, including high oleic chemistry, and the latter was, until recently, the most widely grown cultivar in the U.S. Georgia Browne (Branch, 1994), a Spanish market type, was selected based on its availability, and because it is one of a very few Spanish types grown in the southeastern U.S. Valencia-A (His et al. 1972), a Valencia market type, was selected because of its successful use in previous transformation studies (Cheng et al. 1996, 1997; Egnin et al. 1998; Eapen and George 1994; Li et al. 1997). VC-2 (AgraTech Seed, Golden Peanut Company, LLC), a Virginia market type, was selected because it is widely cultivated in the Virginia-Carolina U.S. peanut growing region.

**Explant Preparation**

The direct shoot organogenesis protocol used followed that described by Sharma and Anajaiah (2000) with modifications described below. For all experiments, prior to use, mature seeds were surface-sterilized by soaking in 70% ethanol for 1 min., followed by a wash for 10 min in 0.1% (w/v) mercuric chloride solution. Following this wash, seeds were rinsed five times in sterile-distilled water and then allowed to soak in sterile-distilled water for four hrs before further use. With forceps and under aseptic conditions, seed coats were carefully removed. Cotyledons were separated into two halves. The cotyledon half containing the embryo axis was designated as "cotyledon A", while the cotyledon without the embryo axis was designated as "cotyledon B". Using a scalpel and forceps, the embryo axis was removed from cotyledon A and discarded. Both cotyledons were then cut into vertical halves to obtain quartered-cotyledon explants (Figure 3-1). The proximal, freshly cut edge of each explant was then embedded into shoot induction medium (SIM; MS salts [Sigma, St. Louis, MO, USA], B5

vitamins, 3% (w/v) sucrose [Fisher Scientific, Hampton, NH, USA], 0.8% (w/v) agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, St. Louis, MO, USA), and either 10, 20, 40, 80, 160, 320, or 640 µM N6-benzyladenine (BA) [Sigma, St. Louis, MO, USA], pH 5.8) at a slight downward angle. Since Sharma and Anjaiah (2000) reported that increased 2,4-D concentrations showed no significant increase in shoot bud formation, 2,4-D concentrations remained at 10 µM for all media formulations. Four cotyledon explants (one whole seed) were placed onto 25 mm Petri dishes containing approximately 50 ml SIM medium.

**Experimental Design**

Each experiment consisted of 40 cotyledon explants (10 seeds). Cultures were sealed and allowed to incubate at 26 ± 1°C under continuous light of 100 µEs$^{-1}$ m$^{-2}$ irradiance for four weeks. Following the four-week shoot induction period, explants were evaluated for direct shoot organogenesis (DSO) on a scale of 1 - 4 for adventitious bud formation (Figure 3-2).  Shoot induction percentage (SI %) was determined for each BA level*cultivar interaction. SI% represented cultures that were capable of moving into the shoot elongation phase (percentage of explants receiving a rating of > 2).

**Evaluation of Cotyledon Explant Source**

Explants from each cultivar were prepared as described above. Cotyledons A and B were cut in half vertically to obtain quartered-cotyledon explants and placed on culture plates containing SIM medium. SI% and DSO rating were determined following a four week culture period.

**Evaluation of Shoot induction and Direct Shoot Organogenesis**

The five previously mentioned cultivars were prepared as described above.

Explants were evaluated for DSO rating and SI% on SIM medium supplemented with

BA at 10 µM (SIM10), 20 µM (SIM20), 40 µM (SIM40), and 80 µM (SIM80). Explant

response was evaluated following a four-week culture period. For cultivars that

responded with a strong linear trend within the 10-80 µM BA range, BA concentrations

were increased until a quadratic (normal) distribution was observed. The assumption

was that shoot induction response should fit a normal distribution, with optimal response

being at the peak of the quadratic curve. Consequently, BA levels for Georgia Green

were tested at 160 µM (SIM160) and 320 µM (SIM320), while BA levels for Valencia-A

were tested at 160 µM, 320 µM, and 640 µM (SIM640). Following a four-week culture

period, explants were evaluated by DSO rating and SI%.

**Regeneration of Mature Plants**

Explants bearing shoot buds were transferred to shoot elongation medium (SEM;

MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific,

Hampton, NH, USA], 0.8% (w/v) agar (Becton, Dickinson and Co., Franklin Lakes, NJ,

USA), and 2 µM BA [Sigma, St. Louis, MO, USA], pH 5.8). Elongated shoots were sub-

cultured twice, every four weeks to fresh SEM (or when shoot length was approximately

2-3 cm in length). Elongated shoots were then placed onto root induction medium (RIM;

MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific,

Hampton, NH, USA], 0.8% (w/v) agar (Becton, Dickinson and Co., Franklin Lakes, NJ,

USA), and 5 µM 1-Naphthaleneacetic acid (NAA) [Sigma, St. Louis, MO, USA], pH 5.8).

Cultures undergoing selection and rooting were maintained at 26°C (± 1°C) under

continuous light of 100 $\mu Es^{-1}\ m^{-2}$ irradiance. Once roots were established, plants were

transferred to pots containing a 2:1 Fafard #2 : sand mixture [Fafard, Agawam, MA, USA]. Plants were hardened under growth chamber conditions maintained at 26 ± 1°C under a 14 h light to10 h dark regime with the light set to 100 $\mu Es^{-1}$ $m^{-2}$ irradiance. Plants reaching maturity were moved to the greenhouse and fertilized and irrigated as needed (Figure 3-3).

**Statistical Analysis**

SI% was determined for each BA level*cultivar by using the frequency procedure (PROC Freq) in SAS software (SAS Institute, 2000). Analysis of variance was carried out on the means for each experimental component (explant type, DSO rating, and SI%) using the Mixed Model procedure (PROC Mixed) in SAS software (SAS Institute, 2000). Statistical significance was determined at $P \leq 0.05$ according to Tukey's HSD mean separation test.

<center>**Results and Discussion**</center>

**Explant Response**

In general, across all cultivars and BA concentrations, explants producing adventitious shoot buds responded as described by Sharma and Anjaiah (2000), but at a lower frequency. Sharma and Anjaiah (2000) reported shoot induction frequencies as high as 95%; the current studies highest incidence of shoot induction was 25%. However, the appearance of those explants developing shoot buds was similar to that described in Sharma and Anjaiah (2000). On SIM, explants turned green and underwent considerable enlargement within the first week of culture initiation. During weeks two and three, multiple shoot buds formed at the proximal cut end of the explants (Figure 3-3A).

In the present study, shoot bud induction was tightly confined to the proximal portion of each explant. Shoot buds developing on the proximal end of the explants were small and too numerous to count (Figure 3-3B). Tiwari et al. (2009) reported a similar response and counted up to 100 buds per explant. Other studies have provided data to explain the highly prolific nature of the proximal region of cotyledon explants. Sujatha et al. (2008), using a direct shoot organogenesis protocol, tested three cotyledon segment types (proximal, middle, and distal) of *Pongamia pinnata*, a tree legume. This study concluded that the proximal cotyledon section, followed by the middle and distal sections, were most responsive in terms of producing shoot buds. These results suggest that there is a gradient of cells within cotyledon tissue that is likely to dedifferentiate, with those cells nearest the proximal region being more likely to become meristematic. Further supporting this observation of cotyledon gradient competence, using serial sections of peanut cotyledons, Victor et al. (1999) saw an increase in meristematic conversion in the epidermal and sub-epidermal cell layers as the sections approached the hypocotyledonary notch region when exposed to thidiazuron and BA.

In Sharma and Anjaiah (2000), whole cotyledon explants were compared to vertically cut, cotyledon halves. Both explants produced shoots at high frequencies, but the number of shoots per responding explant was much higher when the cotyledons were vertically split into halves. The corresponding half of each split cotyledon responded similarly relative to induction frequency and the number of shoots per explant. However, in the present study there was a significant difference in SI% and DSO rating of the two split cotyledon explant sources. A difference in shoot induction

was observed for each type of cotyledon explant examined regardless of cultivar.

Explant A had a higher DSO rating (1.8) and higher SI% (12.8%) than explant B (1.6, and 6.7%, respectively) (P > t = >0.0001) (Figure 3-4). It has been demonstrated across several species that cotyledons have a high capacity for an organogenic growth response (Dunstan and Thorpe, 1986), but, as previously mentioned, this morphogenic potentiality is not uniform across different cotyledonary tissues. Previous work, using *Dalbergia sissoo*, a tree legume (Singh et al. 2002), almond (*Prunus dulcis* Mill.) (Ainsley et al. 2001), and cherry (*Prunus*) (Hokanson and Pooler, 2000), demonstrated that the region of the cotyledon in closest contact with the embryo displayed the highest organogenic capacity. It was observed in the present study, upon embryo axis removal from cotyledon A, that a small amount of embryo axis tissue usually remained at the proximal portion of the cotyledon. Explant A's closer association with the embryo axis, fits the description of Hokanson and Pooler (2000) and provides a plausible explanation for the difference in SI% and DSO rating between the explants.

**Genotype Response**

Based on the findings by Sharma and Anjaiah (2000) that medium supplemented with 20 µM BA led to the highest incidence of adventitious bud formation in peanut, the present study tested BA concentrations ranging 10 - 640 µM to determine the best level for shoot induction response of the five selected cultivars. Cultivars responded to all the BA levels tested producing adventitious shoot buds, but cultivars responded differently to culture treatments (Table 3-1; Figure 3-5).

