IN VIRTO AND *IN VIVO* EVALUATION OF *Arachis paraguariensis* AND *A. glabrata* GERMPLASM

By

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To my late father Isaac O. Ajani

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LIST OF ABBREVIATIONS

2ip	6-γ-γ-(Dimethylallylamino)-purine
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ARF	Auxin Response Factors
Aux/IAA	Auxin/ Indole-3-acetic acid
BAP	6-benzylaminopurine
cGMP	Cyclic Guanosine Monophosphate
CIM	callus induction medium
DNA	Deoxyribonucleic acid
EST	Expressed sequence tag
IBA	Indole-3-butyric acid
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
ITS	Internal transcribed spacers
MS	Murashige and Skoog
N_2O	Nitrous Oxide
NAA	Naphthalene acetic acid
PETG	Polyethylene Terephthalate Glycol
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
SRAP	Sequence-related amplified polymorphism
SSR	Simple Sequence Repeat
TDZ	Thidiazuron
TIR1	Transport Inhibitor Response Protein 1

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Apart from possessing important traits needed for improvement of the cultivated peanut (*Arachis hypogaea* L.), wild *Arachis* species are grown for forage and ornamental purposes. In the first phase of this research, a series of *in vitro* experiments led to the development of high frequency regeneration procedures for *Arachis paraguariensis* Chodat and Hassl. and *Arachis glabrata* Benth.

Both auxin type and concentration in the culture medium had significant influence on root formation although application of auxin may not always be necessary for rooting, it is essential for early emergence of root primordia. Plantlets that were allowed to form roots on medium lacking auxin showed significantly enhanced plantlet vigor and root length, even though root emergence was delayed. For *A. glabrata*, the frequency of regeneration was improved along with reduced production of phenolic compounds when 6-benzylaminopurine (BAP) was substituted with 6-γ-γ-(Dimethylallylamino)-purine (2ip) in the culture medium.

The most suitable regeneration procedure was then combined with the antimitotic agent colchicine to induce chromosome doubling of diploid *A. paraguariensis*. The induced autotetraploids from *A. paraguariensis* had fewer stomata, but more hair-like

trichomes per unit leaf area compared to their diploid precursor. The best results in which 39% and 43% of the explants produced tetraploid plants resulted from treatment with 0.5% colchicine for 4 h and 8 h, respectively. Treating explants with high concentrations of colchicine for 24 h proved to be very lethal.

In the second phase, *in vivo* evaluations of *A. paraguariensis* and *A. glabrata* germplasm with specific emphasis on forage yield, disease resistance and seed production were conducted. *In vivo* evaluation of *A. glabrata* led to the discovery of the seed production potential of the two cultivars ‘UF Peace’ and ‘UF Tito’. Additionally, the result from field evaluation of two genotypes of *A. paraguariensis* confirmed higher resistance to *C. arachidicola* leaf spot than cultivated peanut. The findings from this study should contribute towards genetic improvement of the wild *Arachis* species, as well as the enhancement of gene introgression into cultivated peanut.

CHAPTER 1
INTRODUCTION

Genetic variation describes the variation of alleles which exist among the total genetic loci of a population. On the other hand, genetic variability measures the tendency of individual genetic traits in a population to vary from one another (Sleper and Poehlman, 2006; Smith, 1977). Generally, most efforts of plant breeders are tailored toward the evaluation and preservation of existing variability as well the creation of new genetic variation. The diversity existing within the genus *Arachis* is enormous as observed by the large differences in genome size, botanical characters, and important agronomic traits (Singh et al., 1998a). In addition, wide intraspecific variation has been reported between and within several accessions (Nóbile et al., 2004; Herselman, 2003; Upadhyaya et al., 2011).

Several breeding approaches including sexual hybridization, ploidy manipulation, somatic hybridization, genetic transformation and natural/ induced mutation have been exploited as important means of producing new genetic variation within crop species (Sleper and Poehlman, 2006). While sexual hybridization is mostly applicable to the closely related species that exhibit complete chromosome pairing, the technique of peg or embryo culture may be applied to eliminate partial sterility of F_2 hybrids that are derived from more distant species. Other techniques such as *in vitro* fertilization and somatic hybridization are mostly explored in marginally sexually compatible species with severe F_1 sterility. Furthermore, gene transfer via transgenic methods is currently the only feasible approach for species in the quaternary gene pool (Simpson, 2001; Upadhyaya et al., 2011).

Currently, both conventional and molecular plant breeding methods are increasingly relying on *in vitro* tissue culture as an important tool that provides strategic opportunities for overcoming hybridization barriers (Loberant and Altman, 2010). For instance, results from *in vitro* selection for peanut cell lines with resistance to *Cercosporidium personatum*, the causual agent of late leaf spot, proved that tissue culture techniques could be combined with field evaluations to develop disease resistant cultivars. Development of pest and disease resistant cultivars based on this approach can lead to a reduction in cost, time, space, labor and other resources (Venkatachalam et al., 1998).

Other studies have also demonstrated that embryo and peg tip culture may be very useful for rescuing embryos of hybrids derived from interspecific crosses of *A. hypogaea* and other wild species including *A. villosa* Benth. (Bajaj et al., 1982), *A. duranensis* Krapov. and W.C. Gregory, *A. batizocoi* Krapov. and W.C. Gregory, and *A. valida* Krapov. and W.C. Gregory (Feng et al., 1996). Although, somatic hybridization is yet to be successfully applied to the breeding of *Arachis* species, high frequency of plant regeneration from protoplasts of *A. paraguariensis* Chodat and Hassl was reported by Li et al. (1993). Additionally, several methods utilizing *in vitro* tissue culture regeneration have been described for the production of transgenic peanut plants (Bhatnagar et al., 2010; Anuradha et al., 2006; Athmaram et al., 2006). Although haploid plants have not been derived from any of the *Arachis* species, results from some studies (Bajaj et al., 1981; Croser et al., 2006) indicate that anther culture is a promising tool for the development of haploid plants in this genus. Likewise, *in vitro* induction of tetraploidy is another vital technique that could broaden the available gene

pool, as well as eliminate hybridization barriers that are due to differences in ploidy levels.

This study presents results from detailed *in vitro* and *in vivo* evaluations of two important wild *Arachis* species; *A. glabrata* and *A. paraguariensis*. The rhizoma perennial peanut (RPP) (*A. glabrata* Benth.) is well adapted to the Florida climate and is being cultivated for its high quality forage, as well as for ornamental purposes. Previous evaluations of *A. glabrata* have shown significant phenotypic variation for several agronomic traits including yield potential (Freire et al., 2000; Butler et al., 2006), rate of establishment (Kelly and Quesenberry, 1993; Williams et al., 1997), and winter hardiness (French and Prine, 2006). Since, most accessions of RPP rarely produce seeds (Prine et al., 1981), hybridization has not been effective for breeding this species. The agronomic potential of RPP and the lack of information on its seed production potential were the justification for a major part of this study.

The second species, *A. paraguariensis,* is a potential source of novel genes for the genetic improvement of cultivated peanut. The species is of significant importance because some of its accessions show high levels of resistance to early leaf spot, *Cercospora arachidicola* (Subrahmanyam et al., 1985). Almost all the potential pathways for introgression of desired genes from wild *Arachis* into cultivated peanut involved at least one phase of ploidy manipulation (Simpson, 2001). This is because while the cultivated peanut is tetraploid (2n = 4x = 40), most of its wild relatives are diploid (2n = 2x = 20).

Traditional *in vivo* application of antimitotic agents such as colchicine to plant shoots, meristems, seeds, or seedlings has long been a common method for generating

polyploids (Nebel and Ruttle, 1938). Several studies have shown that *in vitro* chromosome manipulation led to improved efficiency of polyploidy induction, and reduced the occurrence of chimeras compared with *in vivo* methods (Cohen and Yao, 1996; Adaniya and Shirai, 2001). Stalker and Wynne (1979) achieved *in vivo* chromosome doubling of some diploid *Arachis,* while Singsit and Ozias-Akins (1992) obtained chimeric plants in an attempt to induce polyploidy *in vitro*, but there is still no clearly described *in vitro* chromosome doubling procedure for any of the diploid wild *Arachis*.

Certainly, with the availability of a reliable tissue culture regeneration system, coupled with the appropriate method of gene transfer, genes expressing desired traits can be successfully transferred from *A. paraguariensis* and other diploid wild species into cultivated peanut. Given this information, hypotheses, objectives and rationale for this research are hereby presented.

Hypotheses

- *In vitro* tissue culture conditions could be modified to achieve high frequency tissue culture regeneration of *A. paraguariensis* and *A. glabrata*.
- The tissue culture regeneration system derived for *A. paraguariensis* at the initial phase of this study can be successfully combined with antimitotic agent colchicine to derive tetraploid plants.
- Seed production in *A. glabrata* is limited by a high rate of embryo abortion in dense canopies and rhizomes due to competition for energy reserves between sexual (flower/seed) and asexual (rhizome) reproduction.
- There is considerable variation in germination and vigor of seeds of rhizoma peanut cultivars ‘UF Tito’ and ‘UF Peace’.

Objectives

- To optimize *in vitro* tissue culture regeneration procedure for high frequency regeneration of *A. paraguariensis* and *A. glabrata* through defined regeneration pathways.

- To induce tetraploidy in *A. paraguariensis* through *in vitro* tissue culture methods.
- To assess the seed production potential of RPP cultivars UF Tito and ‘UF Peace’.
- To determine the germination percent and vigor of seed produced by the two RPP cultivars.

Rationale

- Very low frequency of tissue culture regeneration using leaf derived explants was observed by McKently et al. (1991) and Vidoz et al. (2004). Therefore, an efficient regeneration procedure for *A*. *glabrata* is lacking. In addition, there is no tissue culture procedure for regeneration of *A. paraguariensis* through a defined regeneration pathway.
- If high frequency of regeneration is achieved through a tissue culture regeneration procedure for *A. paraguariensis* at the initial phase of this study, successful induction of tetraploidy by colchicine should be expressly achieved.
- If the high rate of embryo abortion in dense canopies with extensive rhizomes as a result of competition for resources was the main obstacle to seed production in RPP cv. Florigraze (Niles, 1989; Williams, 1993), then this should be applicable to cultivars ‘UF Tito’ and ‘UF Peace’ as well.
- The result of the study conducted by Venuto et al. (1997) proved that there was considerable variation among five genotypes of RPP for seed germination and seedling vigor. It is therefore likely that such variation would also be observed between cultivars ‘UF Tito’ and ‘UF Peace’.

CHAPTER 2
LITERATURE REVIEW

The Genus *Arachis*

The genus *Arachis* L., belonging to the Fabaceae family, Papilionoideae subfamily, Stylosanthinae subtribe, Aeschynomeneae tribe, has enormous economic and nutritional importance as a source of oil seed, food and fodder. Various *Arachis* spp. spread over more than 2.6 million km^2 of the South American continent. Several of the species have also been introduced to Africa and Asia. Most of the species are perennial or annual legumes that are either trifoliate or tetrafoliate. *Arachis* are peculiarly recognized for their production of underground fruits and there is wide range of genetic diversity within and between the species (Krapovickas and Gregory, 2007).

While cultivated peanut (*Arachis hypogaea* L.) is a major oilseed crop grown in warm and subtropical regions of the world, several of the wild *Arachis* are also important sources of novel genes and therefore possess the potential for use in genetic improvement of cultivated peanut. In addition, several wild species in the sections Rhizomatosae, Arachis, Erectoides, Procumbentes, Caulorrhizae and Triseminatae have been evaluated and found to produce forage that is persistent under grazing and comparable or even superior to other tropical forage legumes in terms of yield and quality (Krapovickas and Gregory, 2007; Stalker and Simpson, 1995)

Origin and Distribution

Indigenous to several parts of South America including Argentina, Brazil, Bolivia, Uruguay, and Paraguay, the goecarpic nature, as well as the numerous types of root systems possessed by *Arachis* are essential for persistence in these diverse environments. Several species were initially described and conserved by the early

European plant explorers who later transported them to other continents (Kochert et al., 1991). It was further germplasm collection by 20th century plant scientists that played a significant role in identifying the origin and geographical distribution of the genus.

Over the years, the categorization of the genetic variability within genus *Arachis* has been facilitated by intensive cooperate efforts of several academic institutions, government bodies and international organizations. There are currently over 15,000 accessions from 93 countries that are being conserved at the RS Paroda Gene Bank in ICRISAT, Patancheru, India. The USDA Southern Regional Plant Introduction Station, Griffin, Georgia holds approximately 8747 accessions. In addition to these, North Carolina State University, Raleigh, N.C. and the Texas A & M University, College Station, TX. maintain a few hundred wild *Arachis* accessions.

The geographical distribution and various types of environments associated with various *Arachis* species were studied by Ferguson et al. (2005) through the use of Geographic Information Systems (GIS). Singh and Simpson (1994) used the gene pool concept in describing *Arachis* gemplasm. Members of the primary gene pool consist of cultivated peanut*, A. hypogaea* and its wild relative *A. monticola*, while the secondary pool represents the diploid species of the section Arachis that are cross compatible with cultivated peanut. The species of other sections that have demonstrated low levels of compatibility with cultivated peanut were placed in the tertiary pool.

Apart from these classifications, interspecific and intraspecific variations in the genus *Arachis* have been described on a molecular basis. Several molecular markers including restriction fragment length polymorphism (RFLP) (Gimenes et al., 2002), amplified fragment length polymorphism (AFLP) (Milla et al., 2005), Simple sequence

repeat (SSR) (Koppolu et al., 2010; Barkley et al., 2007), sequence-related amplified polymorphism (SRAP) (Ren et al., 2010), and internal transcribed spacer (ITS) ribosomal DNA sequencing (Wang et al., 2010; Bechara et al., 2010) have been used to explain these variations. Additionally, high-density oligonucleotide microarray utilizing 49,205 publicly available expressed sequence tags (ESTs) have been developed for cultivated peanut (Payton et al., 2009).

Reproductive Biology

All of the species of *Arachis* produce only subterranean fruit. The perfect papilionaceous flowers of *Arachis* are sessile and are usually borne on a long stalk-like tubular hypanthium consisting of fused lower portions of the calyx, corolla and filaments. The ovary which contains two to three ovules is usually located within the base of the hypanthium. Because the release of pollen from the anthers normally occurs very early in the morning before the flowers open, successful controlled cross-pollination in breeding requires emasculation prior to pollination (Krapovickas and Gregory, 2007).

Although self-pollination is common to all the species, a low occurrence of cross pollination by insects and parthenogenesis is possible. After self-pollination and fertilization, the pedicel curves downward and the cells underneath the ovary start to divide to produce a gynophore or peg that forces the ovary into the ground to form mature fruit. The influence of abiotic factors especially photoperiod and temperature on the reproductive efficiency and allocation of plant resources has been reported specifically for cultivated peanut (Bell et al., 1991). These authors indicate that both temperature and photoperiod have independent effects on growth and development, and that temperature affects the phenology while photoperiod affects the effectiveness of reproduction and the distribution of plant assimilates.

Wild *Arachis* in Forage Systems

Apart from possessing important traits needed for improvement of cultivated peanut, wild *Arachis* spp. are grown for forage and ornamental purposes. Although several wild *Arachis* produce green forage that is palatable to grazing animals, much emphasis has been given to rhizoma perennial peanut (*A. glabrata* Benth.) and *A. pintoi* Krapov. & W.C. Greg. because they are highly persistent under grazing and trampling. They compete well with several weed species and they are resistant to stress factors such as cool temperatures, flooding, diseases, and pest infestations (Freire et al., 2000; Prine et al., 1981).

The Uniqueness of Rhizoma Perennial Peanut

Certainly, both the rhizomatous *A. glabrata* and the stoloniferous *A. pintoi* are sources of high quality persistence forage and are suitable for intensive grazing. Additionally, *A. glabrata* is well adapted to the Florida climate and provides forage with high nutritive value that is similar to alfafa. Its dense rhizomatous masses are also beneficial for stabilizing soils (Freire et al., 2000). The rhizoma perennial peanut was originally added to the USDA national germplasm system in 1936, but intensive research on its evaluation started in the early 1960s.