**Florida-07**. For BA concentrations ranging from 10 - 80 µM, Florida-07's DSO response was quadratic (normal) (p > t = 0.0051) (Table 3-1).  The highest observed DSO rating for Florida-07 was on SIM40 (1.8), which was higher than DSO ratings on

SIM10 (1.5), SIM20 (1.5), and SIM80 (1.5) (Figure 3-5A). The highest observed SI% for Florida-07 was also on SIM40 (7.1%), but was not different than the SI% on SIM10 (0.9%), SIM20 (2.8%), or SIM80 (0.0%) (Figure 3-5B).

**Georgia Green**. Georgia Green had neither a linear nor quadratic DSO trend (p>t = 0.6191, and p>t = 0.8416, respectively), but it had a strong cubic DSO (P > t = 0.0001) (Table 3-1). No biologically relevant cause could be deduced for this trend which was repeatable. SIM40 and SIM10 produced the highest DSO ratings for Georgia Green (2.2, and 2.1, respectively). SIM80 and SIM20 produced similar DSO ratings (1.9, and 1.8, respectively), that were lower than the ratings on SIM40 or SIM10 (Figure 3-5A). No differences were observed in SI% (Figure 3-5B).

**Georgia Browne**. Georgia Browne responded with a strong linear DSO trend for BA concentrations of 10 - 80 µM (P > t = <0.0001) (Table 3-1). The highest DSO ratings were on SIM80 (1.7) and SIM40 (1.6). The DSO rating on SIM80 was higher than ratings on SIM20 (1.5, p>t = 0.0021) or SIM10 (1.5). However, its DSO response on SIM40, was the same as on SIM20 (1.5) and SIM10 (1.5) (Figure 3-5A). The highest SI% was on SIM10 (9.1%), followed by the SI% on SIM80 (9.0) and SIM20 (3.8) (Figure 3-5B).

To normalize the linear DSO response trend between 10 – 80 µM, the BA concentration range was increased with levels of 160 µM and 320 µM. Within the 10 - 320 µM BA range, Georgia Browne had a strong, quadratic DSO trend (P > t = <0.0001) (Table 3-2). Its DSO rating on SIM160 (1.5) was higher than on SIM320 (1.3) (Figure 3-6A). Likewise, the SI% for Georgia Browne was much higher on SIM160 (6.9%) than

SIM320 (0.6%) but neither was higher than those produced on SIM10 and SIM80 (Figure 3-6B).

**Valencia-A**. Valencia-A responded with a strong linear trend within the 10-80 µM BA range (P > t = <0.0001) (Table 3-1). When the BA concentration was extended up to 640 µM a linear trend was still observed (P > t = <0.0001), as well as a weaker quadratic trend (P > t = 0.0021) (Table 3-2). This quadratic trend indicates diminishing returns. Valencia-A had the same DSO rating on SIM80 (1.7), SIM40 (1.7), and SIM20 (1.7), all of which were higher than DSO rating on SIM10 (1.4) (Figure 3-5A). Although the highest SI% was produced on SIM80 (8.1%), this was not different than the SI% on SIM10 (4.6%), SIM20 (5.3%), or SIM40 (4.3%) (Figure 3-5B).

Attempts to normalize the linear DSO response trend were made by increasing BA concentrations to 160 µM, 320 µM, and 640 µM.  Within this 10-640 µM, Valencia-A still responded with a strong linear trend (P > t = <0.0001) (Table 3-2). BA concentrations were not extended beyond 640 µM, because the saturation point was met and medium components precipitated out of solution. No differences were observed in DSO rating between 160 – 640 µM BA (Figure 3-6A). However, Valencia-A's SI% was higher on SIM640 (21.4%) than on SIM160 and SIM320 (Figure 3-6B).

**VC-2**. DSO ratings were similar for VC-2 on all BA concentration tested (Figure 3-4A). Its highest SI% was on SIM10 (22.1%), followed by SIM20 (19.0%), SIM80 (13.9%), and SIM40 (13.4%). Although a decreasing trend was observed for SI%, there was no significant difference among the treatments (Figure 3-4B).

Sharma and Anjaiah (2000) and Tiwari and Tuli (2008) failed to report the statistical difference in SI% between hormone concentrations, but, in general, reported

higher SI% than the present study. Sharma and Anjaiah (2000) and Tiwari and Tuli (2008) also failed to describe the difference in shoot bud appearance (quality). In the present study, regardless of BA concentration, SI% (percentage of explants developing shoot buds) generally appeared to be similar within cultivars. However, DSO rating (measure of quality of shoot buds produced by explants) varied within cultivars. In the present study, the quality of shoot buds at each concentration appeared to be dependent upon BA level (Tables 3-3 and 3-4, Figures 3-5 and 3-6). Similarities in SI% across BA concentrations may suggest that the threshold for growth response may be met at low BA levels. However, based on differences in DSO ratings of the tested BA levels, it is believed that BA concentration plays a significant role in the quality of growth response.

**Cultivar Comparison**

A comparison of the top-performing cultivar*BA level from this study suggest a genotypic influence on growth response (Table 3-3). When comparing tissue culture responses among cultivars, Georgia Green on SIM10 had the highest SI% (24.6%) and the highest DSO rating (2.1), followed by VC-2 on SIM10 (22.1%, 1.8), Valencia-A on SIM640 (21.4%, 1.8), Georgia Browne on SIM80 (9.0%, 1.7), and Florida-07 on SIM40 (7.0%, 1.8) (Table 3-3). Statistically, Georgia Green, Valencia-A, and VC-2 had an equal SI% response, but were higher than Florida-07 and Georgia Browne, which were equal. Georgia Green had the highest DSO rating which was higher than Florida-07, Georgia Browne, Valencia-A, and VC-2, which were all equal (Table 3-3).

Previous studies have only tested Spanish and Virginia market type cultivars. In these studies, Spanish market types, specifically the cultivar JL-24, have performed best in terms of shoot induction response. In the present study, the selected Spanish

market type, Georgia Browne, was one of the poorest performing cultivars. However, it should be pointed out that Georgia Browne is closely related to Georgia Green, a Runner market type, and is not a traditional Spanish market type. Future work should use multiple cultivars from each market type to identify if response is similar at the market type level. However, because of the discrepancy in response by Georgia Browne and Georgia Green, the author feels that shoot induction response is likely genotype dependent.

Earlier studies on peanut organogenesis have also reported a strong genotypic influence on shoot induction (Mroginski et al. 1981, Seitz et al. 1987, McKently et al. 1990, Chengalrayan et al. 2004, Banerjee et al. 2007, Matand et al. 2007). In contrast, Li et al. (1994), Sharma and Anjaiah (2000), and Tiwari et al. (2008, 2009) reported that all tested genotypes responded equally in organogenic response. Tiwari et al. (2009) suggests that this discrepancy in findings may be due to the extent of diversity among the selected genotypes from different studies.

**Regeneration of Mature Plants**

In the present study, data were collected only for shoot induction response, as prolific shoot bud induction is the most critical component for *Agrobacterium* transformation protocols. Although no data were collected post-shoot induction, shoot elongation and rooting portions on the protocol were carried out (Figures 3-3C and 3-3D). Preliminary results indicated that mature plants could be generated for all the tested cultivars at all BA concentrations examined using the described protocol (Figure 3-3E). It appeared that BA in shoot induction medium did not adversely affect shoot elongation and rooting of plantlets, although further testing is required to make a definitive conclusion. Despite phenotypically normal plants being generated in this

study, previous studies have shown that the use of cytokinin growth regulators at high concentrations (0.5-10 mg L$^{-1}$) can lead to residual toxicity which will inhibit or delay the efficiency of shoot elongation and/or root formation (Harris and Hart, 1964; Gray et al.1991; Preece and Imel, 1991, Chandra et al. 2003). Because of this inhibitory effect, cytokinins are usually removed from culture media during later stages of the tissue culture process. Frequently, more than one subculture to a cytokinin-free medium may be required until the level of cytokinin within the tissues has been sufficiently reduced. The need for multiple rounds of subculturing on hormone-free medium suggests that residual cytokinin can persist in adventitious tissue. Based on these previous findings, it was determined that using the lowest BA concentration capable of inducing the desired shoot induction response would be the best option for generating mature peanut plants in future studies. Therefore, Georgia Green and VC-2 on SIM10, Florida-07 on SIM40, Georgia Browne on SIM80, and Valencia-A on SIM640 should be the preferred cultivar*BA concentration combinations used for producing transgenic lines in the future.

## Conclusions

A difference in shoot induction was observed for each type of cotyledon explant examined. Because adventitious shoot bud formation was confined to the proximal region of explants and explant A had a higher SI% and DSO rating, it was concluded that the cotyledon nearest the embryo axis is most likely to de-differentiate and become meristematic. Because shoot induction was higher and of better visual quality for explant A, it was determined that it should be the only explant type used in direct shoot organogenesis for future *Agrobacterium*-mediated transformation studies.

All tested BA levels and cultivars produced adventitious shoot buds, indicating that this protocol is adaptable to a wide array of market types and cultivars. However, there

was a genotype effect because the cultivars responded differently in culture. Georgia

Green on SIM10 had the highest SI% and DSO rating followed by VC-2 on SIM10,

Valencia-A on SIM640, Georgia Brown on SIM80, and Florida-07 on SIM40.

Furthermore, similarities in SI% across BA concentrations indicate that the threshold for

explant growth response can be met at low BA levels. However, differences in DSO

ratings indicate that BA level does play a significant role in the overall quality of the

growth response. Cultivars Georgia Green, Valencia-A and VC-2 appear to be the best

suited for future transformation experiments based on their shoot bud production.
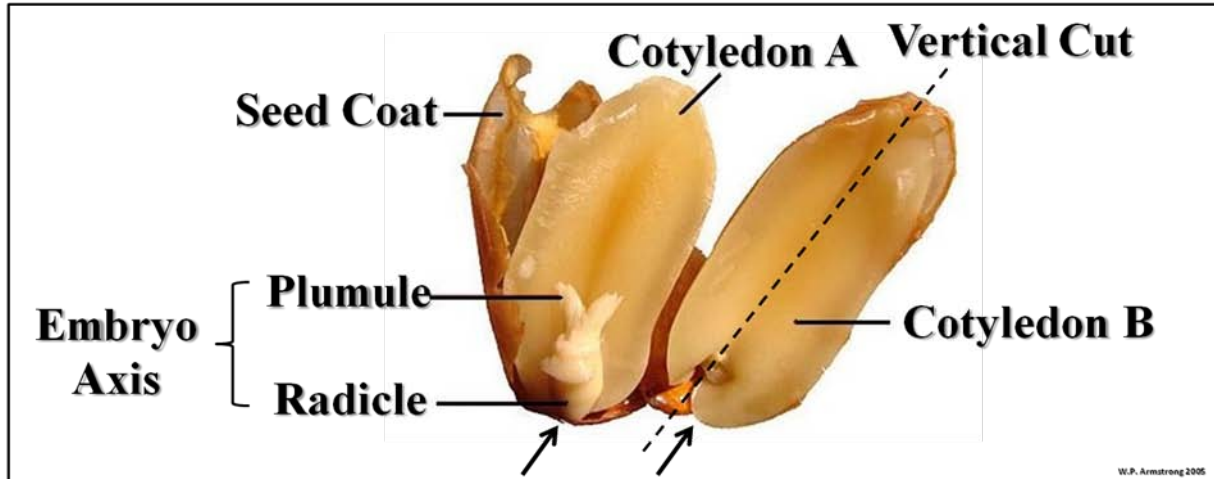
Figure 3-1.  Peanut seed morphology and cotyledon explants preparation. Arrows indicate the proximal end with high regeneration potential. Explants prepared in the following order: (1) Seed coat removed; (2) Cotyledons separated; (3) Embryo axis removed and cotyledon vertically cut, forming explants A; (4) Remaining cotyledon vertically cut, forming explants B. (Photo modified from Armstrong, 2008).
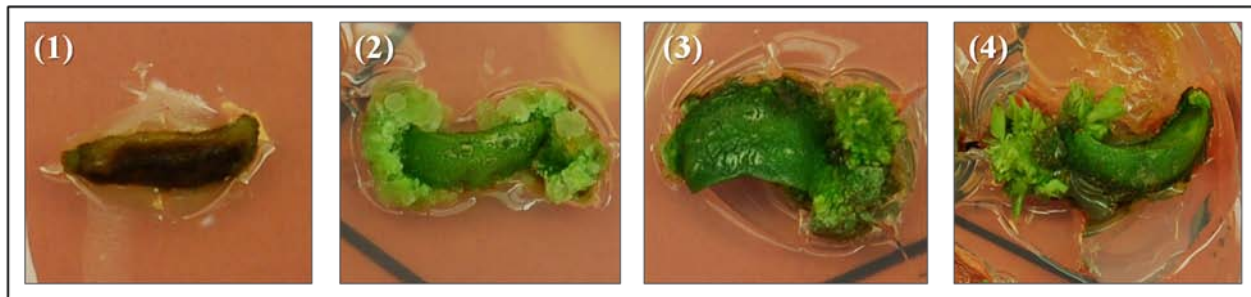


Figure 3-2.  Direct shoot organogenesis (DSO) rating of peanut explants. (1) Slight greening of explants, with no growth; (2) Greening of explants, with callus-like growth, and no adventitious bud formation; (3) Greening of explants, with adventitious bud formation; (4) Greening of explants, with adventitious bud formation, and small leaflet expansion.
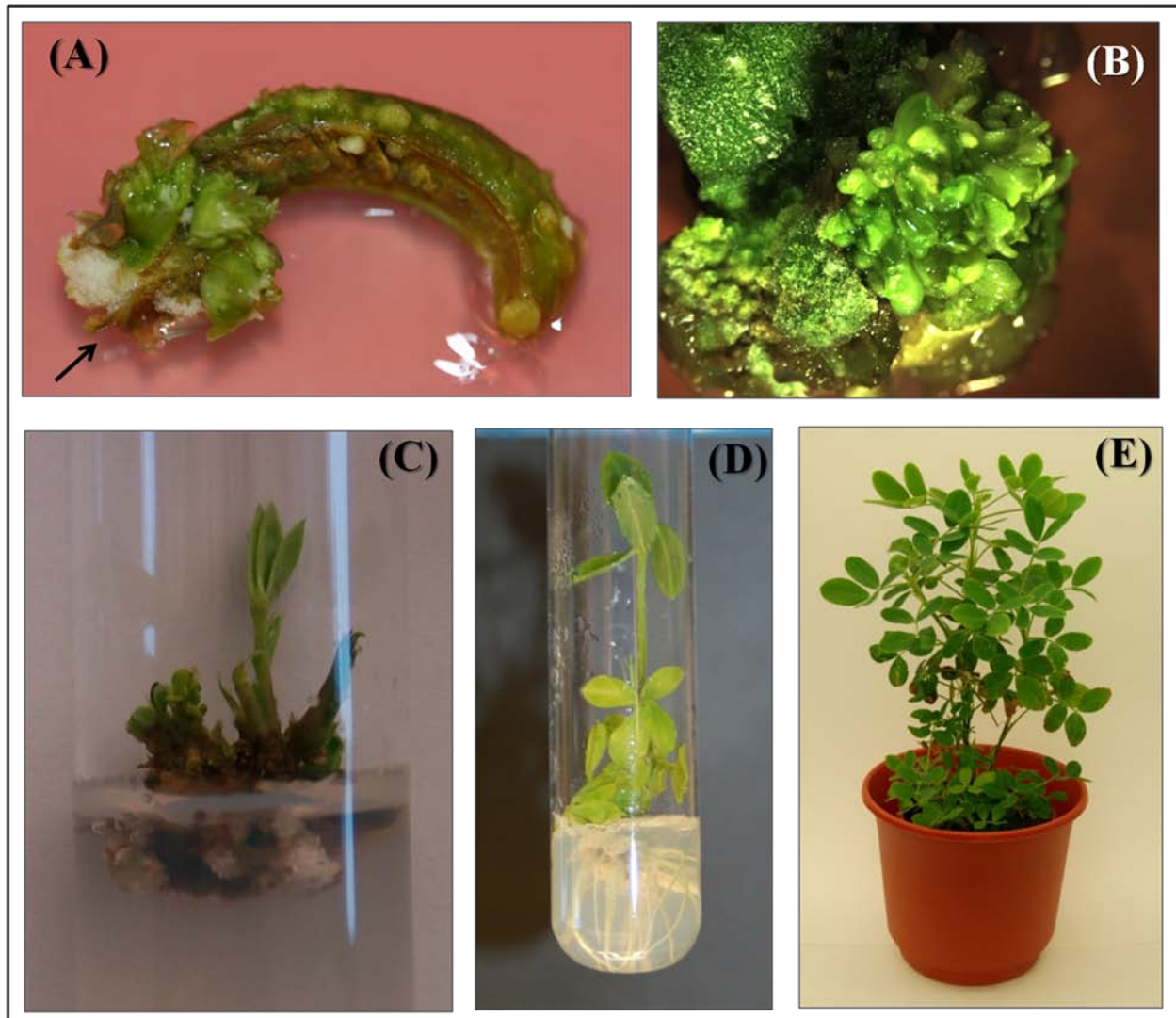
Figure 3-3.  Explant response and regeneration of mature peanut plants. (A) Adventitious shoot buds from cotyledon explants after 3 weeks of culture on shoot induction medium. Arrow indicates the proximal cut end with high regeneration potential. (B) Shoot bud formation on proximal cut end of cotyledon explants after 4 weeks of culture on hoot induction medium (2.5X magnification). (C) Shoot development after 4 weeks on shoot elongation medium. (D) Root development after 4 weeks on root induction medium. (E) Mature plant in soil 16 weeks after initial shoot bud formation.

Table 3-1. Effect of N6-benzyladenine concentrations ranging from 10-80 µM on the peanut cultivar response trend

| Cultivar | Trend* | | |
|---|---|---|---|
| | Linear | Quadratic | Cubic |
| Florida-07 | 0.0985 | 0.0051 | 0.0005 |
| Georgia Browne | <0.0001 | 0.5536 | 0.7933 |
| Georgia Green | 0.6191 | 0.8416 | <0.0001 |
| Valencia-A | <0.0001 | 0.0029 | 0.1533 |
| VC-2 | 0.199 | 0.2278 | 0.9089 |

*Trends determined using orthogonal polynomials in the Estimate statement of the Mixed Procedure of SAS software. Cultivar response trend considered significant at $P \leq 0.05$.