‘Florigraze’ (PI 421707) which is one of the most widely grown cultivars was released after repeated evaluation of an off-type line (Prine et al., 1981). Another important cultivar, ‘Arbrook’ (PI 262817), was later released (Prine et al., 1990) based on adaptation to Florida soil and weather conditions. Later, ‘Arblick’ and ‘Ecoturf’ with low groundcover were released as germplasm by the University of Florida (Prine et al., 2010) for forage and ornamental purposes. Recently, the cultivars ‘UF Tito’ (PI 262826) and ‘UF Peace’ (PI 658214), both with improved yield, persistence, and tolerance to

virus diseases were released by the Florida Agricultural Experimental Station (Quesenberry et al., 2010b).

Limitations to Genetic Improvement of Rhizoma Perennial Peanut

Based on past observations (Venuto et al., 1997; Niles, 1989), most accessions and released cultivars of the section Rhizomatosae produce few, if any seed. Hence, their propagation and germplasm exchange have always been carried out through vegetative means. Consequently, crop improvement efforts in the past have been restricted to improving establishment, evaluating new germplasm collections (Freire et al., 2000), and increasing their performance under grazing (Garay et al., 2004; Rice et al., 1995). However, the approach to genetic improvement of this species might change if enough viable seeds can be induced or produced.

Wild *Arachis* in Gene Introgression

Gene introgression through interspecific hybridization has led to the development of a few important peanut cultivars. Important genes have been transferred from *A. cardenasii* Krapov. & W. C. Greg.*, A. diogoi* Hoehne.*,* and *A. batizocoi* Krapov. & W. C. Greg. into cultivated peanut leading to the development of cultivars ‘COAN’ and ‘NemaTAM’ that are resistant to *Meloidogyne* spp. of the root knot nematode (Simpson and Starr, 2001; Simpson et al., 2003). Another cultivar ‘Tifguard’ with high resistance to both tomato spotted wilt topovirus and root-knot nematode has been released by the USDA (Holbrook et al., 2008).

Furthermore, accessions of *A. diogoi, A. stenosperma* Krapov. & W. C. Greg*., A. duranensis* Krapov. & W. C. Greg.*, A. cardenasii* and *A. correntina* (Burkart) Krapov. & W. C. Greg. have been found to be highly resistant to rust caused by *Puccinia arachidis*, while *A. paraguariensis* Chodat *&* Hassl., *A. diogoi, A. stenosperma* and *A. cardenasii*

are highly resistant to early leaf spot caused by *Cercospora arachidicola* and late leaf spot caused by *Cercosporidium personatum*.

Potential Uses of *Arachis paraguariensis*

Arachis paraguariensis (Section *Erectoides*) is a long-lived perennial wild species with a deep and tuberous taproot. Wide genetic and physiological variations exist between and within accessions of this species. Some accessions are highly resistant to early leaf spot caused by *Cercospora arachidicola*, which is an economically significant and widespread disease of cultivated peanut (Subrahmanyam et al., 1985).

Furthermore, there are accessions that have been found to display resistance to root-knot nematode (*Meloidogyne javanica* (Treub) Chitwood Race-3), an important nematode parasite of cultivated peanut (Sharma et al., 2002). High resistance to attack by tobacco armyworm (*Spodoptera litura* Lepidoptera: Noctuidae), another important pest of peanut has also been reported for this species (Stevenson et al., 1993). In addition to these, *A. paraguariensis* has been described as being very persistent under grazing systems, tolerant to drought, and adaptable to wide soil conditions (Krapovickas and Gregory, 2007).

Various attempts were made to transfer genes coding for important traits from *A. paraguariensis* to the cultivated peanut. But crosses made between A. *paraguariensis* and other wild *Arachis* of the section *Erectoides, Rhizomatosae, Procumbentes, Caulorrhizae Heteranthae,* and *Trierectoides* led to the production of sterile hybrids with low pollen germinability (Rao et al., 2003; Krapovickas and Gregory, 2007).

In a previous report, (Singh, 1998b) made crosses between several accessions of tetraploid *A. hypogaea* and diploid A. *paraguariensis*. Successful pollination leading to fertilization was achieved, but there was impaired development of the resulting

proembryos because they aborted due to hypertrophic growth of nucellar tissue into the embryo sac. Hence, identification of an efficient method for combining the genomes of *A*. *paraguariensis* or other *Arachis* species still needs to be performed, as this could greatly enhance the breeding of *Arachis* species.

Hybridization Barriers

Extensive use of wild *Arachis* for genetic improvement of cultivated peanut has been hampered by several barriers including linkage drag, ploidy barriers and sexual incompatibility (Subrahmanyam et al., 2001). Additionally, there is need for more efficient and reliable tools for validating hybrid identities and monitoring introgressed chromosomal segments. Gene introgression from wild relatives belonging to compatible and incompatible gene pools can expand the genetic base of *Arachis*, and also lead to the development of new and important cultivars.

Introgression Pathways

Several possible options for transferring genes from wild *Arachis* spp. into cultivated peanut have been proposed (Simpson, 2001). However, the decision to follow a particular pathway is largely dependent on the breeding goals, availability of resources, and other essential technologies. The potential pathways for introgression of genes from wild *Arachis* into cultivated peanut are discussed below.

Hexaploid pathway. The hexaploid pathway involves crossing diploid *Arachis* directly with cultivated peanut followed by chemically-induced chromosome doubling of the triploid F_1 hybrid to derive a fertile hexaploid. The hexaploid is then backcrossed to cultivated peanut several times in order to eliminate enough chromosomes to lead to restoration of the tetraploid condition (Simpson, 2001).

Diploid-tetraploid pathway. Several disease-and insect-resistant breeding lines were developed through this pathway (ICRISAT, 1990). It involves crossing two or more diploid wild species followed by induction of tetraploidy in the resulting hybrid before crossing it to cultivated peanut. Backcrossing usually follows to carry out selection for the desired trait. The pathway is most suitable when the progenitor wild species of cultivated peanut are being utilized, as in the case of the development of cultivar 'COAN' (Simpson and Starr, 2001). This pathway can also be modified such that the chromosome number of two wild species is doubled before they are hybridized. If the derived tetraploid hybrid is fertile, it is then crossed to cultivated peanut. Although, several attempts to utilize this modified route have been reported (Simpson, 2001), to date none have been successful due to high levels of sterility in the hybrids.

Transformation pathway. *Agrobacterium* mediated transformation, virus or chemical mediated microinjection, electroporation, particle-bombardment and other transformation methods can be used for directly inserting genes into different species (Sleper and Poehlman, 2006). For traits from species in sections of wild *Arachis* that are not closely related to cultivated peanut, the use of molecular insertion of genes through biotechnology techniques likely will be the only feasible method of gene introgression (Chenault et al., 2008). Currently, efficient methods for producing transgenic peanut plants through *Agrobacterium*-mediated genetic transformation (Sharma and Anjaiah, 2000; Bhatnagar et al., 2010) and particle-bombardment (Ozias-Akins et al., 1993; Niu et al., 2009) have been established, but only limited genotypes of cultivated peanut are amenable to transformation.

In Vitro Tissue Culture of Wild *Arachis*

Tissue culture regeneration refers to the development of organized structures such as roots, shoots, flower buds and somatic embryos from cultured cells or tissues that are called explants. Usually, regeneration is carried out *in vitro* such that the environment and growth medium composition can be altered to achieve high frequency regeneration.

Through the application of tissue culture and biotechnology techniques, the genes controlling disease, insect, and nematode resistance found in wild *Arachis* spp. may be transferred into cultivated peanut. *In vitro* tissue cultures are also capable of inducing stable somaclonal variation (Larkin and Scowcroft, 1981) that can become a useful source of genetic variation. However, the success of such genetic improvement programs is highly dependent on the ability to successfully regenerate plants from tissue cultures. Limited success has been achieved in the regeneration of plants from callus originating from various explants including immature embryos, cotyledons, epicotyls, hypocotyls, and leaves of several *Arachis* species (Gagliardi et al., 2000; Pacheco et al., 2007; Pacheco et al., 2009 ; Pacheco et al., 2008). Plantlet regeneration has also been achieved from short term cultures by *in vitro* organogenesis of a few species of *Arachis* (Gagliardi et al., 2002; Pacheco et al., 2009).

However, some wild species including A. *paraguariensis* and *A. glabrata* still lack efficient tissue culture regeneration procedures that can be successfully employed for somatic hybridization, *in vitro* ploidy manipulation and genetic transformation.

Morphogenic Pathways

Correct identification of developmental pathways of regeneration is important to identify the most suitable system to achieve specific research goals. The three major

developmental pathways for *in vitro* plant regeneration are propagation from pre-existing meristems (shoot culture or nodal culture), shoot organogenesis, and non-zygotic embryogenesis or somatic embryogenesis.

Propagation from pre-existing meristems. The propagation of shoots or nodal segments is a common method for mass production of plantlets through *in vitro* vegetative multiplication. The process usually consist of four stages (Fossard, 1987) starting with the selection and inoculation of explant into nutrient medium, followed by multiplication and growth of culture with repeated subculture. Next is the stage involving rooting and acclimatization of plantlets before the final transfer to soil. Even though, propagation from pre-existing meristems has minute application to plant breeding, it is the major method of plant propagation at industrial level where the goal is typically to propagate and maintain large population of homogenous plantlets (Dodds and Roberts, 1985).

Organogenesis. Shoot organogenesis (adventitious shoot formation) involves propagation from explants without pre-existing meristems through production and subsequent rooting of adventitious shoots (Schwarz and Beaty, 2000). It is a common method of micropropagation and shoots can be directly induced *in vitro* from different explants such as leaf, petiole, stem, and other organs obtained from intact plants. Such shoots can arise directly on explants or indirectly through an intermediary callus phase. The advantages of shoot organogenesis when compared to other *in vitro* regeneration pathways include: 1) efficiency and ease of propagation, 2) more synchronous shoot production than somatic embryogenesis, 3) importance of its often single-cell origin in

genetic transformation, and 4) the relatively higher *ex vitro* survival of derived plantlets (Preece, 2000).

In general, the cellular or tissue origin of shoots derived from organogenesis appears controversial. There are reports that have used histological and morphological analysis to describe the formation of adventitious shoots of plants from different cellular origins. The model proposed by Broertjes and Van Harten (1978), Broertjes and Keen (1980), and Verma and Mathur (2011) stating that directly-regenerated adventitious shoot meristems are always formed from one or a few daughter cells originating from a single cell has not been widely accepted. There was an opposite proposition that such regenerated plants are of multicellular and multihistogenic origin (Norris et al., 1983; Zhu et al., 2007).

Since the exact origin of shoots derived from organogenesis has led to inconsistent observations, it is recommended that definition of the origin of shoots emerging from explants should not be generalized. On the other hand, investigations should be carried out with the aid of histological and molecular tools as required to determine the exact origin for each genotype or species. This approach will be worthwhile especially when there is need to determine the most suitable pathway for specific crop improvement methods such as genetic transformation and ploidy manipulation.

Non-zygotic embryogenesis. This is an important pathway for producing somaclonal variants, developing artificial seeds, and synthesizing metabolites. Non-zygotic embryogenesis involves regeneration of embryos from cells, tissues or organs other than the zygote (Steward et al., 1958). Embryos can be initiated either directly

from explants without callus formation, in a manner usually described as direct embryogenesis or indirectly when callus masses form on explants before embryos are developed.

The developmental patterns of non-zygotic embryos *in vitro* have been described based on studies in *Arabidopsis* (Zimmerman, 1993; Yasuda et al., 2000). First, there is asymmetric division followed by formation of a globular structure which transforms into an oblong. Next is the formation of a heart-shaped structure, then torpedo and cotyledonary shape structures, and finally the mature embryo. Non-zygotic embryos originate from a single cell, and are bipolar with both shoot and root poles. They also lack vascular connections between the callus mass or explant thus making their separation easy (Stephen et al., 2000)

Signal Transduction

Definitely, cellular dedifferentiation is characterized by remarkable changes in the pattern of gene expression; there are specific genes involved in dedifferentiation, acquisition of competence and induction of *in vitro* regeneration (Henry et al., 1994). It is also possible that genes are regulated by epigenetic mechanisms, including certain chromatin effects. Shoot regeneration efficiency in tissue culture has been described as a quantitative trait that often varies between plant species and within a plant species among subspecies, varieties, cultivars, or ecotypes. Consequently, shoot regeneration often becomes difficult especially when a different regeneration procedures have to be developed for different genotypes within the same species (DeCook et al., 2006). The quantitative trait loci (QTL) associated with variation in shoot regeneration efficiency in *Arabidopsis* were identified by Schiantarelli et al. (2001), while candidate genes for regeneration in several species have also been defined through the use of QTL

mapping (Bolibok and Rakoczy-Trojanowska, 2006; Taguchi-Shiobara et al., 2006) and microarray analyses (Bao, 2009).

Totipotency, genotype, and explant source. The concept of totipotency is fundamental in understanding the process of *in vitro* tissue culture regeneration. It is the potential of living cells to express the total genetic potential of the parent plant in regenerating to whole new individuals. Totipotency is highly dependent on genotype, explant type, and the presence of appropriate stimuli (Dodds and Roberts, 1985).

There are easy-to-regenerate species, as well as recalcitrant ones. Several legumes and woody species have been described as recalcitrant. Generally, *Arachis* was described as one of these recalcitrant leguminous species (Cheng et al., 1994; Mroginski et al., 2004). But advancements in tissue culture research in recent years have led to establishment of various regeneration procedures for cultivated peanut and many of the wilds *Arachis* spp. (Dunbar and Pittman, 1992; Faustinelli et al., 2009; Li et al., 1993; Pacheco et al., 2009).

Often the effects of genotype on developmental pathways, growth rates, and callus characteristics are observed *in vitro*. Similarly, the amount and type of endogenous plant hormone possessed by different plant genotypes influence their tissue culture response. The explant source and age has been found to be crucial for successful tissue culture regeneration as well. As a rule, explants obtained from less differentiated, immature tissues are easier to regenerate while explants derived from highly differentiated tissues are less responsive *in vitro*. In reality, the capacity of cells to become competent for regeneration may be completely lost once they over-mature. Moreover, the orientation and positioning of explants on the culture medium often

becomes important for absorption of nutrients and growth regulators (Papafotiou and Martini, 2009).

Endogenous and exogenous growth regulators. Endogenous growth regulators or phythohormones are signaling molecules produced within the plant in extremely low concentrations. These molecules play very important roles in regulating cellular processes including the formation of somatic embryos, shoots, stems, leaves and flowers. Other processes such as seed growth, time of flowering, senescence, the direction of tissues growth, plant longevity, and death are also heavily regulated by these growth regulators (Gan et al., 2004). Growth regulators are usually produced by plants within the meristematic zone where high concentration of actively dividing cells exists before any specialized tissue is differentiated. They are then transported to other plant parts to induce specific changes or they are deposited in cells for future release and utilization.

Exogenous growth regulators may be artificially synthesized and are used to regulate plant growth *in vitro* or *in vivo*. The type and quantity of plant growth regulators (PGRs) in the tissue culture medium is critical for determination of the morphogenic pathway of cultured cells. The five main classes of PGRs that are commonly used in plant cell and tissue culture are auxins, cytokinins, gibberellins, abscisic acid, and ethylene.

Certainly, auxins and cytokinins affect most key developmental episodes in plants. They both have pronounced and synergistic effects on plant growth and development especially in apical dominance and the development of roots and shoots (Eklöf et al., 1997). Specifically, cytokinin signaling modulates auxin signaling and transport for

determining root meristem size in *Arabidopsis* (Dello Ioio et al., 2007). The balance of auxin and cytokinin in the medium is therefore important for determination of the morphological fate of explants. Different organ types can also be achieved by regulating the auxin-to-cytokinin ratio. Ordinarily, a relatively high auxin to cytokinin ratio promotes regeneration of roots, while the reverse leads to the production of shoots. Typically, explant cells will proliferate to form callus when they are exposed to a nutrient medium containing approximately the same level of auxin and cytokinin.

Environmental stimuli. Various environmental factors, such as temperature, light conditions, and humidity may function as signals thereby causing groups of cells to redefine their fate. Hence, abiotic factors inside culture vessels and in growth chambers can influence cellular and physiological processes involved in tissue culture regeneration. These factors interact with the cellular factors to produce either a positive or negative effect.

In general, temperature has to be optimal and constant while light intensity should be adequate and of the right quality and duration. When humidity is too high, plantlet growth can be retarded (Chen, 2004). The design of the culture vessel is very much associated with ventilation, humidity, and accumulation of gasses such as ethylene that could hinder shoot or root elongation and also induce leaf abscission in plantlets (Kumar et al., 2009). Additionally, the type of support matrix can determine how water relations, humidity, temperature and some other physical factors are manifested. Clearly, there is a complex interaction within and between the physical, chemical and physicochemical environment of explants.