Table 3-2. Effect of N6-benzyladenine concentrations ranging from 10-320 µM for Georgia Browne and 10-640 µM for Valencia-A on the peanut cultivar response trend

| Cultivar | Trend* | | |
|---|---|---|---|
| | Linear | quadratic | Cubic |
| Georgia Browne | 0.0625 | <0.0001 | 0.0668 |
| Valencia-A | <0.0001 | 0.0021 | 0.4706 |

* Trends determined using orthogonal polynomials in the Estimate statement of the Mixed Procedure of SAS software. Cultivar response trend considered significant at $P \leq 0.05$.
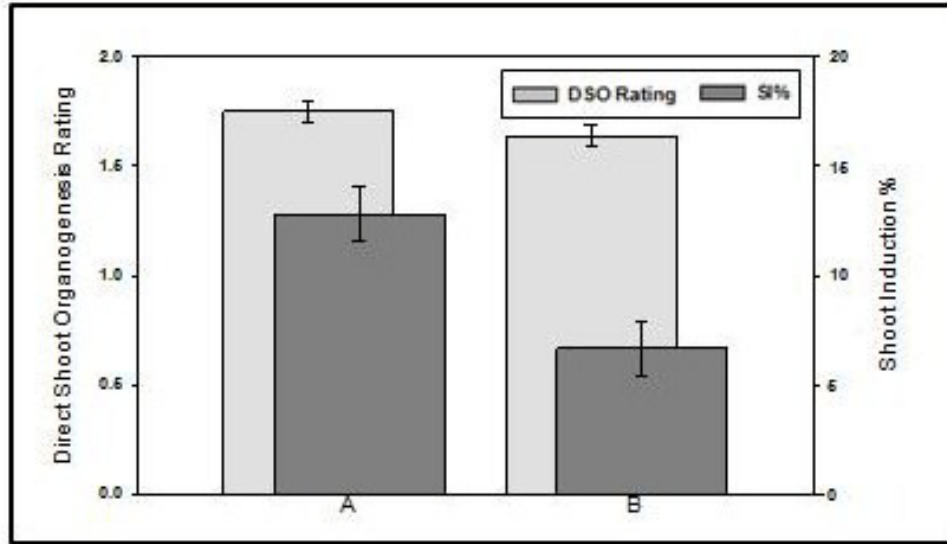
Figure 3-4. Shoot organogenesis response from two types of peanut cotyledon explants (A) Explant derived from cotyledon with embryo axis previously attached, (B) Explant derived from cotyledon without embryo axis previously attached.
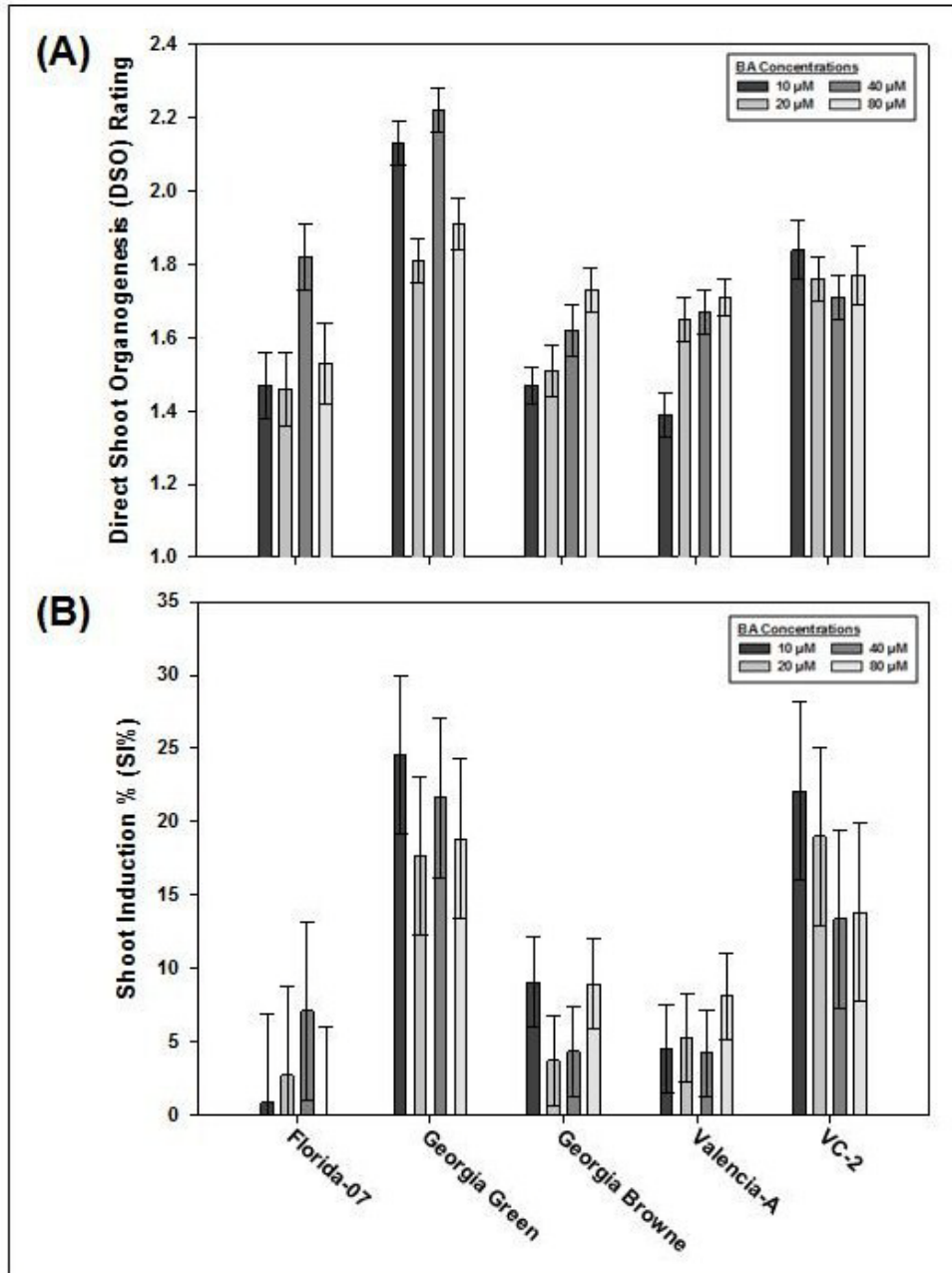
Figure 3-5.  Effect of N6-benzyladenine concentration ranging form 10 - 80 μM on (A) direct shoot organogenesis rating of peanut cotyledon explants, and (B) shoot induction %. Each value is a mean ± SE.
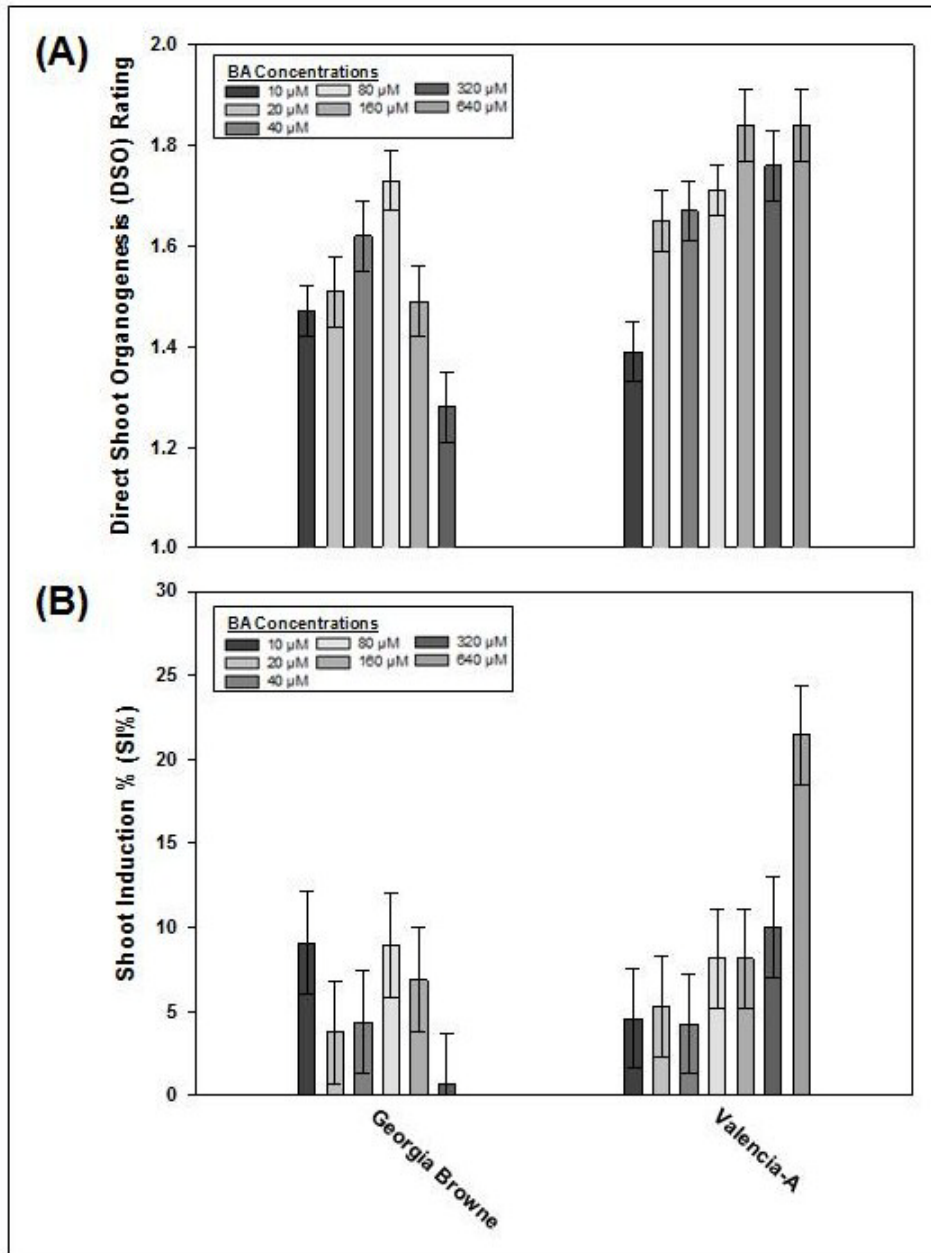
Figure 3-6.  Effect of N6-benzyladenine concentration ranging from 10 - 320 µM for peanut cultivars Georgia Browne and 10-640 µM for Valencia-A on (A) direct shoot organogenesis, and (B) shoot induction %. Each value is a mean ± SE.

Table 3-3.  Comparison of top-performing cultivar* N6-benzyladenine concentration combinations in peanut tissue culture of quartered, de-embryonated cotyledon explants.

| Cultivar | Market Type | N6-benzyladenine (µM) | DSO Rating | SI% |
|---|---|---|---|---|
| Florida-07 | Runner | 40 | 1.8 ± 0.1 b | 7.1 ± 6.1 b |
| Georgia Green | Runner | 10 | 2.1 ± 0.1 a | 24.6 ± 5.4 a |
| Georgia Bowne | Spanish | 80 | 1.7 ± 0.1 b | 9.00 ± 3.1 b |
| Valencia-A | Valencia | 640 | 1.8 ± 0.1 b | 21.4 ± 3.3 a |
| VC-2 | Virginia | 10 | 1.8 ± 0.1 b | 22.1 ± 6.1 a |

*Mean DSO rating ± SE and SI% ± SE for cultivar*treatment following 4 week culture period.
**Means within cultivars followed by the same letter are not different at the $P \leq 0.05$.

TRANSIENT EXPRESSION OF *UIDA* (B-GLUCURONIDASE) IN PEANUT
COTYLEDON EXPLANTS

## Abstract

Peanut is susceptible to a variety of abiotic and biotic stressors. In the U.S., foliar

and soilborne diseases/pests are the most prevalent of these stressors and annually

lower yields and profits for growers. Outside of pesticides, the primary means to

overcoming these stressors is conventional breeding. Conventional breeding for

disease resistance has been a slow endeavor due to the lack of genetic diversity

available in cultivated peanut. Recently, interest has increased in using transgenic

approaches to complement traditional breeding for improved agronomic performance in

peanut. Sharma and Anajaiah (2000) reported the development of a highly efficient

peanut transformation protocol via *Agrobacterium*-mediated transgene delivery.

However, this protocol was optimized for JL-24, an Indian peanut cultivar not readily

available in the U.S.  In the present study, the protocol described by Sharma and

Bhatnagar-Mathur (2006) was tested using two readily available U.S. cultivars (Georgia

Green and VC-2) and four *Agrobacterium* strains (ABI, C58C1, GV3101, and LBA4404)

harboring the *CaMV 35S-uidA* gene construct. It was hypothesized that the protocol

described by Sharma and Bhatnagar-Mathur (2006) could be used to successfully

transform these selected cultivars. The purpose of this study was to identify

*Agrobacterium* strains that ould successfully infect the selected cultivars. Following

inoculation and co-cultivation of explants, a histochemical β-glucuronidae (GUS) assay

analysis was performed to test for transient expression of the *uidA* gene. The only

explants testing positive for *uidA* expression were those infected with *Agrobacterium*

strain ABI. It was concluded that *Agrobacterium* strain ABI must be used for future transformation experiments.

**Introduction**

Throughout a growing season, peanut is exposed to many biotic and abiotic stressors that can lower yields and profits for growers. In the U.S., foliar and soilborne diseases/pests are the most prevalent of these stressors. Domestically, the most prevalent biotic stressors of peanut include tomato spotted wilt virus (TSWV; Tospovirus vectored by thrips), root-knot nematode (*Meloidogyne arenaria* (Neal) Chitwood race 1), White Mold (*Sclerotium rolfsii* Sacc.), *Cylindrocladium* black rot (*Cylindrocladium parasiticum* Crous, Wingfield and Alfenas), *Sclerotinia* blight (*Sclerotinia minor* Jagger), Rust (*Puccinia arachidis* Speg.), early leaf spot (*Cercospora arachidicola* S. Hori), and late leaf spot (*Cercospiridium personatum* (Berk and M. A.Curtis) Deighton). Outside of pesticides the primary means to overcoming these diseases is conventional breeding. Conventional breeding for disease resistance has been a slow endeavor due to the lack of genetic diversity available in cultivated peanut. Recently, interest has increased in using transgenic approaches to complement traditional breeding for improved agronomic performance and disease resistance in peanut. Routine peanut transformation would allow breeders to have access to otherwise unavailable genetic resources.

Peanut has been successfully transformed using both particle bombardment and *Agrobacterium*-mediated transformation (see Chapter 1 for review). Recently, as an alternative to lengthy bombardment methods, protocols using faster, direct organogenesis and *Agrobacterium* have been investigated. Transformation by *Agrobacterium* is believed to be superior to bombardment because integration patterns

tend to be "cleaner", meaning whole gene constructs integrate into the host genome with low copy number. Additionally, and perhaps most favorable, tissue culture requirements tend to be far less intensive in terms of sub-culturing and time to plant maturity. This reduction in time and handling lessens the likelihood for contamination and somaclonal variation, and therefore, loss of putative transgenics. Once established, protocols are far less labor intensive and more economically sound.

Sharma and Anjaiah (2000) reported the development of a direct shoot organogenesis and transformation protocol via *Agrobacterium*-mediated transgene delivery. However, this protocol was optimized using cv. JL-24, an Indian cultivar not readily available in the U.S. Likewise, many of the earlier studies reporting the successful transformation of peanut via *Agrobacterium* used cultivars not readily available or economically important in the U.S (Venkatachalam 1998, 2000; Rohini et al. 2000, 2001; Khandelwal et al 2003, 2004; Anurahda et al. 2006, 2008; Bhatnagar-Mathur t al. 2007; Tiwari 2008, 2009). Very few readily available domestic peanut cultivars have been transformed (Franklin et al. 1993; Eapen and George, 1993; McKently et al. 1995; Cheng 1996, 1997; Li et al. 1997; Egnin et al. 1998; Dodo et al. 2007; Yin et al. 2007). Within these studies that have reported successful peanut transformation, the number of cultivars used has been relatively narrow; the Indian cultivar most commonly transformed via *Agrobacterium* has been JL-24 followed by TMV-2, while in the U.S. it has been Valencia-A.

Sharma and Anjaiah (2000) report a protocol which results in a high production of transgenics. It was hypothesized that the protocol described by Sharma and Bhatnagar-Mathur (2006) could be expanded to successfully transform U.S. cultivars. The purpose

of this study was to identify *Agrobacterium* strains virulent to the candidate cultivars, Georgia Green and VC-2.

## Materials and Methods

### *Agrobacterium* Strain and Gene Construct

Peanut transformation experiments were conducted using a modified protocol described by Sharma and Bhatnagar-Mathur (2006). For transformation and transient expression experiments, *Agrobacterium* strains ABI, C58C1, GV3101, and LBA4404 harboring *CaMV 35S-uidA* expression cassette were tested (*CaMV 35S-uidA*, constitutively expressed promoter from Cauliflower Mosaic virus linked to *uidA*, a reporter gene derived from *E. coli* which encodes for β-glucuronidase (GUS). A single colony of an *Agrobacterium* strain was incubated in 20 ml of yeast extract peptone medium (YEP; 10 g L-1 Yeast Extract [Fisher Scientific, Waltham, Massachusetts, USA], 10 g L-1 Bacto Peptone [Sigma, St. Louis, MO, USA], 5 g L-1 NaCl [Fisher Scientific, Waltham, Massachusetts, USA]) and grown overnight on a shaker at 200 rpm at 28°C to an OD600 of 0.5-0.8. An overnight culture (10 ml) was pelleted by centrifugation at 600 g for 10 min. Pelleted cells were resuspended in 30 ml of 0.5X MS medium (Murashige and Skoog 1962). The suspension was then incubated at 4°C for 1 hr prior to explant inoculation.

### Explant Preparation and Inoculation

Mature dry seeds of Georgia Green and VC-2 were surface-sterilized in a 0.1% (w/v) mercuric chloride solution for 10 min, rinsed five times with sterile water, and soaked in sterile distilled water overnight. Using sterile technique, seed coats were removed, cotyledons were separated, and embryo axes were removed. Cotyledons were sliced vertically to obtain quartered cotyledon explants. Explants were briefly

immersed (1-2 sec) into an *Agrobacterium* suspension culture at room temperature for inoculation. Explants were then blotted on sterile filter to remove excess suspension solution. The proximal, freshly cut edge of each explant was embedded into shoot induction medium (SIM; MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific, Hampton, NH, USA], 0.8% (w/v) agar [Becton, Dickinson and Co., Franklin Lakes, NJ, USA], 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) [Sigma, St. Louis, MO, USA], and 10 µM N6-benzyladenine (BA) [Sigma, St. Louis, MO, USA], pH 5.8) at a slight downward angle. Explant/bacterial co-cultivation lasted a period of three days. Co-cultivation conditions were set to 26°C (± 1°C) under continuous light of 100 µEs$^{-1}$ m$^{-2}$ irradiance.