The Challenges of *In Vitro* Tissue Culture of Wild *Arachis*

The problems facing tissue culture regeneration of *Arachis* species are specific for each genotype, explant type, and regeneration pathway and cannot be oversimplified. For instance, tissue culture of some wild *Arachis* spp. including *A. glabrata* has not been very successful due to lack of a suitable explant source. On the other hand, A. *paraguariensis* possesses very high morphogenic potential; successful regeneration from protoplast and callus cultures (Li et al., 1993; Still et al., 1987) support its potential suitability as an ideal model system for studying *in vitro* regeneration and morphogenesis of leguminous species. However, failure of regenerated shoots to produce roots either *in vitro* or *ex vitro* has constituted a major limitation to the production of a large number of surviving plantlets.

For most breeding programs utilizing *in vitro* tissue culture techniques, the final aim is successful establishment of derived plantlets in the field or in the greenhouse. However, a substantial number of plantlets do not survive due to stress encountered during the acclimatization process. Lack of roots or poor formation prior to acclimatization can also result in a low survival rate. More importantly, there is need to identify specific challenges that are being faced by each of the *Arachis* spp. and devise appropriate methods for overcoming them.

Root formation. Some plants easily form roots *in vitro* while others do not. Events involved in formation of a root during embryogenesis or organogenesis are controlled by interactions of many exogenous and endogenous factors such as sugars, calcium, auxins, cytokinins, polyamines, ethylene, nitric oxide, hydrogen peroxide, carbon monoxide, cyclic guanosine monophosphate (cGMP), and peroxidase. Specifically, auxin signaling promotes the expression of cytokinin signaling inhibitors,

while cytokinin signaling also promotes the expression of auxin signaling inhibitors (Bishopp et al., 2011). Adventitious roots are post-embryonic; their formation involves a process of dedifferentiation, in which determined cells change their morphogenetic pathway and then act as precursor cells for the root primordia.

It has been clearly established that auxin response factors (ARF) transcription factors and Aux/ IAA proteins play crucial roles in auxin signaling. These two proteins facilitate the auxin gene expression response. The Aux/IAA proteins normally function as repressors by binding ARF transcription factors to prevent the activation of promoters and other control sequences of genes that are turned on or off by auxin. The binding of auxin to TIR I receptor leads to the formation of a unique complex that binds proteins that attaches ubiquitin to Aux/IAA proteins for their targeted destruction. Hence gene transcription can commence (Teale et al., 2006; Kepinski and Leyser, 2002). Even though it is more cost effective to root plantlets *ex vitro* than *in vitro*, it is not always a feasible option for leguminous species that are difficult to root and acclimatize.

Acclimatization and plantlet survival. Certainly, an acclimatization process is required for most *in vitro* derived plantlets to ensure high survival rates. The *ex vitro* environment generally has significantly lower relative humidity, higher light intensity and is non-aseptic. Hence, plantlets undergo numerous stresses during transplanting. Besides, the negative carryover effects of synthetic growth regulators on acclimatization can also exert a negative impact on plantlet survival rates (Valero-Aracama et al., 2010). Rooting is an important determinant of *ex vitro* survival but *in vitro* derived roots are prone to damage during acclimatization. Moreover, shoots are usually exposed to

12-24 hours of photoperiod prior to acclimatization to improve their photosynthetic efficiency and also to prepare them for *ex vitro* acclimatization.

In Vitro **Ploidy Manipulation**

Polyploidy occurs when the number of chromosomes in a cell becomes multiplied. It is a key evolutionary mechanism that often leads to creation of new species from different progenitors. There are two common forms of polyploidy. Autopolyploidy is the formation of a polyploid with more than one diploid chromosome set from the same species genome while allopolyploidy involves the combination of multiple genomes into one single nucleus. Several autopolyploids occur spontaneously or naturally through the fusion of 2n gametes. However, it is also possible to chemically induce polyploidy by using antimitotic agents such as colchicine, oryzalin or trifluralin (Quesenberry et al., 2010a). Chemical induction of polyploidy has been successfully utilized to transfer useful traits between several species (Stalker and Simpson, 1995). Doubling of chromosome numbers in this way can also be used to facilitate the crossing of diploid, tetraploid and hexaploid species and to improve fertility of some hybrids (Adams and Wendel, 2005; Castillo et al., 2009).

Autopolyploids often display polysomic inheritance because they have more than two copies of each chromosome with equal chance of recombining at meiosis. Therefore, crossovers can occur between more than two homologues, resulting in multivalent arrangements of homologous chromosomes at metaphase I and chromosome abnormal segregation at anaphase (Burnham, 1966). Consequently, anomalous chromosome segregation may lead to partial sterility. But some autopolyploids are nearly completely fertile with mostly bivalent configurations observed at meiosis I. The formation of bivalents in fertile autopolyploids is a random event, and

appears to be under genetic regulation (Cifuentes et al., 2010). Nevertheless, the underlining genetic basis for the reduction in formation of multivalents still needs to be investigated. Colchicine induced polyploidy can produce changes in plant morphology and is considered suitable for crop improvement. Gene duplication occurs as a result of polyploidy and such duplicated genes may be expressed equally or unequally. Likewise, silencing of one copy of a duplicated gene is a common response to polyploidy (Adams and Wendel, 2005).

Ploidy Detection

The use of flow cytometry for ploidy analysis in plant breeding is widely accepted. The technique can easily be used to distinguish parent plants, screen offspring after interploidy crosses, monitor ploidy levels during seed multiplication or evaluate occurrence of polyploidisation (Leus et al., 2009). Flow cytometric analysis involves the estimation of DNA content by using the fluorescence and light scatter properties of distinct particles such as cells, nuclei, and chromosomes during their passage inside a narrow, liquid stream. The nuclei to be analyzed are usually released into cell homogenates by chopping or by lysis of protoplasts. Flow cytometry of nuclear DNA is appropriate for cell cycle analysis because the nuclear DNA content reflects the position of each cell within the cell cycle. Hence it is possible to determine the fraction of the cell population in the G1, S and G2 phases of the cell cycle. Usually, computer software is used for such analysis to derive a DNA content distribution curve with corresponding peaks. For nuclear genome size determination, the G1 peak corresponding to the unknown sample nuclei is compared relative to nuclei isolated from another plant of known DNA content.

Knowledge of leaf stomata size and frequency can be used to define incidence of tetraploidy. Typically, the stomata in the tetraploids are clearly larger and more dispersed than those of the same diploid species. Observations on the size and frequency of stomata on leaf surfaces have suggested that they can be successfully utilized as rapid indirect methods for preliminary identification of ploidy level in several species (Beck et al., 2003; Quesenberry et al., 2010a). This is most useful when screening a large number of plants for polyploidy.

Finally, traditional chromosome cytological examination is usually carried out for verification of induced polyploids. Ordinarily, chromosomes from preparations of root tips or pollen mother cells are observed and counted under the microscope. For instance, a normal tetraploid plant should have two times the number of chromosome of the diploid. Counting of chromosomes is laborious and prone to errors especially in species having a large number of chromosomes of small size. Results from a study on chromosome doubling of *Trifolium* species with the use of N_2O (Taylor et al., 1976) revealed that ploidy level classification based on pollen size was almost as effective as subsequent cytological verification via counting of chromosomes.

CHAPTER 3
OPTIMIZING *IN VITRO* REGENERATION CONDITIONS FOR *Arachis paraguariensis*

Tissue culture and biotechnology techniques are central to several innovative concepts for overcoming hybridization barriers between cultivated peanut (*Arachis hypogaea* L.) and several of its wild relatives. Of significant importance is the diploid *Arachis paraguariensis* Chodat & Hassl, a wild species with novel traits including disease, insect, and nematode resistance. Although tissue culture of cultivated peanut has witnessed a remarkable improvement in the past few decades, many of the wild *Arachis* species appear recalcitrant to similar methods (Srinivasan et al., 2010). Limited success has been achieved in the regeneration of plants from mature leaflets (Dunbar and Pittman, 1992), protoplasts (Li et al., 1993), immature zygotic embryos (Sellars et al., 1990), stamens (Still et al., 1987), cotyledons, embryonic axes and embryonic leaflets of *A. paraguariensis* (Pacheco et al., 2009).

Identification of the tissue culture regeneration pathway and the specific factors influencing morphogenetic potential of a given species is important for developing a highly efficient regeneration system. This is because during the process of cellular dedifferentiation, the developmental fate of explants can easily be altered by endogenous hormones as well as several environmental factors including stress, exogenous growth regulators, light, temperature and humidity. The formation and activities of meristems are highly dependent on these factors. Consequently, the morphogenic pathway for the tissue culture explant is highly flexible and open to alternative routes.

Generally, the two common pathways of plant tissue culture regeneration are organogenesis and non-zygotic (somatic) embryogenesis. During organogenesis

adventitious organs or axillary buds form directly or indirectly on the explant. Non-zygotic embryogenesis involves a notable developmental pathway with anatomical and physiological features of embryos that are highly comparable to zygotic embryos (Zimmerman 1993; Mandal and Gupta, 2002). Due to the single cell origin of non-zygotic embryos, they are preferred in several regeneration systems for clonal propagation, ploidy manipulation, gene transfer and synthetic seed production. However, tissue regeneration via organogenesis has proved to be advantageous for studying regulatory mechanisms of plant development (Hicks, 1994).

The role of thidiazuron (TDZ) in bio-regulation of *in vitro* morphogenesis is well recognized. For instance, the auxin 2,4-D has been used to induce embryogenesis in many cultivars of peanut, however, replacing 2,4-D with TDZ lead to the development of the most efficient genotype-independent peanut non-zygotic embryogenic system. (Saxena et al., 1992; Sharma and Anjaiah, 2000). In addition, Murthy et al. (1995) reported that culturing seedlings for just two days on TDZ-supplemented medium was sufficient to induce embryogenesis. The only report currently existing on *in vitro* non-zygotic embryogenesis of *A. paraguariensis* utilized immature zygotic embryo explants but the induction of embryogenesis did not commence until 120 days after culture initiation (Sellars et al., 1990). A major problem in previous studies utilizing organogenesis was difficulty in regenerating shoots to produce roots either *in vitro* or *ex vitro*. Still et al. (1987) first reported that root formation of *A. paraguariensis* plantlets rarely occurred in agar or liquid cultures, therefore, grafting to stems of rooted seedlings was the method used to obtain plants from regenerated shoots. In another study, Li et al. (1993) observed shoots that failed to form roots or normal plantlets after 90 days of

culture initiation. Consequently, porous root-cubes saturated with nutrient medium and auxin were used before rooting could be achieved.

Flowering *in vitro* is a phenomenon that has been reported in *A. paraguariensis* tissue cultures (Still et al., 1987; Li et al., 1993), but the environmental and genetic factors involved are unknown. Apart from being a significant event for potential gene transfer methods such as *in vitro* fertilization, flowering *in vitro* can be used as an experimental system for studying the molecular mechanisms of flowering (McDaniel et al., 1991; Kane et al., 2000) in *Arachis.* Therefore, this study was designed to: 1) optimize an *in vitro* regeneration procedure for *A. paraguariensis* through clearly defined morphogenic pathways, 2) increase rooting efficiency of the regenerated shoots over that previously achieved, and 3) identify the influence of photoperiod on *in vitro* flowering.

Materials and Methods

Explant Source and Sterilization

Seeds of *A. paraguariensis* were obtained from the USDA Plant Genetic Resources Conservation Unit, Griffin Georgia, a component of the Germplasm Resources Information Network (GRIN) National Plant Germplasm System (NPGS). Identification and further information on the origin of the six genotypes of *A. paraguariensis* used for this study are presented in Table 3-1. Shelling of seeds was done manually before they were surface-sterilized by rinsing in 70% ethanol for 1 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. Thorough washing with sterile-distilled water was performed before seeds were soaked in sterile water for 4 h in a laminar airflow chamber. The seed coat was then carefully removed before each type of explant was derived. The quartered-seed explants were obtained

after removal of the seed coat and each seed was aseptically dissected into four equal longitudinal pieces. The deembryonated cotyledon explants were obtained after the removal of the entire embryonic axis from each seed followed by longitudinal dissection into four equal sections.

Culture Media and Environment

All media formulations consisted of MS basal salts (Murashige and Skoog, 1962) supplied by Sigma-Aldrich, St. Louis, MO. Vitamins (Sigma # G1019) were as in Gamborg B5 medium (Gamborg et al., 1968). All media also contained 30gL^{-1} sucrose (Sigma # S5390), 0.8% (w/v) agar (Sigma # A7921), and were supplemented with different concentrations and combinations of auxin, cytokinins. The pH of each medium was adjusted to 5.8 using 0.1 N KOH prior to autoclaving at 121^{o}C with 1.06 kg cm^{-2} pressure for 20 min while stock solutions of each plant growth regulator were first filter-sterilized through a double 0.2 µm filter before being added to the autoclaved media in sterile bottles. Routinely, 1 mlL^{-1} of Plant Preservative Mixture (PPM^{TM}, Plant Cell Technology, Washington DC, USA) was added to the culture medium.

Liquid medium (25 ml) was dispensed into each 2.5 × 10 cm petri dish while each Phytatray^{TM} culture vessel (Sigma # P5929) contained 50 ml of medium formulation. Initial culture incubation of explants was performed at 26±1ºC under continuous lighting provided by cool white fluorescent lamps at 60 µmol$m^{-1}s^{-1}$ light intensity. A medium (MS0) that lacked growth regulator was used as the control treatment for the explant regeneration experiments.

Experimental Design and Data Collection

This study consisted of five separate experiments that were designed as follows:

- **Tissue culture regeneration (Experiment I)**. 6 (genotypes) x 2 (explants) x 3 (2,4-D concentrations) x 3 (BAP concentrations) factorial experiment in a completely randomized design with sub-sampling with 2 replications

- **Tissue culture regeneration (Experiment II).** 6 (genotypes) x 3 (TDZ concentrations) x 4 (BAP concentrations) or 4 (2ip concentrations) factorial experiment in a completely randomized design with sub-sampling having 4 replications

- ***In vivo* evaluation.** Two genotypes evaluated in a randomized complete block design with 2 replications

- ***In vitro* rooting experiment**. 6 (genotypes) x 3 (auxin types) x 3 (auxin concentrations) x 2 (culture vessel) factorial experiment in a randomized complete block design with 4 replications

- ***In vitro* flowering experiment**. 6 (genotypes) x 3 (photoperiod) factorial experiment in a completely randomized design with sub-sampling having 2 replications

Statistical Analysis

SAS PROC MIXED (SAS Institute, 2010) was used for all data analysis according to a mixed effects model while mean separation was performed using the Tukey's Honestly Significant Difference Test (P = 0.05). In the tissue culture regeneration experiments, each petri dish contained four explants or shoots to constitute a subsample. Means for shoot characteristics for each medium-genotype-replicate were computed and analyzed. Data on the effect of medium on regeneration and shoot characteristics were analyzed according to a completely randomized design with sub-sampling. Growth regulator concentration and explant type were treated as fixed effects while genotype, replicate and the interactions of the growth regulators with growth regulator and genotype were treated as random effects. A separate analysis within each genotype was also implemented to confirm the results of the overall analysis.

The data on the effect of culture vessel, auxin type, and auxin concentration on rooting, survival, and plantlet characteristics were analyzed according to a randomized

complete block design with each replicate being a block. Culture vessel, auxin type and auxin concentration were treated as fixed effects whereas genotype, replicate and the interactions were considered as random effects.

In order to determine the effect of photoperiod on flowering, the data were subjected to analysis according to a completely randomized design with sub-sampling. Genotype and photoperiod were treated as fixed effects while replicate and the interactions were analyzed as random effects. The test for significance of treatment effects for the greenhouse and field evaluation studies were carried out according to a mixed model while means were separated based on Tukey's Honestly Significant Difference Test (P = 0.05).

Histological Analysis

Histological studies were performed at the University of Florida College of Medicine Electron Microscopy Core Facility according to the method of Yeung and Saxena, (2005). Callus masses at different developmental stages were fixed in a mixture of 1.6% paraformaldehyde and 2.5% glutaraldehyde in a 0.05M phosphate buffer at pH 6.8 for 24 h. Tissues were then dehydrated using methyl cellosolve followed by two changes of absolute ethanol before infiltration and embedding in glycol methacrylate-based plastic molding cups. Serial sections were obtained with a glass knife using a Leica 2040 rotary microtome with a retractable return stroke. Thin sections were mounted on glass slides and stained with toluidine blue while photographs were taken with Nikon light microscope.