**Transient Expression in Cotyledon Explants and Histochemical GUS-assay**

Explants of Georgia Green and VC-2 were inoculated with *Agrobacterium* strains ABI, LBA4404, GV3101, and C58C1 harboring the *CaMV 35S-uidA* construct. Explants were placed onto SIM medium as previously described. Following co-cultivation, explants were assayed for transient GUS expression. Explants were removed from SIM medium and rinsed in 70% EtOH for 5 min, followed by a 5 min rinse in sterile water. Explant pieces were placed into a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% Triton X-100, and 1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) and vacuum infiltrated for 5 min. Explant pieces were then placed at 37°C overnight under constant agitation. Explants were visually examined for "blue" GUS sectors indicating *uidA* expression.

<div align="center">

**Results and Discussion**

</div>

Transient expression of *uidA* was used in the first peanut transformation experiments to identify strain virulence. Lacorte et al. (1991) used several

*Agrobacterium* strains to induce *uidA*-expressing tumor masses on peanut seed and seedling explants. Lacorte et al. (1991) reported strain A281 to be the most virulent strain tested. In a similar study, Franklin et al. (1992) reported *uidA* expression in callus tissue following infection with *Agrobacterium* strains EHA101 and LBA4404. Georgia Green and VC-2 explants inoculated with *Agrobacterium* strains C58C1, GV3101, and LBA4404 harboring the *CaMV 35S-uidA* plasmid showed no signs of transient *uidA* expression following GUS-histochemical analysis. Prior to the current study, no reports have been made which indicate that strains GV3101 or C58C1 have been used in peanut transformation studies. However, LBA4404 has been successfully used in several studies testing transient and stable in peanut (Venkatachalam et al. 1998, 2000; Rohini et al. 2000, 2001; Yin et al. 2007). Explants of VC-2 and Georgia Green inoculated with ABI showed transient expression, with several "blue" sectors observed on the cut surface of the explants. Eighty explants per cultivar were inoculated with ABI, 41% of the Georgia Green explants and 43% of VC-2 explants were positive for *uidA* expression (Table 4-1, Figure 4-1). Prior to this study, no peanut transformation studies have been reported using strain ABI. ABI was identified as being the most virulent strain of those tested. The development of blue sectors on explants is a clear indication of nuclear delivery of the *CaMV 35S-uidA* expression cassette. C58C1, GV3101, and LBA4404 lacked the necessary host-"pathogen" virulence required for transformation. However, using a similar protocol, Yin et al. (2007) produced stable transgenics using LBA4404. The discrepancy of this study with the current study can only be explained by differences in cultivar; Yin et al. (2007) used Baisha 1016 peanut. Because *Agrobacterium* strain ABI was the only strain to produce GUS positive, blue-sectors

upon assaying, it was determined that ABI was the only viable strain for use in future transformation experiments using Georgia Green and VC-2.

To further determine the optimal *Agrobacterium*/cultivar combination, attempts were made to quantify *uidA* expression through use of a 4-methylumbelliferyl β-D-glucuronide (MUG) assaying and quantitative real-time PCR (qRT-PCR). Because of the high lipid content of peanut seed, protein extracts from explants were of extremely low quality. These low quality extracts did not allow for the detectable hydrolytic conversion of MUG into glucuronic acid and 7-hydroxyl-4-methylcoumarin (MU). qPCR analysis, using *uidA* specific primers, was also unsuccessful despite positive GUS assay staining observed in control explants. No detectable traces of *uidA* expression were observed. The discrepancy between the GUS assay and the qRT-PCR results can be explained by the accumulation of stable, GUS protein being translated from a non-detectable amount of *uidA* mRNA transcripts within a cell.

## Conclusions

Results from the transient expression study indicate the nuclear delivery of *CaMV 35S-uidA* gene construct. Because transient *uidA* expression was only observed in explants inoculated with *Agrobacterium* strain ABI and not C58C1, GV3101, and LBA4404, it was concluded that strain ABI was the best option for use in future stable transformation experiments when using Georgia Green and VC-2 explants. Based on the findings of this study, attempts were made to produce mature, transgenic peanut lines expressing for *CaMV 35S-uidA, DR5-uidA*, and *SAG12-IPT*. Results to these experiments can be found in Appendix A.

Table 4-1.  Transient expression of *CaMV 35S-uidA* in peanut cotyledon explants

| *Agrobacterium*-strain | Cultivar | SIM[1] | # GUS + | # GUS - |
|---|---|---|---|---|
| ABI | Georgia Green | 80 | 33 | 47 |
| | VC-2 | 80 | 34 | 46 |
| C58C1 | Georgia Green | 80 | 0 | 80 |
| | VC-2 | 80 | 0 | 80 |
| GV3101 | Georgia Green | 80 | 0 | 80 |
| | VC-2 | 80 | 0 | 80 |
| LBA4404 | Georgia Green | 80 | 0 | 80 |
| | VC-2 | 80 | 0 | 80 |

[1]Number in column represents the total number of explants which were inoculated and onto SIM for 3 day co-cultivation.

Figure 4-1.  Arrows indicate transient *uidA* expression on the proximal end of de-embryonated, quartered cotyledon explants of peanut cv. Georgia Green. Explants were inoculated with *Agrobacterium* strain ABI harboring the *CaMV 35S-uidA* expression cassette.

APPENDIX A
TRANSFORMATION OF PEANUT WITH *SAG12-IPT* FOR A 'STAY GREEN'
PHENOTYE

**Introduction**

Several studies have developed transgenic plants expressing for the *SAG12-IPT*

chimeric gene to delay the onset of leaf senescence ('Stay Green'). Engineering plants

to retain leaves, even under pathogen attack, could potentially negate some of the

undesirable effects associated with pathogen infection.  Preliminary data (M. Jones and

D. Clark, University of Florida) indicated that transgenic petunia expressing *SAG12-IPT*

had a delayed leaf senescence response (Jandrew, 2002). Transformants also

appeared to develop fewer chlorotic spots and gained tolerance to petunia leaf spot

disease caused by *Cercospora petunia* (Jandrew 2002) (refer to Chapter 1, Figure 1-1).

Similar results were reported by Swartzberg et al. (2008), in which tomato plants

transformed with *SAG12-IPT* displayed suppressed symptoms of the disease caused by

*Botrytis cinerea*. It is hypothesized that the same tolerance response can be

incorporated into peanut lines expressing for *SAG12-IPT*.

Transient expression of *uidA* reported in Chapter 4 suggest that *Agrobacterium*

strain ABI possesses the virulence required to produced mature, stable transgenic

peanut lines. Likewise, several previous studies report the successful transformation of

peanut using *Agrobacterium* strain LBA4404. Yin et al. (2007), using LBA4404, Georgia

Green explants, and a similar direct shoot organogenesis protocol developed multiple

independent transgenic plants. Based on these findings, it was hypothesized that

Georgia Green, VC-2, and Valencia-A could be successfully transformed. The current

study attempted to integrate the *CaMV 35S-uidA, DR5-uidA*, and *SAG12-IPT*

expression cassettes in independent peanut lines.

## Materials and Methods

### *Agrobacterium* Strain and Gene Constructs

Peanut transformation experiments were conducted using a modified protocol described by Sharma and Bhatnagar-Mathur (2006). *Agrobacterium* strains LBA4404 and ABI harboring the *CaMV 35S-uidA* (previously described in Chapter4), *DR5-uidA* (*DR5-uidA*, an auxin-inducible promoter linked to β-glucuronidase gene), or *SAG12-IPT* (*Sag12-IPT*, senescence-specific promoter linked to isopentyl transferase gene) expression cassette were used in experiments for stable transformation (Figure A-1). A single colony of *Agrobacterium* was incubated in 20 ml of yeast extract peptone medium (YEP; 10 g L$^{-1}$ Yeast Extract [Fisher Scientific, Waltham, Massachusetts, USA], 10 g L$^{-1}$ Bacto Peptone [Sigma, St. Louis, MO, USA], 5 g L$^{-1}$ NaCl [Fisher Scientific, Waltham, Massachusetts, USA]) and grown overnight on a shaker at 200 rpm at 28°C to an OD600 of 0.5-0.8. An overnight culture (10 ml) was pelleted by centrifugation at 600 g for 10 min. Pelleted cells were resuspended in 30 ml of 0.5X MS medium. The suspension was then placed at 4°C for 1 hr prior to explant inoculation.

### Explant Preparation and Inoculation

Mature dry seeds of Georgia Green, VC-2, and Valencia-A were surface-sterilized in a 0.1% (w/v) mercuric chloride solution for 10 min, rinsed five times with sterile water, and soaked in sterile distilled water overnight. Using sterile technique, seed coats were removed, cotyledons were separated, and embryo axes were removed. Cotyledons were sliced vertically to obtain quartered cotyledon explants. Explants were briefly immersed (1-2 sec) into an *Agrobacterium* suspension culture at room temperature for inoculation. Explants were then blotted on sterile filter to remove excess suspension solution. The proximal, freshly cut edge of each explant was embedded into shoot

induction medium (SIM; MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific, Hampton, NH, USA], 0.8% (w/v) agar [Becton, Dickinson and Co., Franklin Lakes, NJ, USA], 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) [Sigma, St. Louis, MO, USA], and either 10 µM or 640 µM N6-benzyladenine (BA) [Sigma, St. Louis, MO, USA], pH 5.8) at a slight downward angle. Georgia Green and VC-2 explants were placed onto shot induction medium (SIM) supplemented with 10 µM BA, while Valencia-A was placed on to SIM supplemented with 640 µM BA. Explant/bacterial co-cultivation lasted a period of three days. Co-cultivation conditions were set to 26°C (± 1°C) under continuous light of 100 µEs-1 m-2 irradiance. Following co-cultivation, explants were sub-cultured to fresh SIM medium supplemented with 50 mg L$^{-1}$ timentin and 50 mg L$^{-1}$ kanamycin. Explants remained on this SIM medium for 3-4 weeks.

**Regeneration of Mature Plants**

Explants bearing shoot buds were transferred to shoot elongation medium (SEM; MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific, Hampton, NH, USA], 0.8% (w/v) agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), and 2 µM BA [Sigma, St. Louis, MO, USA], pH 5.8) containing 50 mg L$^{-1}$ timentin and 50 mg L$^{-1}$ kanamycin for selection. Following three weeks under selection, surviving shoots were sub-cultured twice, every 4 weeks to SEM supplemented with 100 mg L$^{-1}$ kanamycin. Elongated shoots (approximately 2-3 cm in length) were then placed onto root induction medium (RIM; MS salts [Sigma, St. Louis, MO, USA], B5 vitamins, 3% (w/v) sucrose [Fisher Scientific, Hampton, NH, USA], 0.8% (w/v) agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), and 5 µM 1-Naphthaleneacetic acid (NAA) [Sigma, St. Louis, MO, USA], pH 5.8). Cultures undergoing selection and rooting

were maintained at 26°C (± 1°C) under continuous light of 100 µEs-1 m-2 irradiance.

Once roots were established, plants were transferred to pots containing a 2:1 Fafard #2

: sand mixture [Fafard, Agawam, MA, USA]. Plants were hardened undergrowth

chamber conditions. Plants reaching maturity were moved into greenhouse conditions

and fertilized and irrigated as needed. Plants that reached maturity underwent genomic

PCR screening and when appropriate, GUS-assay analysis.

**Genomic DNA Analysis**

Using the CTAB extraction method, genomic DNA was isolated from putative

transgenic lines that survived tissue culture selection to maturity. From $T_0$ plants, freshly

expanding compound leaves were collected and immediately frozen in liquid nitrogen.

Small quantities of tissue (< 300 mg) were homogenized in microcentrifuge tubes using

a pellet pestle. Precipitated DNA was air-dried and resuspended in sterile distilled

water.

PCR amplification was carried out using gene specific primers. Putative *Sag12-*

*IPT* transgenic plants were screened with primers that flanked the *Sag12* promoter and

the IPT gene, producing a 1000 bp product (Forward: 5'-

GATTTGATTAAGCTTTTAACTTGC-3', Reverse: 5'-GCCCGCCGTTGGCCTCATGAT-

3'). Putative *CaMV 35S-uidA* plants were screened with primers which annealed to the

uidA gene only, producing an 819 bp product (Forward: 5'-

CCCCAACCCGTGAAATCAAA-3', Reverse: 5'- GTTCGCCCTTCACTGCCACT-3').

Thermal cycler conditions were set as such: 95°C for 1min (denaturation), 60°C for  30 s

(annealing), °C for  1 min (extension), for 30 cycles, and held at 4°C until recovery. The

amplified products were assayed by electrophoresis in 1% agarose gels in 1X TAE.

**GUS Assay**

Explants were removed from SIM medium and rinsed in70% EtOH for 5 min,
followed by a 5 min rinse in sterile water. Explant pieces were placed into a solution
containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% Triton X-
100, and 1 mg ml$^{-1}$ 5-bromo-4-chloro-3- indolyl glucuronide (X-gluc) and vacuum
infiltrated for 5 min. Explant pieces were then placed at 37°C overnight under constant
agitation. Explants were visually examined for "blue" GUS sectors indicating *uidA*
expression.

## Results and Discussion

In this experiment, explants of VC-2 and Georgia Green were inoculated using
Agrobacterium strain LBA4404. In total, 400 individual explants of VC-2, and 320
explants of Georgia Green were inoculated with *Agrobacterium* strain LBA4404
harboring various gene constructs (*SAG12-IPT, 35S-uidA, DR5-uidA*). Under selection,
approximately 1% of the Georgia Green explants and 4% of the VC-2 explants survived
selection and yielded mature plants, none of which were transgenic (Table A-1).
Likewise, attempts to transform Georgia Green, VC-2, and Valencia-A via
*Agrobacterium* strain ABI harboring the *SAG12-IPT* plasmid were also unsuccessful
(Table A-2). Under selection, 3% of Georgia Green and 3% of VC-2 explants inoculated
resulted in mature plants. None of the Valencia-A explants inoculated resulted in the
development of mature plants.

Sharma et al. (2000) reported shoot bud induction efficiencies to be nearly 96%
and transformation efficiencies of those explants to be 55% when using cultivar JL-24
and *Agrobacterium* strain C58. Because JL-24 and strain C58 were not readily
available, cultivars Georgia Green, VC-2 and Valencia-A were used in these initial

transformation experiments. Shoot bud induction efficiencies in VC-2, Georgia Green, and Valencia-A (22%, 25%, and 21%, respectively) were much lower than those reported by Sharma et al. (2000). Transformation efficiencies in the present experiment were not as high as those reported by Sharma et al. (2000) because of the dramatic difference in shoot induction frequencies. Another possible explanation is poor cultivar/*Agrobacterium* strain interaction.

## Conclusions

Although no transgenic peanut lines were developed in this study, the author of this paper is optimistic that use of this protocol with the selected cultivars will lead to the generation of multiple independent transgenic lines. Consistent transient expression of *CaMV 35S-uidA* has been observed in explants, meaning that expression cassettes are being delivered to the nucleus of cells of explants (refer to Chapter 4). Transgene integration is a rare event and occurs at very low frequencies, even within crops with established transformation systems. Given this fact, and the fact that past studies report peanut being recalcitrant to transformation, it is not surprising that transgenic lines were not generated in the present study. However, as tissue culture conditions are further improved and other highly virulent *Agrobacterium* strains are identified, the routine transformation of Georgia Green, VC-2, and Valencia-A peanut should become a reality.

Further work will be required to improve shoot bud induction frequencies, which will likely improve overall efficiencies to produce mature transgenic plants. The use of other *Agrobacterium* strains should be explored which may be more virulent than those tested. Although JL-24 is not readily available domestically, efforts should be made with this cultivar to duplicate Sharma and Anajaiah's (2000) result.
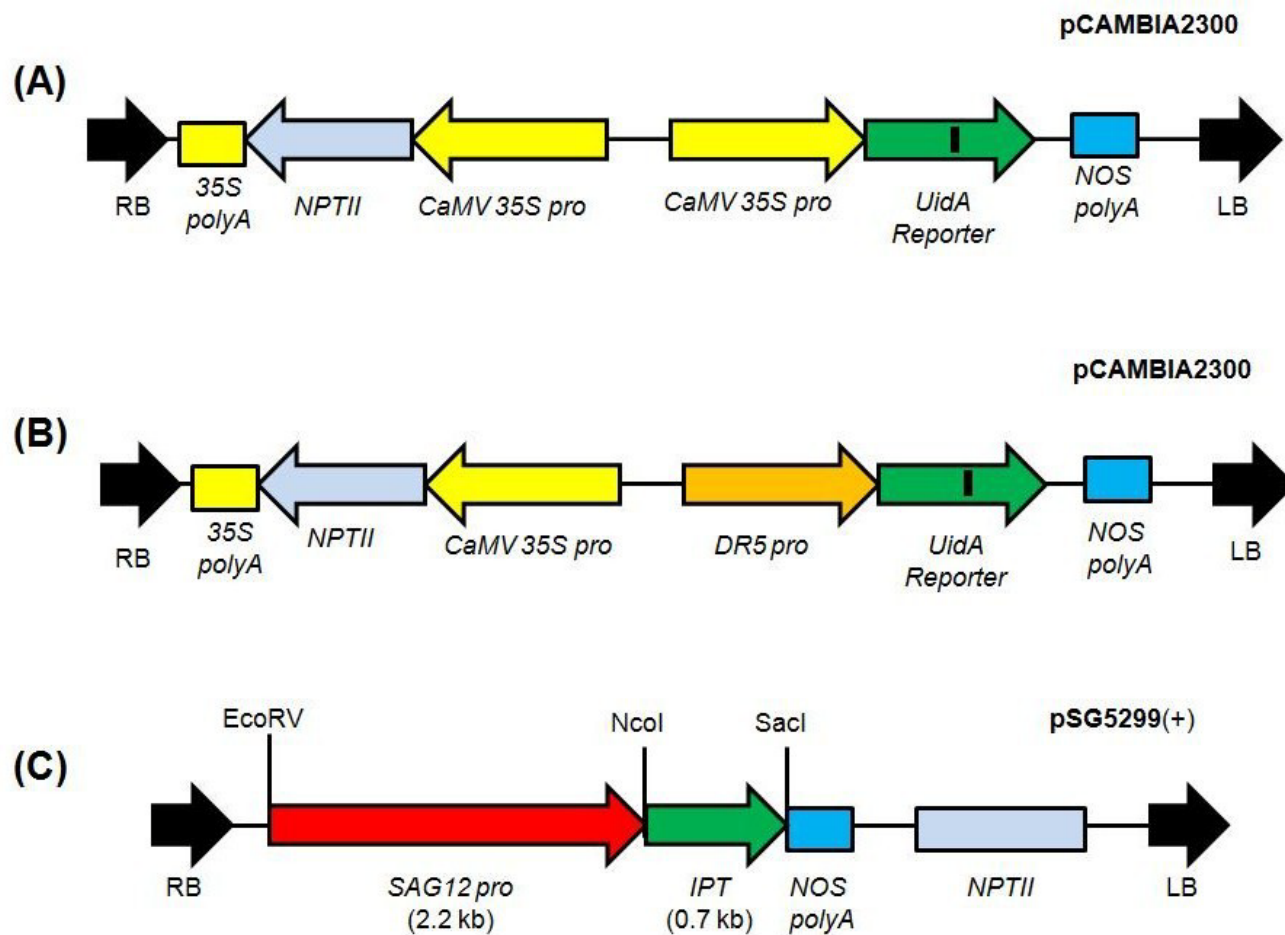
Figure A-1. Expression cassettes used for transformation of de-embryonated, quartered cotyledon explants of peanut, (A) *CaMV 35S-uidA*, (B) *DR5-uidA*, and (C) *SAG12-IPT*.