Tissue Culture Regeneration

Each explant was carefully implanted on the appropriate medium inside petri-dishes with their cut ends embedded in the medium. Observations for greening,

swelling, and formation of callus, leaf, or globular structures on the explants were recorded for each subsample, while the frequency of shoot and root formation, as well as plantlet survival, and were observed throughout the regeneration studies.

Experiment I

In the first experiment, deembryonated cotyledon and embryonic axis explants were established on MS medium containing 2, 4-D (0, 2.2 and 4.4 mgL^{-1}) or BAP (0, 2.2 and 4.4 mgL^{-1}) alone or combined in a factorial manner inside 2.5 x 10 cm Petri dishes. After callus formation, the maturation, conversion of embryo and rooting took place on MS medium without any growth regulator inside PhytatrayTM culture vessels.

Experiment II

The second experiment involved testing of quartered-seed explant on MS medium containing TDZ (0, 2.2 and 4.4 mgL^{-1}) alone, or in combination with either BAP (1.1, 2.2 and 4.4 mgL^{-1}) or 2ip (1.1, 2.2, 4.4 mgL^{-1}) inside 2.5 x 10 cm petri dishes. Elongation of buds was performed inside Phytatray^{TM} culture vessels containing 50 ml of MS medium that was supplemented with 2 mgL^{-1} BAP and 1 mgL^{-1}. However, the plantlets were rooted on MS medium devoid of growth regulator under the same conditions except that the photoperiod was reduced to 16 h.

***In Vitro* Rooting**

Rooting was tested on semi-solid MS medium supplemented with IAA, IBA or NAA each at concentrations of 0.2, 0.6 and 1 mgL^{-1}. These media were compared in 11.4 cm × 8.6 cm × 10.2 cm polyethylene terephthalate glycol (PETG) vessels and in 2.5 cm × 15 cm glass tubes. Micro-shoots (Figure 3-1a) of 1-2 cm height were transferred into each vessel type. Each PETG vessel contained four micro-shoots on 50 ml of medium, while there was only one micro-shoot on 10 ml of medium per glass tube. The micro-

shoots were derived according to the regeneration procedure developed from the previous regeneration experiment utilizing MS salts with vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar, 2.2 mgL^{-1} TDZ and 4.4 mgL^{-1} BAP. The number of rooted shoots and the number of roots per plantlet were recorded after six weeks of culture initiation. The maximum root length was determined from the mean of the three longest roots per plantlet. Afterwards, plantlets were transferred into Jiffy® peat pellets (Jiffy, Lorain, OH.) and acclimatized for two weeks in a humidity chamber. The acclimatized plants were transplanted into pots containing Metro-mix 300 (Sun Gro Horticulture, Canada Ltd.) and sand in a ratio of 1:1 (v/v). After four weeks, the number of surviving plants were counted.

***In Vitro* Flowering**

Regenerated micro-shoots derived from quartered-seed explants maintained on semisolid MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar, 2.2 mgL^{-1} TDZ and 4.4 mgL^{-1} BAP were used for this study. Micro-shoots of 1-2 cm height (four per culture vessel) were placed into Phytatray™ culture vessels containing 50 ml of MS medium with 3% sucrose (w/v) and 0.8% (w/v) agar but without growth regulators. Cultures were maintained in three separate growth chambers at 26 ± 2°C under 12, 16 or 24 h photoperiods with illumination of 60 $\mu molm^{-1}s^{-1}$ provided by daylight-type florescent lamps. After the appearance of the first flower bud, observations and data collection were performed weekly until the plantlets ceased to flower. Flowering plantlets were acclimatized and transplanted into pots filled with Metro-mix 300 and sand in a ratio of 1:1 (v/v) (1:1) in the greenhouse.

In Vivo Evaluation

After two to three roots have been formed, the plantlets derived from the experiment utilizing TDZ (Experiment II) were transplanted into Jiffy® peat pellets and acclimatized for two weeks inside a humidity chamber before they were transferred to Metro-mix 300 and sand in a ratio of 1:1 (v/v) in 16.5 (depth) × 16 cm (diameter) pots under greenhouse conditions.

One hundred an eight plants that had already been acclimatized and established inside pots in the greenhouse were transplanted to the field in the summer of 2009. The field study was carried out at the Plant Science Research and Education Unit, Citra, Florida in a randomized complete block design with two replications. A cultivated peanut (*A. hypogaea*) variety, 'Florunner,' and seedlings of *A. paraguariensis* that had never been subjected to any *in vitro* tissue culture procedure, were planted as control treatments.

Evaluation of the plants was carried out in 2009 and 2010. Leaf spot disease caused by *C. arachidicola* was rated using the Florida peanut leaf spot scoring system (Chiteka et al., 1988) where 1 = no disease, 2 = very few leaf spots in canopy, 3 = few leaf spots in lower and upper leaf canopy, 4 = some leaf spotting in lower and upper canopy with light defoliation (<10%), 5 = leaf spots noticeable in upper canopy with some defoliation (<25%), 6 = leaf spots numerous with significant defoliation (<50%), 7 = leaf spots numerous with heavy defoliation (<75%), 8 = numerous leaf spots on few remaining leaves with severe defoliation (<90%), 9 = very few remaining leaves covered with leaf spots and severe defoliation (<95%), and 10 = plants defoliated or dead.

Harvesting of seeds was performed manually by hand-lifting of the pedicels. The pods were removed, washed and air dried in a cool, dry area. The seed yield of plants

grown inside pots in the greenhouse was then compared with the yield attained by plants that were established in the field.

Results

Two experiments were designed to investigate the roles and interactions of different genotypes, explant sources, and growth regulators on tissue culture regeneration of *A. paraguariensis.* The study was also aimed at identifying the different regeneration pathways involved during the *in vitro* regeneration process.

Experiment I - The Role of 2,4-D in Induction of Embryogenesis

Indirect non-zygotic embryogenesis was achieved after the induction of embryogenic callus from the deembryonated cotyledon explants. Greening and swelling of explants began after 24 h of explant incubation followed by callus formation after 2-wk. The calli displayed great variability in color, texture and proliferation rate depending on the culture medium. Sub-culturing of callus masses was carried out every 2-wk, however, embryo formation did not commence until after one month of culture initiation. When the callus masses were observed under a stereomicroscope, embryogenic masses with proliferating shoots were observed. Consequently, the calli were transferred onto MS medium lacking growth regulators for maturation.

Minimal callus formation was observed on the embryonic axis explants after 2 wk of culture incubation on the MS medium formulations containing 2,4-D and BAP, but no embryos or shoots were formed. The calli that formed turned black and died after 4-wk of culturing. Likewise, no callus or shoot formation was observed on any of the explants that were cultured on the MS medium (control). The explants however remained fresh until the entire medium dried up.

The effects of 2,4-D and BAP concentrations and combinations on tissue culture regeneration from deembryonated cotyledon explants of *A. paraguariensis* are presented in Table 3-2. Induction of non-zygotic embryos was observed in all the concentrations of 2,4-D that were tested. However, the earliest embryo initiation occurred when deembryonated cotyledons were cultured onto MS medium containing 4.4 mgL^{-1} 2,4-D or 2.2 mgL^{-1} BAP. The highest number of plantlets was recovered from medium that was supplemented with 4.4 mgL^{-1} 2,4-D and 4.4 mgL^{-1} BAP. All the plantlets produced lacked vigor, exhibited poor root formation, and were short. For example heights ranged from 1.7-2.8 cm (Table 3-2). Consequently, only a few of them survived initial acclimatization, and survived post-acclimatization stress in the greenhouse.

Experiment II - The Role of TDZ in Induction of Embryogenesis and Organogenesis

Callus formation was observed after 24h of culturing the quartered-seed explants onto all the media containing 4.4 mgL^{-1} of TDZ in combination with any of the levels of BAP tested (Figure 3-1b). Green globular structures were observed on callus masses as early as one week after culture initiation (Figure 3-1c). These globular structures were distinct (Figure 3-1d) and after a week of further development, it was easy to remove them as individuals from the explant. Histological analysis revealed that the structures were bipolar somatic embryos lacking a suspensor cell, but having a well-defined protoderm (Figure 3-1e). Embryos differentiated to form shoots after they were transferred to MS medium lacking growth regulators. Table 3-3 shows the effects of TDZ and BAP concentrations and combinations on shoot regeneration across all six genotypes. The results indicate that the application of 4.4 mg-1 of TDZ in combination

with the same or lower levels of BAP was necessary for formation of non-zygotic embryos. However, combining 1.1 mgL^{-1} of BAP to the optimal concentration of TDZ resulted in the highest mean number (14.8 ± 1.6) of plantlets per explant cultured and the tallest plantlets (7.3 ± 0.7 cm) at acclimatization. Besides, the time (6 ± 0.3 days) taken for buds to appear was relatively short.

In general, the explants on media that were supplemented with TDZ and BAP were more prolific with regard to callus, globular structure and shoot formation than the explants cultured on media containing TDZ alone or in combination with 2ip (Table 3-3 and Table 3-4). Interestingly, the application of TDZ in combination with 2ip (Table 3-4) resulted in the overall highest number of plantlets recovered per explant of 20.2 ± 1.8 but with higher mean number of days (13.9 ± 1.0) to bud initiation than for the treatment utilizing BAP.

The regeneration pathway for tissues originating from the experiment utilizing TDZ in combination with BAP differs depending on the concentration, but not the genotype. Regeneration of the explants cultured on medium containing 2.2 mgL^{-1} of TDZ in combination with any of the concentrations of BAP did not follow a specific pathway; non-zygotic embryo as well as organogenic callus developed simultaneously on the same explant. Nonetheless, regeneration via shoot organogenesis was observed in the explants that were cultured on medium containing TDZ only. Contrariwise, the histological analysis of tissues from the experiment utilizing TDZ in combination with 2ip revealed that the regeneration pathway was purely non-zygotic embryogenesis.

Rooting and Post-Acclimatization Survival of *In Vitro*-derived Plantlets

The results from the rooting experiment revealed that the culture vessel type has a significant (P = 0.05) influence on rooting efficiency across all the genotypes studied.

Across all the auxin treatments, plantlets in wide PETG vessels showed a significantly higher rooting (68%) and *ex vitro* survival (38%) than those in glass-tubes 17% and 6% respectively (Figure 3-4). *In vitro* flowering was observed in several of the shoots even though they lacked roots (Figure 3-2c). The PETG vessel also gave plantlets with a higher number of roots per plantlet and were faster to initiate rooting (14.9 vs 27.3) than the glass tubes. Additionally, roots of plantlets grown in PETG vessel were significantly longer than those in glass tubes (3.7 vs 1.2 cm) across all the genotypes and auxin treatments (Figure 3-5).

The influence of auxin type and concentrations on root formation inside PETG vessel is presented in Table 3-5. The treatment which gave the most desirable rooting performance based on a combination of number of roots, maximum root length and number of days to root initiation was the 0.2 mgL^{-1} of IBA. The control treatment of MS medium devoid of auxin gave the maximum root length (7.1 ± 1.2 cm), and the highest number of days to rooting of 26.6± 1.8.

The *ex-vitro* survival of plantlets in response to the various auxin treatments inside PETG vessel is shown in Figure 3- 6. Highest post acclimatization survival was recorded for the non-treated control and the IBA or IAA treatment of 0.2 mgL^{-1}. The rooting response and survival of the six genotypes were evaluated across all the auxin treatments inside PETG vessel and the result is presented in Figure 3-7. Only PI 468365 appeared to be a difficult-to-root genotype with 40% rooting and 17% survival rates.

The addition of 1 $mgL^{-1}$of NAA to the medium led to early emergence of root and the highest number of root per plantlet, nonetheless, the roots produced by the plantlets

were thick and short (Figure 3-2b) while there was also severe leaf abscission of the plantlets (Figure 3-2d). Consequently, these plantlets failed to survive during *ex-vitro* acclimatization and further transplanting to soil. Conversely, plantlets that were allowed to form roots on MS medium without any growth regulator were vigorous with long flexible roots (Figure 3-2e).

The Effect of Photoperiod on *In Vitro* Flowering

High frequency of flowering was observed for all the genotypes that were studied *in vitro* (Figure 3-3a-b). Each flower appeared normal and was borne on an erect pedicel (Figure 3-3c). A pollen germination study was therefore conducted to determine if the pollen was fertile. All the pollen samples tested germinated well *in vitro*. A further confirmation of the plantlet's fertility was the occurrence of *in vitro* peg formation (Figure 3-3d).

The effect of photoperiod on *in vitro* flowering in the five genotypes is summarized in Table 3-6. The highest flowering percentage of 65% recorded across all the genotypes for plantlets exposed to 12 h photoperiod is indicative that flowering induction actually occurred. However, the overall mean flowering frequency across all the genotypes and photoperiod treatments was 48%. The effect of photoperiod treatments on the time taken to initiate flower buds starting from after the explant incubation is displayed in Figure 3-8. The result suggested that the length of photoperiod had a significant influence (P=0.05) on the time taken for the plantlets to initiate flower buds *in vitro*. Beside, flowering response differed among the five genotypes (Figure 3-9), but the plantlets exposed to the12 h photoperiod were the earliest to initiate flower buds. This result was consistent across all the genotypes that were studied.

Performance of Plantlets in the Greenhouse and in the Field

The result from two years combined data for *C. Arachidicola* leaf spot disease incidence in field-cultivated plantlets and seedlings of *A. paraguariensis* is presented in Figure 3-10. A cultivated peanut (*A. hypogaea* L.) variety Florunner was planted along as a control treatment. Although there was no difference in disease incidence scores among the genotypes and between plantlets and seedlings, nevertheless, there was a wide variation in the incidence *C. Arachidicola* leaf spot between *A. paraguariensis* and *A. hypogaea*.

Both the *in vitro* regenerated plantlets and the *in vivo* derived seedlings produced seeds in the greenhouse and in the field. Contrary to the situation in the greenhouse, seed harvesting was laborious in the field because the field-cultivated plants had very long pedicels (Figure 3-3e). Seed formation was observed in the greenhouse cultivated plants as early as three mo after transplanting. However, the plantlets and seedlings in the field did not produce seeds until the following Spring. Hence the first harvesting of seeds was carried out when the seedlings and the plantlets had been established in the greenhouse and in the field for 9 months. Only mature pods (Figure 3-3f) were harvested for a comparison of the seed yield of *in vitro*-derived plantlets and the germinated seedlings. From the results in Figure 3-11, Grif 15201 had higher ($P< 0.001$) seed yield than Grif 15208 in the greenhouse and in the field for both plantlets and seedlings. The mean yield over genotypes did not differ between the plantlets and seedlings in the greenhouse, but the seedlings in the field produced more seeds than the *in vitro* derived plantlets.

Discussion

This study was undertaken with the objective of developing an efficient and rapid protocol for the regeneration of *A. paraguariensis* through defined morphogenic pathways followed by a field evaluation of the *in vitro* derived plantlets. The study also examined the roles of auxin and culture vessel on rooting and post-acclimatization survival of plantlets. Finally, the effect of photoperiod on the induction of *in vitro* flowering was investigated.

Though some tissue culture regeneration procedures have been developed for *A. paraguariensis*, scanty information is available on the morphogenic pathways involved. The importance of making use of the appropriate tissue culture regeneration pathway for breeding techniques such as embryo rescue, ploidy manipulation and genetic transformation cannot be overemphasized. A number of explants including Stamens (Still et al., 1987), mature leaflets (Dunbar and Pittman, 1992), protoplast (Li et al. (1993) and embryonic axis (Sellars et al., 1990) have been used with limited success in tissue culture regeneration of *Arachis*. Therefore preliminary studies were conducted to develop and identify the most suitable explant. The morphogenic response observed in the quartered-seed explant was high and rapid. Further observations showed that the quartered–seed can be advantageous in shoot regeneration of these six genotypes of *A. paraguariensis* as compared to other explants reported earlier. However, the simultaneous occurrence of organogenesis and embryogenesis on the same callus mass that was observed might be due to the nature of the explant since it is made up of tissues from the embryonic axis as well as the cotyledon.

The cytokinin-like action of TDZ has proved to be more effective than BAP for inducing shoot regeneration in the cultivated peanut (Gill and Saxena 1992). Besides, Li

et al. (1994) reported that it is possible to achieve organogenesis in peanut after a short period of culturing explants onto medium containing TDZ. The result from the present study suggests that continuous exposure of explants to TDZ is not required for shoot induction as bud initiation from the callus mass was observed after 5 days of explant incubation in medium containing TDZ. Furthermore, prolonged exposure of explants to TDZ has been found to inhibit shoot formation and rooting of plantlets in *A. hypogaea* (Akasaka et al., 2000). Hence, explants should be removed from culture medium containing high concentration of TDZ immediately after bud formation in order to prevent the inhibitory action of TDZ on shoot elongation. The present work shows that culturing quartered-seed explant for one week onto MS medium that have been supplemented with 4.4 mgL^{-1} TDZ and 1.1 to 2.2 mgL^{-1} of BAP or 2ip is effective for high frequency regeneration via non-zygotic embryogenesis. The use of 2ip in combination with TDZ led to the recovery of more plantlets; however, bud initiation was delayed.

It has been shown in several studies involving different species that genotype plays a critical role during *in vitro* regeneration (Bailey et al., 1993; Srinivasan et al., 2010). Yet, it is desired that a regeneration protocol be applicable to a wide range of genotypes or even species. The protocols developed in this study were suitable across six different genotypes of *A. paraguariensis*. Further research might be needed to determine the applicability of the protocols to other genotypes or species.

Two of the genotypes with varying morphology were selected for greenhouse and field studies. The seed production potential and resistance to *C. Arachidicola* leaf spot disease in *in vitro* derived plantlets and *in vivo* derived seedlings were evaluated. The

result revealed the wide variation in seed production potential of the two genotypes. Hereafter, future research is needed to identify the genotypes that are high yielding so that seed-derived explants can be made available for tissue culture regeneration.

Based on the results from the rooting experiment, the use of PETG vessel significantly enhanced the rooting of plantlets in the presence or absence of auxin treatment. Similar results have been reported by Lucchesini and Mensuali-Sodi (2004); during rooting, the use of ventilated vessels in comparison with the closed ones enhanced development of roots, and doubled the dry weight of *Phyllirea latifolia* plantlets. Water relations in the tissue culture vessel is dependent on factors such as the culture medium and environmental factors including temperature, photosynthetic photon flux, and the exchange of gas in and out of the culture chamber. In addition, a high auxin to cytokinin ratio at the site of root initiation has been found to be capable of promoting early root induction (Gaspar et al., 1996). Specifically, the use of NAA at concentrations between 0.5 and 1.0 mg L^{-1} has been effective for rooting plantlets of wild *Arachis* species (Gagliardi et al., 2000). However, the results from this study revealed that supplementing the rooting medium with 1 $mg^{-1}$of NAA led to severe leaf abscission as well as inhibition of root elongation. Besides, there was a great deal of callus proliferation at the base of the shoots. Consequently, it is suggested that plantlets be allowed to form root on MS medium lacking any growth regulator when there is no need for rapid root emergence, otherwise, the plantlets should be rooted on a medium containing 0.2 mgL^{-1} of IBA.

An *in vitro* flowering system is a convenient tool to study specific aspects of flowering, and floral organ development (Wang et al., 2002). Different cytokinins,

sucrose concentrations, photoperiod, and subculture time have been used to promote flowering *in vitro* in many species (Vu et al., 2006; Narasimhulu and Reddy, 1984). *In vitro* flowering has been reported in *A. paraguariensis* (Still et al., 1987; Li et al., 1993), however, the factors influencing the event are yet to be understood. In the present study, the high frequency of flowering recorded across all the genotypes for plantlets exposed to 12 h photoperiod could be attributed to short day length induced *in vitro* flowering, an event that has also been reported in *Kinnow mandarin* culture (Singh et al., 2006)

In summary, high frequency and rapid regeneration procedures have been established for tissue culture regeneration of *A. paraguariensis* via indirect shoot organogenesis and non-zygotic embryogenesis. Supplementing the culture medium with 4.4 mgL^{-1} TDZ in combination with 2.2 mgL^{-1} 2ip resulted in the overall highest number of plantlets recovered per explant but it took 2 wk for buds to form on explants. It is also possible to recover a moderately high number of plantlets per explant cultured on medium containing 4.4 mgL^{-1} TDZ in combination with 1.1 to 4.4 mgL^{-1} BAP within a week of culture initiation. Root formation in the *in vitro* derived plantlets has been improved over what was achieved in previous studies. Wide PETG vessel is recommended at elongation and rooting stage of *in vitro* regeneration while supplementation of the medium with 0.2 mgL^{-1} IBA may be carried out to induce early emergence of roots. Finally, *in vitro* flowering in *A. paraguariensis* appeared to be influenced by of photoperiod; 12 h photoperiod was most effective for induction of *in vitro* flowering.

Further observations in the greenhouse revealed that some morphological variation existed among the *in vitro* derived plantlets that originated from quartered-seed explants through indirect organogenesis. These variations was most easily seen in the shape of leaves and the branching habit of the plants and are probably due to somaclonal variation (Larkin and Scowcroft, 1982) that could be genetic or epigenetic in origin. Since this is a common phenomenon especially in plants regenerated via the callus phase, further assessment using molecular markers should reveal the origin and the genetic stability of these plantlets.

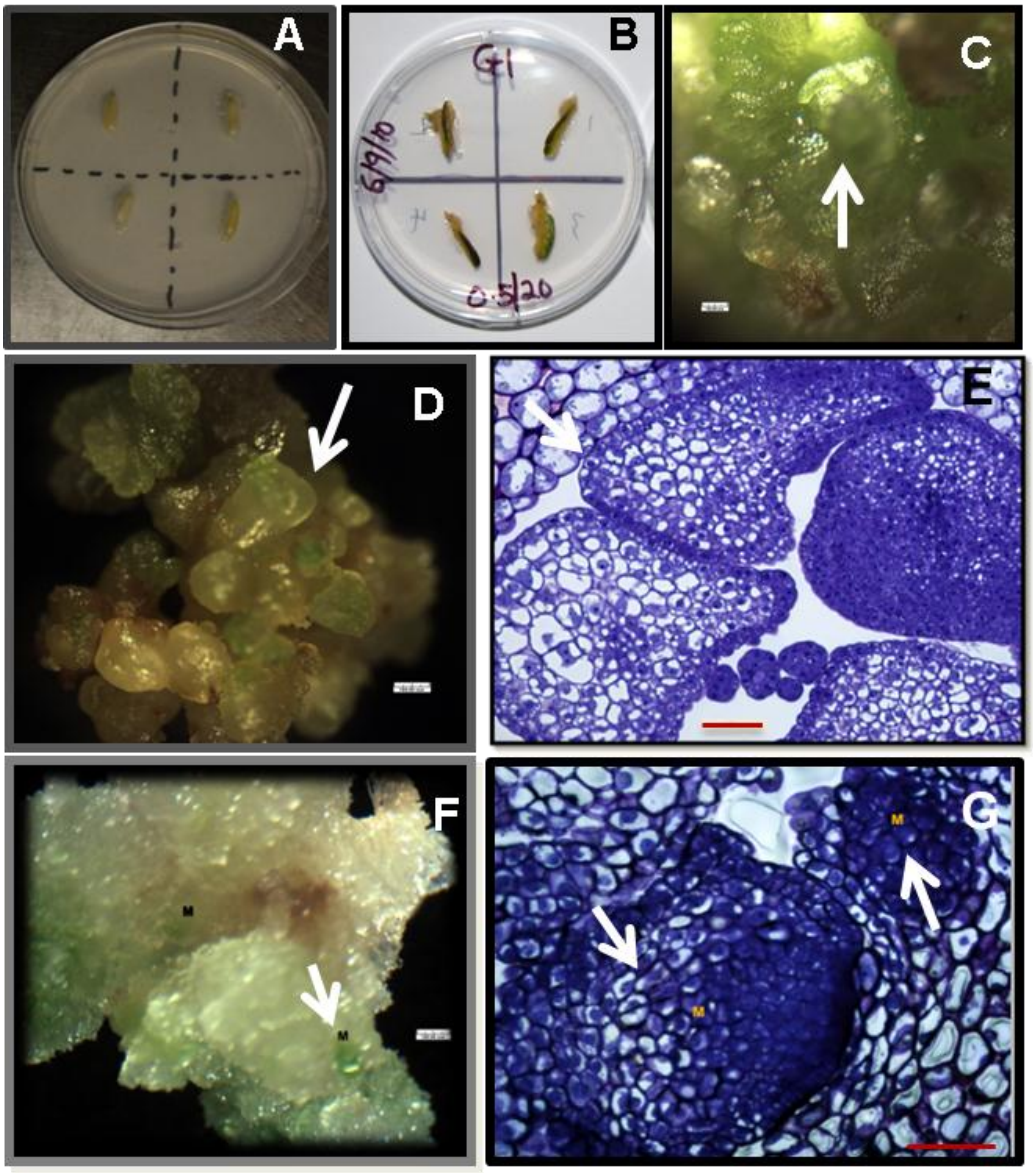

Figure 3-1.*In vitro* regeneration of *A. paraguariensis* via non-zygotic embryogenesis and organogenesis. (A) Quartered-seed explants culture on medium containing 4.4 mgL^{-1} TDZ and 2.2 mgL^{-1} 2ip. (B) Callus formation on explants after 24hr of culture initiation. (C) Green globular structures on the callus mass after one wk of culture initiation, bar= 1mm. (D) Distinct globular structures on explants after 3 wk of culture, bar=1mm. (E) Histological analysis of embryogenic tissue revealing bipolar somatic embryo with a well-defined protoderm, and without suspensor cell, bar=100µm. (F) Organogenic callus mass from explant culture on medium containing 4.4 mgL^{-1}TDZ , bar=1mm. (G) Histological analysis of organogenic tissue showing the formation of a meristematic dome and vascular connection between explant and the emerging organ, bar=100µm.

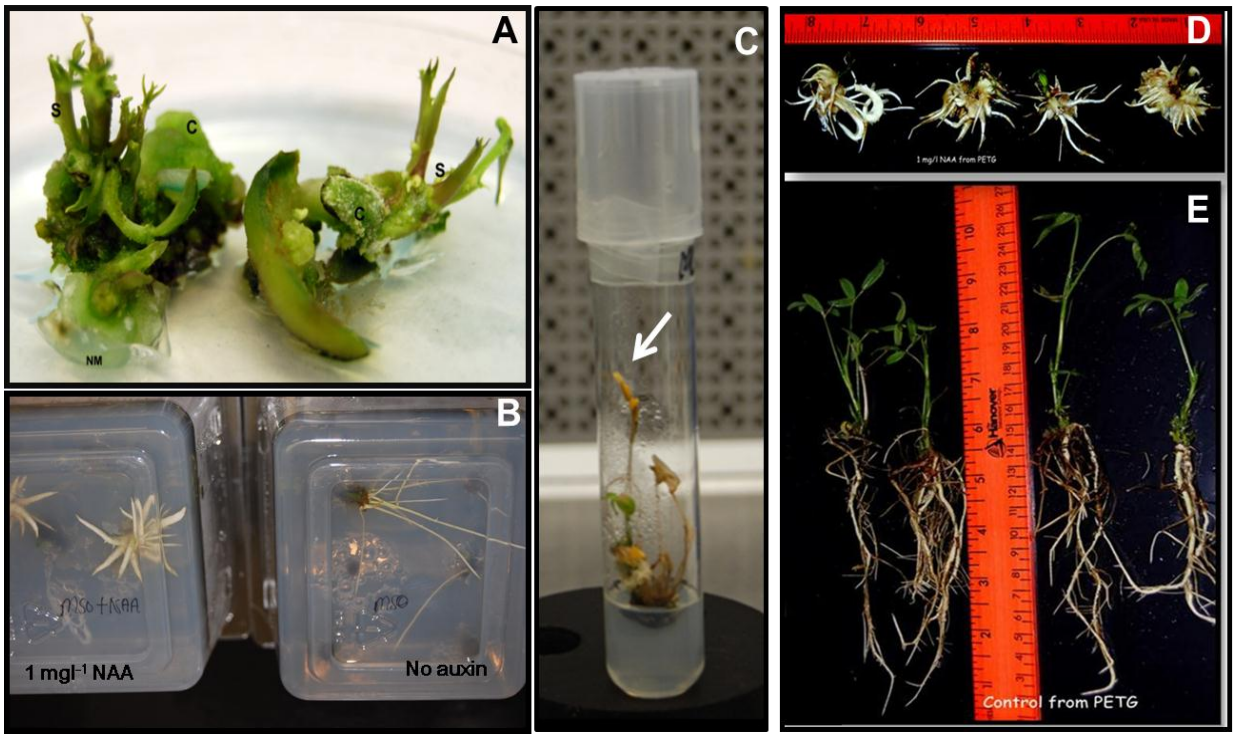

Figure 3-2. *In vitro* rooting of *A. paraguariensis* as affected by auxin and culture vessel treatments. (A) Micro-shoots used for the rooting experiment were excised from quartered-seed explants that were cultured on MS medium containing 2.2 mgL^{-1} TDZ and 4.4 mgL^{-1} BAP, S=shoot, NM=nutrient medium, while C= callus. (B) Roots from plantlets growing on medium containing 1 mgL^{-1} NAA were thicker and shorter than those cultured on medium devoid of auxin. (C) A plantlet inside a glass-tube on medium containing auxin flowered without root formation. (D) Plantlets treated with 1 mgL^{-1} of NAA suffered severe leaf abscission as well as inhibition of root elongation. (E) Plantlets cultured on MS medium without growth regulator were vigorous with long flexible roots.

Figure 3-3. *In vitro* flowering, peg formation and *ex-vitro* seed formation in *A. paraguariensis*. (A-B) High frequency *in vitro* flowering observed in genotype Grif 15201 and PI 262842 respectively. (C) The flowers were normal with viable pollen. (D) Peg formation *in vitro* was observed in genotype Grif 15201. (E) *In vitro* regenerated plantlets produced seeds after transplanting to pots in the greenhouse. (F) Mature pods and seeds of *A. paraguariensis* were obtained 3 months after transplanting.