Table A-1. Assay results of transformation attempts of peanut using *Agrobacterium* strain LBA4404

| Construct | Cultivar | Selection | | | | *Ipt/uidA* PCR | GUS assay |
| | | SIM[1] | SEM1[2] | SEM2[3] | RIM[4] | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *CaMV 35S-UidA* | VC-2 | 80 | 12 | 4 | 3 | - | - |
| *SAG12-IPT* | VC-2 | 80 | 19 | 3 | 0 | n/a | n/a |
| *DR5-uidA* | VC-2 | 80 | 20 | 7 | 6 | - | - |
| *SAG12-IPT* | VC-2 | 80 | 33 | 8 | 0 | n/a | n/a |
| *SAG12-IPT* | VC-2 | 80 | 16 | 10 | 6 | - | n/a |
| *CaMV 35S-uidA* | Georgia Green | 80 | 6 | 1 | 1 | - | - |
| *DR5-uidA* | Georgia Green | 80 | 13 | 6 | 0 | n/a | - |
| *SAG12-IPT* | Georgia Green | 80 | 9 | 5 | 0 | n/a | n/a |
| *SAG12-IPT* | Georgia Green | 80 | 12 | 5 | 1 | - | n/a |

[1]Number in column represents the total number of explants which were inoculated and cultured on SIM. [2]Number in column represents the total number of explants which developed adventitious shoot buds and were moved to SEM (SEM1). [3]Number in column represents the total number of individual shoots were sub-cultured to fresh SEM (SEM2). [4]Number in column represents the total number of shoots that developed roots on RIM.

Table A-2.  Assay results of attempted transformation of peanut using *Agrobacterium* strain ABI harboring *SAG12-IPT*

| Cultivar | SIM[A] | SEM1[B] | SEM2[C] | RIM[D] | *IPT* PCR |
|---|---|---|---|---|---|
| | | Selection | | | |
| Georgia Green | 80 | 16 | 9 | 2 | - |
| VC-2 | 80 | 11 | 5 | 2 | - |
| Valencia-A | 80 | 12 | 3 | 0 | n/a |

[A]Number in column represents the total number of explants which were inoculated and cultured on SIM. [B]Number in column represents the total number of explants which developed adventitious shoot buds and were moved to SEM (SEM1). [C]Number in column represents the total number of individual shoots were sub-cultured to fresh SEM (SEM2). [D]Number in column represents the total number of shoots that developed roots on RIM.

APPENDIX B
PEANUT TRANSFORMATION STUDIES

Table B-1. List of published *Agrobacterium*-mediated peanut transformation studies.

| Cultivar | Explant | Trait | Promoter | Strain | Reference |
|---|---|---|---|---|---|
| Tatu, Tatui, Tatu branco, Tupa, Penapolis | Epicotyls | β-Glucuronidase | ATC1 | T37, A281, Bo542, A136 EHA101, LBA4404 | Lacorte et al. 1991 |
| Okrun | Hypocotyls | β-Glucuronidase | CaMV 35S | ASE1 | Franklin et al. 1993 |
| New Mexico 'A' | leaf sections | β-Glucuronidase | CaMV 35S | EHA105 | Eapen and George 1994 |
| Florigiant, NC-7, Florunner, F435AT | embryo axes | β-Glucuronidase | MAS | EHA105 | McKently et al. 1995 |
| New Mexico 'A' | leaf sections | β-Glucuronidase | CaMV 35S | EHA105 | Cheng et al. 1996, 1997 |
| New Mexico 'A' | leaf sections | Nucleocapsid gene from TSWV | CaMV 35S | EHA105 | Li et al. 1997 |
| New Mexico 'A', Florunner, Georgia Runner, Sunrunner, Southrunner | Epicotyls | β-Glucuronidase β-Glucuronidase | CaMV 35S | EHA101 | Egnin et al. 1998 |
| VRI-2, TMV-7 | Cotyledon | β-Glucuronidase | CaMV 35S | LBA4404 | Venkatachalam et al. 1998, 2000 |
| TMV-2 | embryo axis attached to one cotyledon | β-Glucuronidase | CaMV 35S | LBA4404 | Rohini and Rao 2000 |
| JL-24 | de-embryonated cotyledon | β-Glucuronidase | CaMV 35S | C58 | Sharma and Anjaiah 2000 |
| TMV-2 | embryo axis attached to one cotyledon | Peanut clump virus coat protein | CaMV 35S | LBA4404 | Rohini and Rao 2001 |
| TMV-2 | Plumule of embryo axes | Tobacco chitinase | CaMV 35S | EHA105 | Khandelwal et al. 2003, 2004 |
| JL-24 | embryo axis attached to one cotyledon | Rinderpest virus hemagglutinin β-Glucuronidase | none | GV2260 | Anuradha et al. 2006 |
| JL-24 | de-embryonated cotelydon | DREB1A | CaMV 35S, rd29A | C58 | Bhatnagar-Mathur et al. 2007 |
| Georgia Green | Hypocotyls | Ara h2 | CaMV 35S | EHA105 | Dodo et al. 2007 |
| Baisha 1016 | de-embryonated cotelydon | FAD2 | CaMV 35S | LBA4404 | Yin et al. 2007 |
| JL-24 | embryo axes | mustard defensin (BjD) | CaMV 35S | EHA105 | Anuradha et al. 2008 |
| JL-24 | de-embryonated cotelydon | synthetic Cry1 EC | CaMV 35S | EHA101 | Tiwari et al. 2008 |

Table B-2. List of published peanut transformation studies using particle bombardment.

| Cultivar | Explant | Trait | Promoter | Reference |
|---|---|---|---|---|
| | leaflets from mature embryos | β-Glucuronidase | CaMV 35S | Clemente et al. 1992ab |
| Toalson, Florunner | somatic embryos | β-Glucuronidase | CaMV 35S | Ozias-Akins et al. 1993 |
| Florunner, Florigiant | shoot meristems of embryo axes | β-Glucuronidase Phosphinothricin resistance (bar) Nucleocapsid gene from TSWV | CaMV 35S | Brar et al. 1994 |
| MARC-1, Forunner, Toalson | somatic embryos | *crylA c* | CaMV 35S | Singsit et al. 1997 |
| Florunner, Georgia Runner, MARC-1 | somatic embryos | β-Glucuronidase | (vsp B, CaMV 35S | Wang et al. 1998 |
| Florunner, Georgia Runner, MARC-1 | somatic embryos | Nucleocapsid gene from TSWV | CaMV 35S | Yang et al. 1998, 2004 |
| Gajah, NC-7 | somatic embryos | β-Glucuronidase | CaMV 35S | Livingstone and Birch 1999 |
| VC-1, AT120 | somatic embryos | Luciferase (*luc*) Nucleocapsid protein gene from TSWV β-Glucuronidase | CaMV 35S | Magbanua et al. 2000 |
| Luhua 9, YueYou 116 | somatic embryos | β-Glucuronidase | CaMV 35S | Deng et al. 2001 |
| Okrun | somatic embryos | Rice chitinase | CaMV 35S | Chenault et al. 2002, 2003, 2005 |
| Okrun | somatic embryos | Alfalfa glucanase Nucleocapsid gene from TSWV | CaMV 35S | Chenault and Payton 2003 |
| Georgia Runner | embryonic axes | Mercury resistance (*merA*) | *AtACT2* | Yang et al. 2003 |
| Gajah, NC-7 | somatic embryos | Peanut stripe virus coat protein | CaMV 35S | Higgins et al. 2004 |
| Georgia Green, MARC-1 | somatic embryos | Green fluorescent protein Mercury resistance (*merB*) | CaMV 35S | Joshi et al. 2005 |
| NC-7, Wilson, Perry | somatic embryos | Barley oxalate oxidase | CaMV 35S | Livingstone et al. 2005 |
| JL-24 | somatic embryos | BTVP2 | CaMV 35S | Athmaram et al. 2006 |
| Georgia Green | somatic embryos | Bcl-xL | CaMV 35S | Chu et al. 2007 |

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BIOGRAPHICAL SKETCH

Scott Burns was born in 1983 in Marietta, Georgia to Mike and Neva Burns. Scott attended elementary, middle, and high school in his hometown, Canton, Georgia. Scott graduated from Cherokee High School in 2002. Upon completing high school, Scott enrolled at the University of Georgia.  He graduated from UGA in 2006 with a Bachelor of Science degree in applied biotechnology. While working as an undergraduate research assistant at UGA, Scott developed an interest in using genetic approaches to improve agricultural performance in agronomic crops. Upon completion of his undergraduate degree, Scott took an internship position managing a production greenhouse for the Walt Disney Company in Orlando, Florida. After completing his internship, Scott enrolled in at the University of Florida in 2007, serving as a graduate assistant in the Agronomy Department. Scott will earn a Master of Science in agronomy from UF in 2010. His master's degree research focused on using genetic strategies to develop novel sources of tolerance to peanut leaf spot disease.