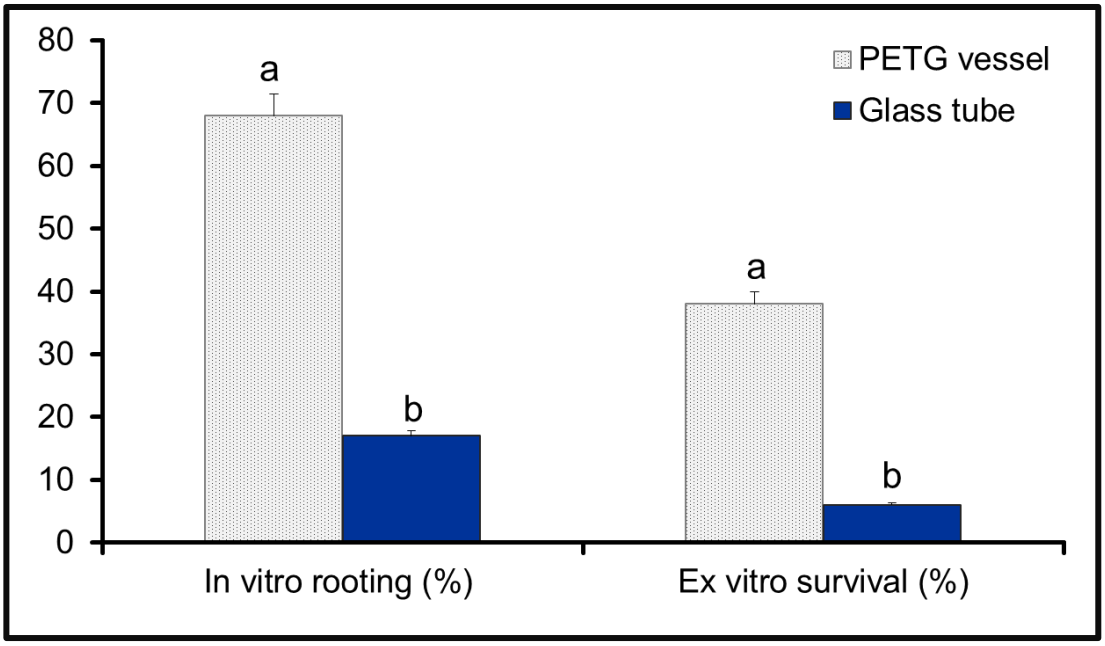

Figure 3-4. The influence of culture vessel type on the rooting efficiency across six genotypes of *A. paraguariensis*. Mean percentage followed by the same letter(s) within each parameter (%) are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

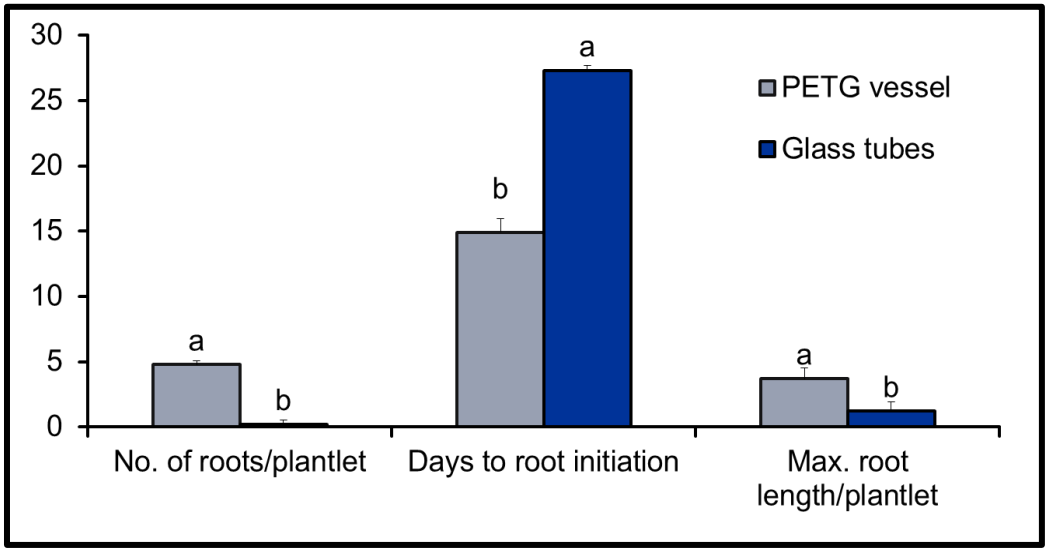

Figure 3-5. The effect of culture vessel type on the root characteristics of *in vitro* derived plantlets. Means followed by different letter(s) within each parameter are significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

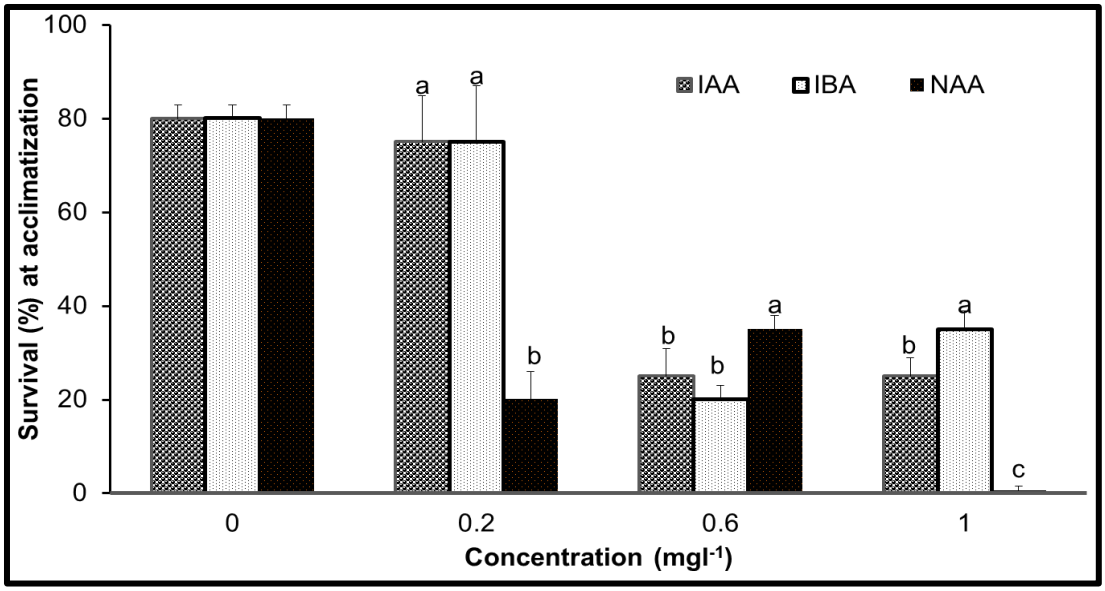

Figure 3-6. *Ex vitro* survival of rooted shoots in response to various auxin treatments at 6 wk after culture initiation in PETG vessel. Means followed by the same letter(s) within each concentration of auxin are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

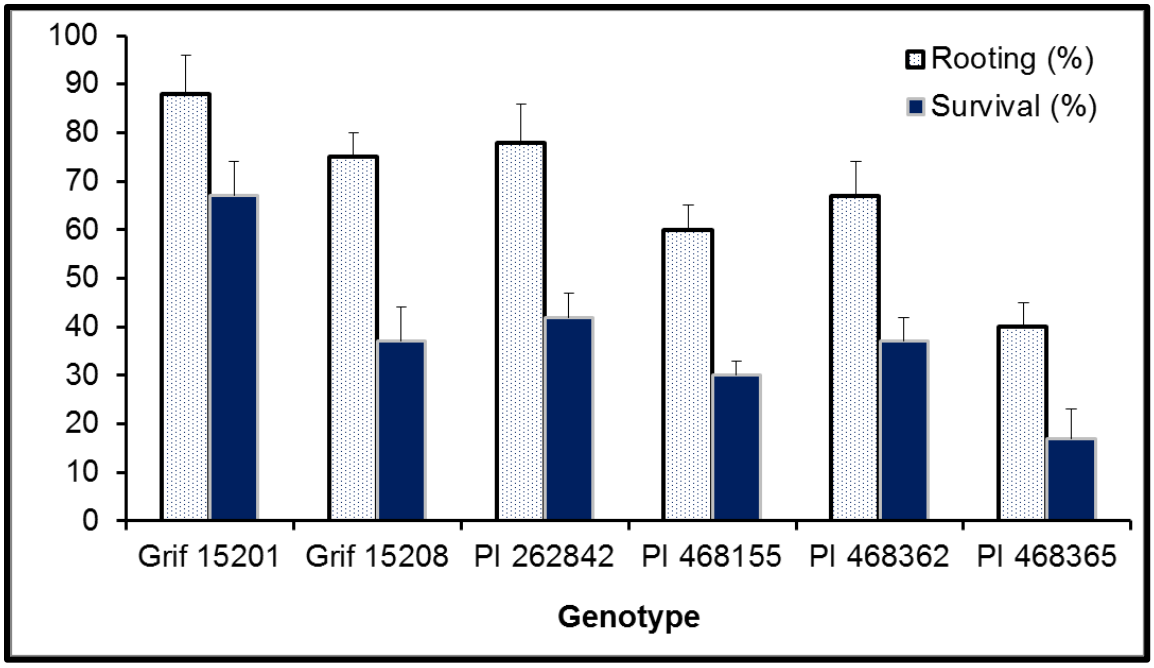

Figure 3-7. The rooting response and survival of six genotypes of *A. paraguariensis* across all the auxin treatments inside a PETG vessel. Bar = SE.

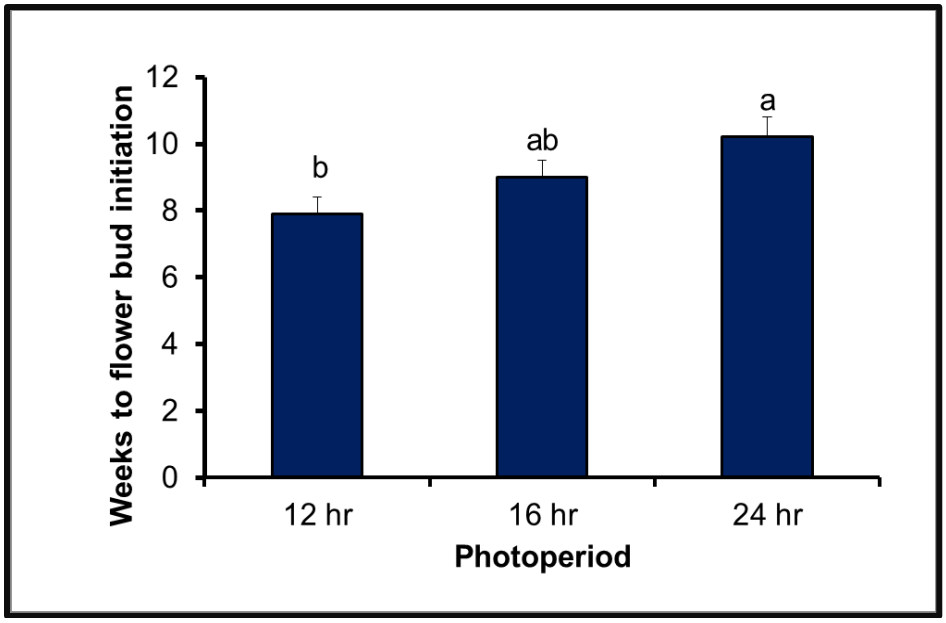

Figure 3-8. The time to initiate flower buds *in vitro* across five genotypes of *A. paraguariensis* as affected by photoperiod. Means followed by the same letter(s) are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

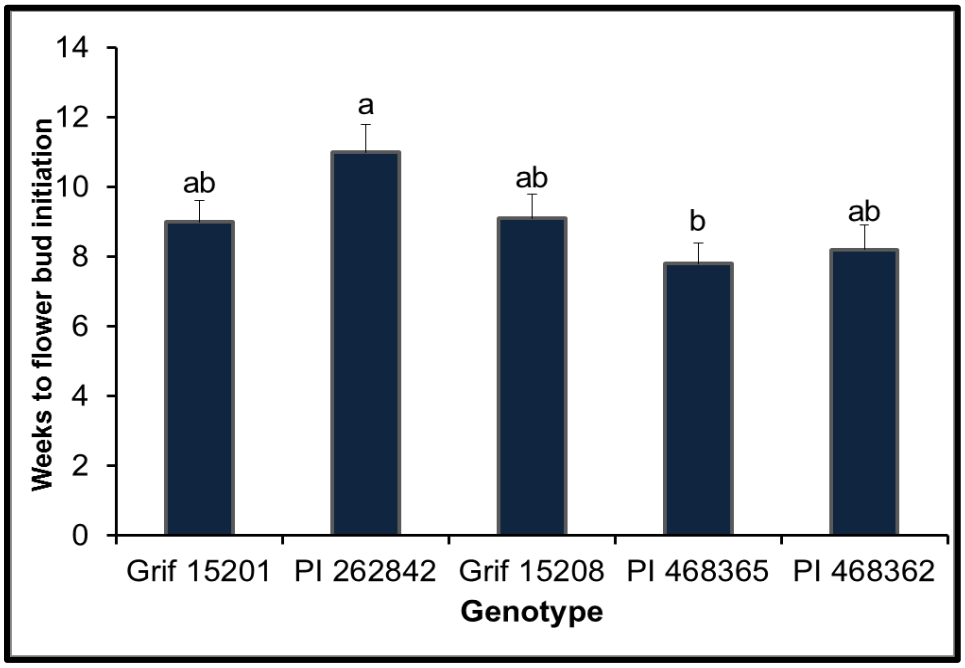

Figure 3-9. *In vitro* flowering response of five genotypes of *A. paraguariensis*. Means followed by the same letter(s) are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

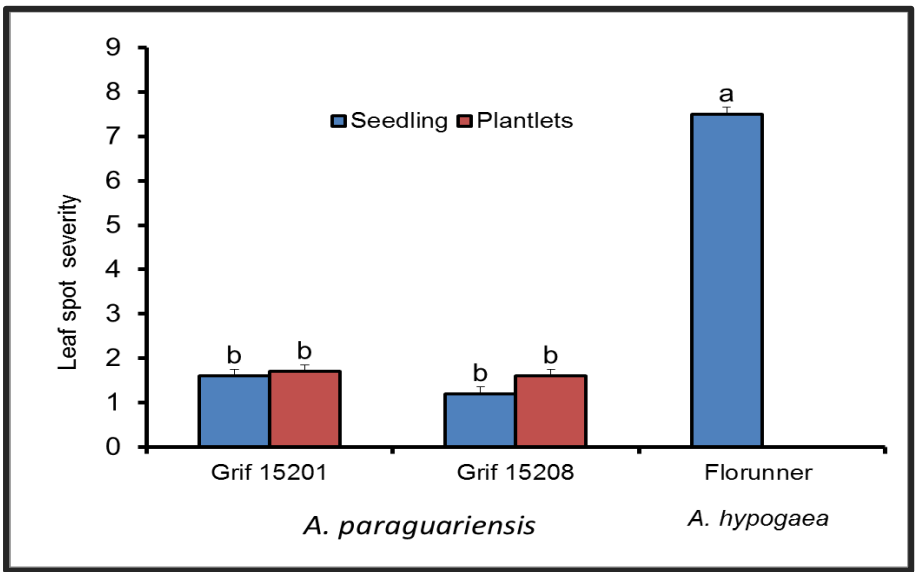

Figure 3-10. Leaf spot disease scores for two genotypes of *A. paraguariensis* and a cultivated peanut susceptible variety Florunner. Means followed by the same letter(s) are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

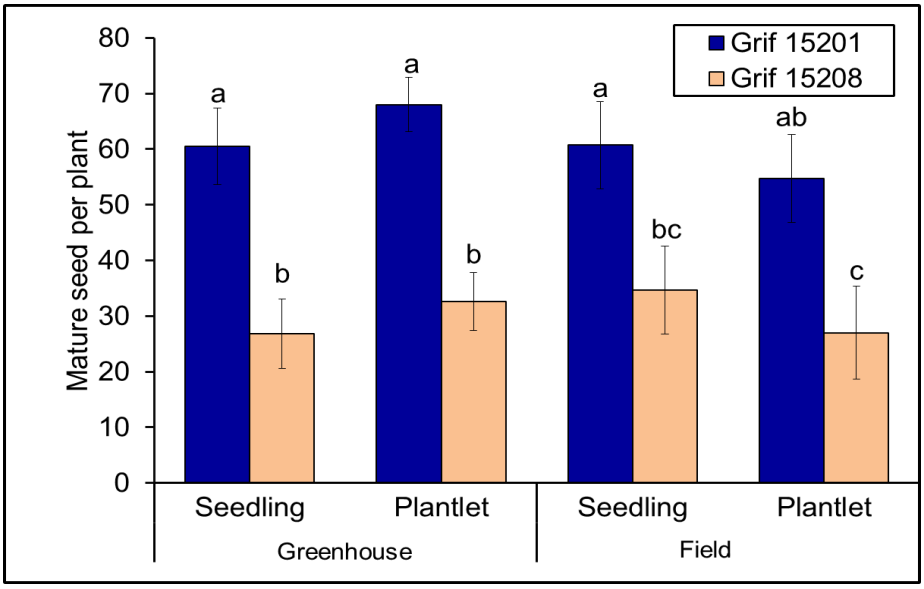

Figure 3-11. Seed yield of *in vitro* derived plantlets and germinated seedlings of two genotypes of *A. paraguariensis* inside pot in the greenhouse and in the field. Means followed by the same letter(s) within each section are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

Table 3-1. The identity and geographical origin of the six genotypes of *A. paraguariensis* used for the study.

NPGS/GRIN Accession No	Taxonomic authority	Origin
Grif 15201	*A. paraguariensis* Chodat & Hassl.	Paraguay
Grif 15208	*A. paraguariensis* Chodat & Hassl.	Paraguay
PI 262842	*A. paraguariensis* subsp. *paraguariensis*	Brazil
PI 468155	*A. paraguariensis* subsp. *paraguariensis*	Brazil
PI 468362	*A. paraguariensis* subsp. *paraguariensis*	Paraguay
PI 468365	*A. paraguariensis* subsp. *paraguariensis*	Paraguay

Table 3-2. The influence of 2,4-D and BAP concentrations and combinations on tissue culture regeneration from deembryonated cotyledon explant of *A. paraguariensis.*

2,4D (mgL^{-1})	BAP (mgL^{-1})	No. of days to embryo initiation	No. of plantlets recovered/ explant	Height (cm) at acclimatization
0	0	-	0	0
0	2.2	-	0	0
0	4.4	-	0	0
2.2	0	-	0	0
2.2	2.2	81a	1.3b	1.7a
2.2	4.4	62b	2.3b	2.5a
4.4	0	78a	3.7b	2.7a
4.4	2.2	36c	3.8b	2.4a
4.4	4.4	41c	8.1a	2.8a

Each value represents the mean 2 replicates. Means followed by the same letter(s) within each column are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

Table 3-3. The effects of TDZ and BAP concentrations and combinations on shoot regeneration across all the six genotypes.

TDZ (mgL^{-1})	BAP (mgL^{-1})	No. of days to bud initiation	No. of plantlets/ explant	Height (cm) at acclimatization	Regeneration pathway
0	0	-	0	0	NR
0	2.2	-	0	0	NR
0	4.4	-	0	0	NR
2.2	0	5c	2.0c	2.5b	SO
2.2	1.1	10ab	4.6c	2.3b	O+E
2.2	2.2	10ab	4.1c	2.6b	O+E
2.2	4.4	10ab	3.5c	3.1b	O+E
4.4	0	11a	3.9c	2.8b	SO
4.4	1.1	6bc	14.8a	7.3a	E
4.4	2.2	6bc	10.4b	6.2a	E
4.4	4.4	6bc	8.1b	6.3a	E

Means followed by the same letter(s) within each column are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.
SO = Shoot organogenesis; O+E= Organogenesis and Embryogenesis (simultaneously on the same callus mass); E= Embryogenesis (non-zygotic); NR = No response.

Table 3-4. The effects of TDZ and 2ip concentrations and combinations on tissue culture regeneration across all the six genotypes.

TDZ (mgL^{-1})	2ip (mgL^{-1})	No. of days to bud initiation	No. of plantlets/ explant	Height (cm) at acclimatization	Regeneration pathway
0	1.1	-	0	0	NR
0	2.2	-	0	0	NR
0	4.4	-	0	0	NR
2.2	1.1	11b	15.7b	8.1ab	E
2.2	2.2	13ab	7.3c	7.1ab	E
2.2	4.4	15a	18.7ab	8.5ab	E
4.4	1.1	14ab	18.9ab	10.8a	E
4.4	2.2	14ab	20.2a	7.8ab	E
4.4	4.4	12ab	19.7ab	5.6b	E

Means followed by the same letter(s) within each column are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.
E= Embryogenesis (non-zygotic); NR = No response.

Table 3-5. Root formation as affected by auxin type and concentration after 6 weeks of culture in PETG vessel.

Auxin	Conc. (mgL^{-1})	No of roots/ plantlet	Max. root length (cm) at acclimatization	Days to root initiation
IAA	0.2	7.1ab	5.6abc	11.3c
	0.6	1.7b	2.0cd	11.9bc
	1	6.0b	3.2bcd	12.0bc
IBA	0.2	5.4b	6.4ab	12.2bc
	0.6	1.4b	1.8d	16.5b
	1	2.8b	5.0abcd	14.6bc
NAA	0.2	4.3b	2.5cd	11.5c
	0.6	4.8b	2.5cd	16.2bc
	1	13.6a	1.6d	11.3c

Means followed by the same letter(s) within each column are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test.

Table 3-6. The effects of photoperiod on *in vitro* flowering in five genotypes of *A. paraguariensis.*

Photoperiod (h)	Genotype	No. of plantlets evaluated	No. of flowered plantlets	Flowering frequency (% ± SE)
12	Grif 15201	40	34	85 ± 7
	Grif 15208	40	25	63 ± 5
	PI 262842	40	37	93 ± 6
	PI 468362	40	20	50 ± 6
	PI 468365	40	14	35 ± 4
Mean		40	26	65 ± 5
16	Grif 15201	40	24	60 ± 6
	Grif 15208	38	25	66 ± 4
	PI 262842	40	30	75 ± 5
	PI 468362	40	16	40 ± 5
	PI 468365	40	8	20 ± 1
Mean		40	21	52 ± 4
24	Grif 15201	39	16	41 ± 4
	Grif 15208	40	10	25 ± 5
	PI 262842	40	21	53 ± 2
	PI 468362	40	2	5 ± 2
	PI 468365	38	4	11 ± 3
Mean		39	11	27 ± 3
Overall	Mean	40	19	48 ± 3

CHAPTER 4
IN VITRO INDUCTION OF TETRAPLOIDY IN *Arachis paraguariensis*

At least one step involving ploidy manipulation is required along many of the potential pathways for introgression of disease resistant genes from wild *Arachis* species into cultivated peanut (*Arachis hypogaea* L.). This is because while cultivated peanut is tetraploid (2n = 4x = 40), many of its wild relatives are diploid (2n = 2x = 20) species (Simpson, 2001; Mallikarjuna et al., 2004). Results from previous studies (Stalker and Wynne, 1979; Simpson, 2001) indicate that creating autotetraploids or amphidiploids and subsequently crossing the tetraploid plants with *A. hypogaea* can be a more efficient pathway to germplasm introgression than producing triploid hybrids through direct crossing of *A. hypogaea* to the wild species.

Arachis paraguariensis is a wild species from the section Erectoides. Some of its accessions are highly resistant to early leaf spot, an economically important and prevalent fungal disease of cultivated peanut caused by *Cercospora arachidicola* (Subrahmanyam et al., 1985). Tolerance to root-knot nematode, *Meloidogyne javanica* Race 3, which is an important nematode parasite of cultivated peanut (Sharma et al., 1999), as well as resistance to tobacco armyworm (*Spodoptera litura*, Lepidoptera: Noctuidae), another important peanut pest (Stevenson et al., 1993) have also been reported in several accessions of *A. paraguariensis*. However, attempts (Singh, 1998b; Rao et al., 2003) to generate a fertile hybrid after crosses with cultivated peanut have not been successful due to hybridization barriers.

Induced chromosome doubling has been useful for facilitating the crossing of diploid, tetraploid and hexaploid species and also to improve the fertility of hybrids (Brubaker et al., 1999). The induction of tetraploidy through the use of colchicine is a

suitable method to be explored because it does not require special equipment and it is relatively safe (van Harten, 1998). Importantly, the method often leads to the disruption of cellular mitosis through inhibition of the spindle fibers that segregate replicated chromosomes into daughter cells. In many cases, the resulting polyploids show changes in plant morphology (Taylor and Quesenberry, 1996), and such plant materials can be used as genetic bridges for the transfer of desirable features into other species (Recupero et al., 2005). Traditional application of colchicine *in vivo* to plant shoots, meristems, seeds, or seedlings has long been a well-known method for generating polyploids (Nebel, 1938), nevertheless, research has shown that *in vitro* chromosome manipulation is a more efficient way of inducing polyploidy than the *in vivo* methods (Cohen and Yao, 1996; Adaniya and Shirai, 2001).

Autotetraploids of several wild *Arachis* species have been produced (Simpson, 2001; Singh, 1986) via *in vivo* colchicine treatment. However, there is no clearly described procedure for *in vitro* chromosome doubling of several diploid *Arachis* species including *A. paraguariensis*. Consequently, the first objective of this study was to develop an *in vitro* chromosome doubling procedure for *A. paraguariensis* while the second objective was to evaluate the fertility and morphology of the induced autotetraploids.

Materials and Methods

Plant Materials

One year-old mature greenhouse grown plants were the source of mature seed-derived explants that were used in this study. Seeds harvested from the plants were washed, air-dried and shelled manually before storage in an air-tight container at 4°C for 6 months.

Tissue Culture Initiation and Establishment

Seeds were surface-sterilized by rinsing in 70% ethanol for 1 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. Thorough washing with sterile-distilled water was performed before seeds were soaked in sterile water for 4 h in a laminar airflow chamber. The seed coat was then surgically removed and each cotyledon with the embryonic axis still attached was cut into vertical halves to obtain the quartered-seed explant. The callus explant was derived by culturing quartered-seed explants onto semi-solid callus induction medium (CIM) containing MS inorganic salts, B5 vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar, 4.4 mgL^{-1} TDZ and 2.2 mgL^{-1} 2ip. Green and compact callus masses each weighing 0.2 g were selected as explants after 3 wk of culture incubation. Plantlets for deriving shoot tips were obtained by allowing some of the callus masses to develop into shoots on MS medium lacking growth regulators. Each shoot tip, consisting of a terminal bud plus two expanded leaves, was then excised.

A fresh stock solution of colchicine (Sigma) was prepared by dissolving an appropriate amount of powdered colchicine in 2 ml of 95% ethanol. The different explants were then immersed in the aqueous solutions of colchicine at concentrations of 0.05%, 0.1%, 0.2% and 0.5% (w/v) for 4, 8 , 16, 20 and 24 h while controls were held in sterile, distilled water for similar durations. Shoot regeneration of the quartered-seeds was allowed to take place on semi-solid CIM inside Phytatray^{TM} culture vessels for 2 wk. The shoots were then sub-cultured onto MS basal medium without growth regulators. Treated callus and shoot-tip explants were cultured separately onto MS basal medium without growth regulators. When two to three roots formed, each plantlet was

transplanted into a Jiffy® peat pellet and acclimatized in a humidity chamber for 2 wk. Hardened plants were later transferred into soilless mix in a greenhouse.

Culture Condition

The pH of all media was adjusted to 5.8 prior to autoclaving while the plant growth regulator solutions were filter-sterilized through a double 0.2 µm filter before they were added to the autoclaved media in sterile bottles. Each Phytatray™ (Sigma) culture vessel contained four explants on 50 ml of medium formulation while culture incubation was performed at 26±1ºC under a 16-h photoperiod provided by cool white fluorescent lamps at 60 µE/m^2/s light intensity.

Ploidy Determination by Flow Cytometry

Flow cytometric analysis of tissue samples from regenerated *A. paraguariensis* diploid plants was carried out using a Partec PAII Ploidy Analyzer® according to the method of Quesenberry et al. (2010a). Approximately 0.5 cm^2 of leaves, root, and a small piece of soft meristematic tissue were excised. In order to discharge the intact nuclei, a standard edge razor blade was used to chop the tissue inside a plastic petri dish containing 500 µl of extraction buffer (CyStain® UV Precise P). The nuclei were then washed into the tilted side of the dish and siphoned up with a micro-pipet before being filtered into an analysis vial with the aid of 50 µm filter. After the addition of 1500 µl of staining buffer (Partec DAPI: 4, 6-diamidino-2-phenylindole) to the vial, samples were allowed to stain for 3 min before analysis. The untreated diploid control was sampled and used as a known 2x reference standard, while samples from *in vitro* regenerated cultivated peanut (*A. hypogaea*) and rhizoma peanut (*A. glabrata*) were used as known 4x reference standards. The reference standards were run each time plants were processed for analysis while the gain of the ploidy analyzer was always

adjusted to produce G2 peaks at near 200 and 400 for the diploid and tetraploid standards respectively. At least 10,000 nuclei per sample were analyzed at least twice on different days.

Morphological and Fertility Observations

Stoma and trichome observations were made on plants that were identified as tetraploids based on their flow cytometry profiles while the samples used as control were obtained from plants that have not been treated with colchicine. The frequency and size of stoma and trichome from upper and basal leaves from greenhouse plants were determined by coating the abaxial side of leaves with clear transparent nail polish. The polish was allowed to dry before being peeled off, and dry mounted on microscope slides. For each leaf position, the number of stomata and trichomes per 1 mm^2 of 20 plants were determined with the aid of an ocular micrometer of a compound light microscope. Additionally, the morphology of 20 putative tetraploid, and 20 untreated diploid plants were assessed. Leaf measurements of breadth/width ratios were based on the means of 2 upper and 2 basal leaves from 6 months old greenhouse plants while the plant height was measured from the soil level to the base of the apical bud. Observations for flowering, peg and seed formation were also recorded.

Experimental Design, Data Collection and Analysis

The experiment was laid out in a split plot with 4 (colchicine concentration) x 5 (treatment duration) factorial main plots and 3 explant-types (quartered-seed, callus and shoot tip) as the subplots. Observations for callus shoot and root formation as well as plantlet survival were carried out throughout the *in vitro* regeneration studies. Ploidy level analysis was first performed at 2 months after culture initiation and repeated 2 months after ex-vitro acclimatization. The DNA histograms and nuclear DNA content

were estimated on the basis of a linear scale with the aid of FlowJo V.7.6.3 for Windows (Treestar, Ashland, OR, USA). Data on frequency of tetraploid induction were analyzed using GLIMMIX procedure in SAS statistical program (SAS Institute, 2010). A factorial logistic regression model was used since the dependent variable (tetraploidy) is dichotomous. The explant type was considered as a fixed effect while colchicine concentration, treatment duration and their interaction were the random effects. Moreover, the plant morphology data was analyzed separately using a mixed model approach.

Results

Tetraploid Induction by Colchicine Application to Quartered-Seeds and Shoot-Tips

After the exposure of quartered-seed explants to the various concentrations and treatment durations of colchicine, the overall frequency of regeneration obtained was lower than that of the water-treated controls, especially when higher levels of colchicine were applied for longer durations. The time required for shoot formation was also significantly increased (Table 4-1). All the quartered-seed explants survived every level of colchicine concentrations for the 4 h and 8 h treatment durations. However the best results in which 39% and 43% of the explant produced tetraploid plants were 0.5% colchicine for 4 h and 8 h, respectively. The proportion of induced tetraploids and explant lethality followed a linear pattern with increasing colchicine concentrations at 4 h and 8 h treatment durations. Besides, application of the two highest concentrations of colchicine to explants for 24 h proved to be lethal to the quartered-seeds (Figure 4-2).

The shoot-tip explants yielded a relatively low frequency of plantlet formation and tetraploid induction. The three tetraploid plants that were derived out of the 25 plantlets

that regenerated (Table 4-1) did not survive *ex vitro* acclimatization due to poor root formation.

The representative flow cytometric DNA histograms of DAPI-stained nuclei preparations of the diploid and the induced tetraploid *A. paraguariensis* are shown in Figures 4-3a and 4-3b, respectively. The dominant peak indicates the ploidy level.

Overall, quartered-seed explants gave the highest frequency of plantlet regeneration and tetraploid induction, as well as the lowest mortality rate (Table 4-1). Additionally, the flow cytometric analysis of induced tetraploids from quartered-seeds revealed that the plants were true-to-type with absence of chimeras. Therefore, quartered-seed proved to be the best explants for *in vitro* induction of tetraploidy in *A. paraguariensis*.

Regeneration of Mixoploids from Colchicine-Treated Callus

The capabilities of the different explants to induce tetraploidy *in vitro* is summarized in Table 4-1. The lowest frequency of plantlet regeneration and highest explant mortality rate were observed from the colchicine-treated callus explants. Only chimeras were produced despite exposure to the various colchicine treatments. Results from the flow cytometric analysis revealed two patterns of peaks for the chimeric plants. The histograms for two of the plants revealed approximately equal amount of 4x and >5x nuclei (Figure 4-2c) while the other three plants displayed profiles having three peaks. The first of the three peaks corresponds to G1 nuclei of the 2x cells, and was followed by a peak that represents both the G2/M nuclei of 2x cells and the G1 nuclei of the 4x cells while the third peak corresponds to the G2/M nuclei of 4x cells (Figure 4-2d).

Morphology, Fertility and Survival of Induced Tetraploid and Mixoploid

The flow cytometry analysis of DAPI-stained cell nuclei of the induced tetraploid agreed with the stomata and trichome density observations. In other words, increasing the ploidy level from 2x to 4x in *A. paraguariensis* resulted in fewer stomata but more trichomes per unit leaf area (Figure 4-4a-d). Additionally, the upper leaves of both the diploid and tetraploid plants have a significantly higher stomata density than the basal leaves (Figure 4-5) while there was no significant difference in plant height and leaf breadth/width between the diploid and tetraploid plants. Flower size (Figure 4-7e) and density of trichomes (Figure 4-5) on upper leaves were the noticeable and consistent morphological differences observed between the two ploidy levels.

All the diploid plants generated during this study were fertile and produced viable seeds inside pots after six months of transplanting to the greenhouse, but flowering was observed in only two of the tetraploid plants. Flowers on the tetraploids were larger than those of the diploids (Figure 4-7e) but their fertility could not be verified due to lack of sufficient pollen.

During the summer months of 2010, all the tetraploid and mixoploid plants were adversely affected by severe heat stress. Temperature fluctuations a result of faulty air conditional occurred in the greenhouse where the plants were kept. Brown spots first appeared on leaves of the affected plants followed by the rolling up of the leaf margins, and finally, total defoliation results (Figure 4-7a-c). Tissue samples from plants showing these symptoms were submitted to the University of Florida Plant Disease Clinic for analysis, but, no disease pathogens were isolated from the samples. Interestingly, all the diploid plants recovered from the heat stress (Figure 4-7d) while only nine of the tetraploids remained alive.

Discussion

The flow cytometry results concurred with stoma and trichome observations, thus highlighting the practicality of flow cytometry for the analysis of DNA content as well as ploidy level in *Arachis* species. Although changes in ploidy level are often accompanied by morphological variations (Taylor and Quesenberry, 1996), the only noticeable and consistent morphological differences between the diploid and the induced tetraploid *A. paraguariensis* were flower size and the density of trichomes on the upper leaves. Hence, the use of morphological features alone may not be ideal for identification of putative tetraploids. Ploidy determination using stoma and trichome characteristics alone could also be misleading because only ploidy level of the L1 meristem layer can be determined. Other methods such as root tip chromosome counts and chloroplast counts in guard cell have been portrayed as very consistent and reliable for ploidy determination (Singsit and Ozias-Akins, 1992; Quesenberry et al., 2010a). Nevertheless, these methods require certain degree of technicality and often prove to be laborious.

The inverse relationship between colchicine concentration and explant survival was anticipated as well as the delayed shoot formation in colchicine-treated explants. Colchicine as an anti-mitotic agent is capable of acting on dividing cells by breaking down the spindle fibers during C-mitosis so that the chromatids lay within the same cell without subsequent cell plate formation. Usually, the tissue has to recover from the interference before the chromosome number is altered. *In vitro* colchicine treatment during callus phase of tissue culture regeneration has been effective for chromosome doubling in other species (Quesenberry et al., 2010a; Wu and Mooney, 2002), however, the colchicine-treated callus explant used in this study failed to yield any true tetraploid

plants. Additionally, the tissue culture regeneration follows a pathway of non-zygotic embryogenesis which due to its single cell origin should decrease the possibility of chimerism, yet all the 5 plants derived from the callus explants were chimeras. The failure to induce tetraploidy in the callus explants might be associated with the developmental stage of the callus explants at the time of treatment especially because nodular shoot buds had already been formed on the callus at the time of colchicine application. Callus at this stage was selected because it is more compact and can remain solid for several hours in the aqueous solution of colchicine. Future studies should aim at determining the optimal stage of colchicine application during callus induction and proliferation so as to increase the frequency of polyploidy induction.

The chimeric plants obtained from colchicine-treated callus were identified based on their flow cytometric profiles but the exact type of chimerism was not verified. The occurrence of chimerism is quite typical in somatic polyploidization. It is important to identify the type of chimera produced from colchicine-induced tetraploid since the apical meristem is made up of 3 distinct cell layers usually referred to as LI, LII and LIII. The most stable type of chimera is periclinal resulting from mutation in the entire portion of one or more meristem layers. (Zhu et al., 2010).

In this study, an efficient procedure for production of tetraploid *A. paraguariensis* was demonstrated. The flow cytometry analysis of DNA allows rapid evaluation of hundreds of putative tetraploids. The tetraploid plants grown in the greenhouse have larger flower, as well as higher stomata and trichome density than the diploid plants. Nonetheless, there is still the need to evaluate the stability and fertility of the induced tetraploids.

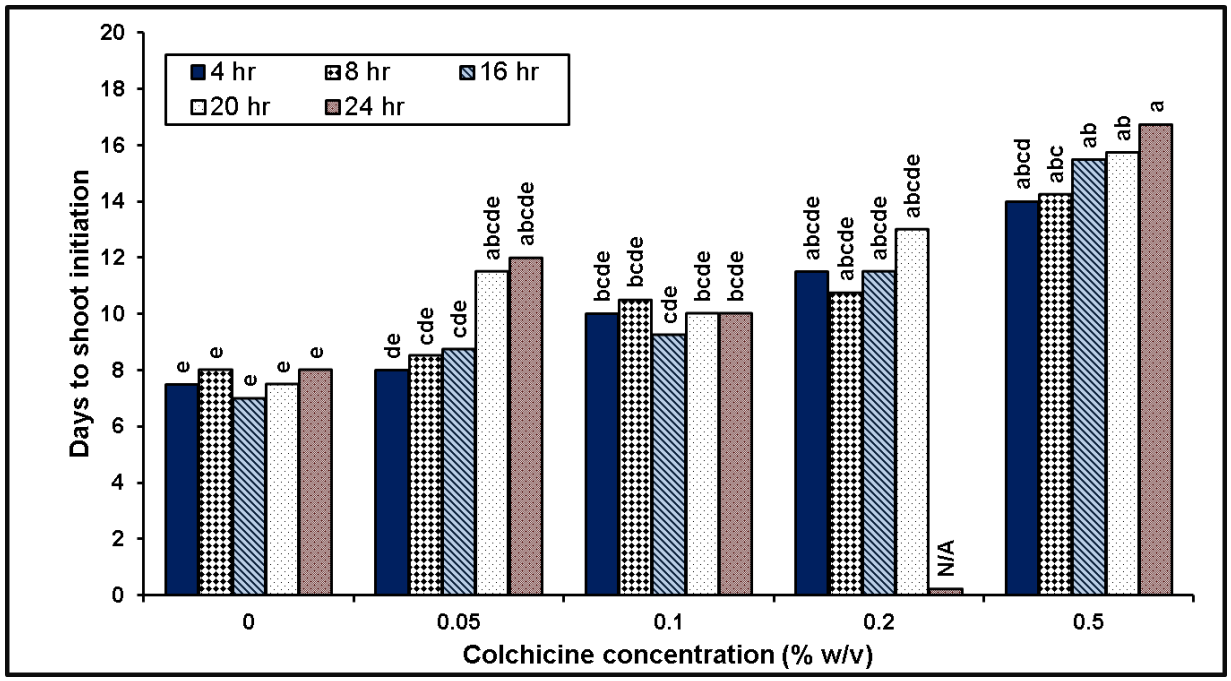

Figure 4-1. The effect of colchicine concentration and treatment duration on timing of *in vitro* shoot formation from quartered-seed explants of *A. paraguariensis*. Different letters on top of each bar indicate a significant difference according to Tukey's Honestly Significant Difference Test (P = 0.05). Error bars represent SE. NA = data not available due to death of explant materials.

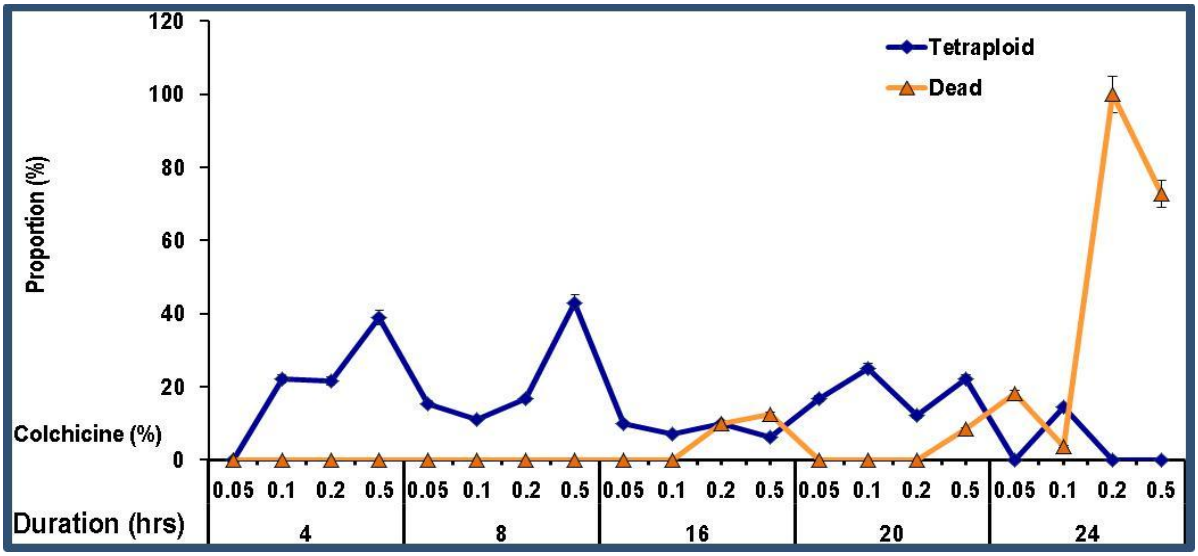

Figure 4-2. Effect of colchicine concentration and treatment duration on tetraploid induction and quartered-seed explants viability (Mean ± SE) in *A. paraguariensis*

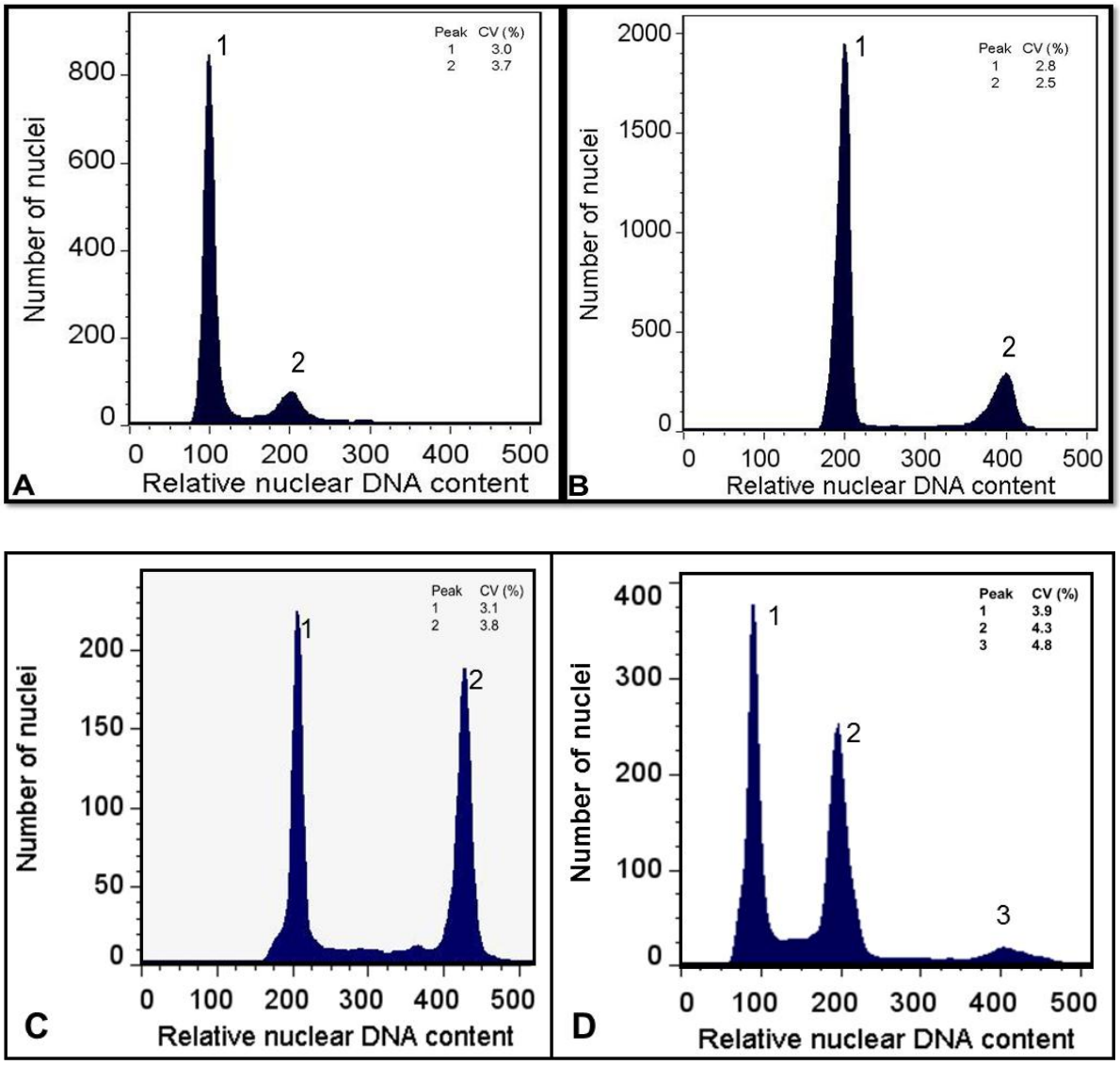

Figure 4-3. Representative flow cytometric analysis showing DNA histograms of *A. paraguariensis*; (A) diploid with normal 2n ploidy, (B) induced tetraploid with 4n ploidy, (C) mixoploid with 4n+>5n ploidy, and (D) mixoploid with 2n+4n ploidy. The G1 peak of the diploid was approximately on channel 100 while the tetraploid showed a G1 peak on channel 200. The G2 peak of the diploid was approximately on channel 200 while the tetraploid showed a G2 peak on channel 400.

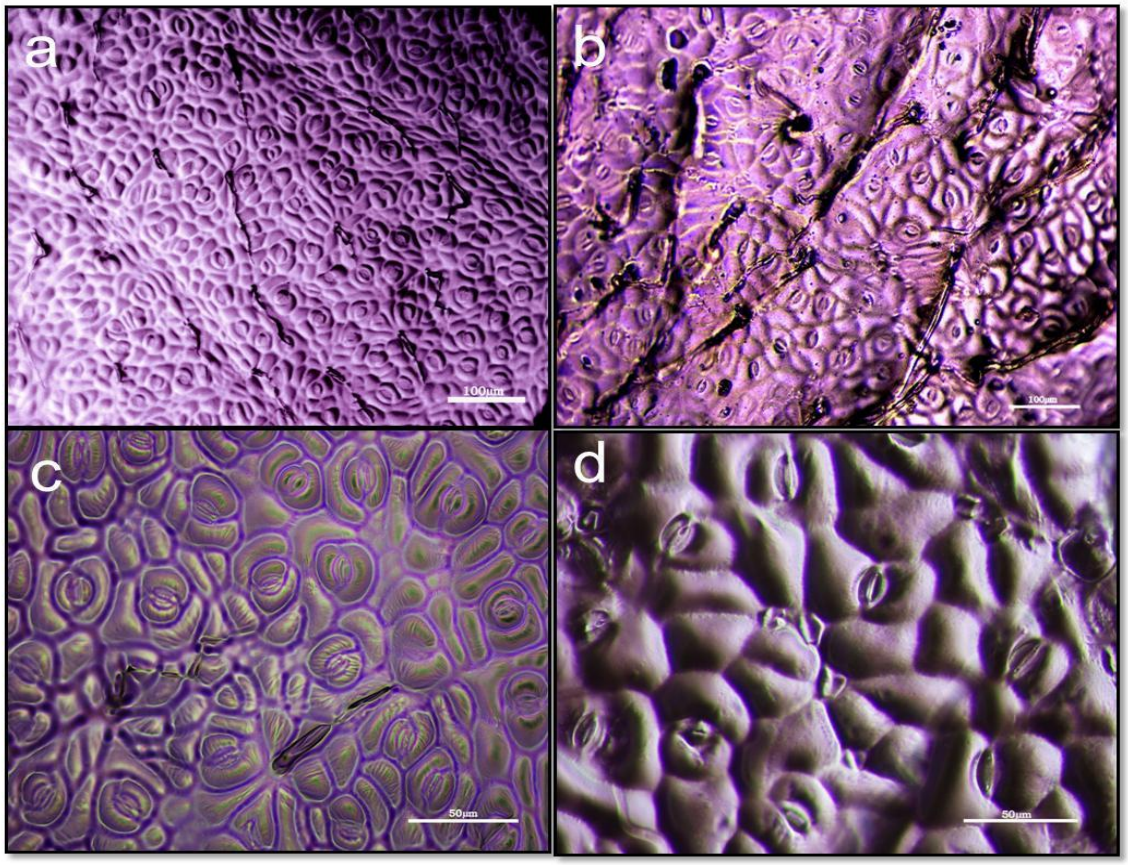

Figure 4-4. Imprints of *A. paraguariensis* leaf showing trichomes from 2n = 2x = 20 (a) and from 2n = 4x = 40 (b), stomata from 2n = 2x = 20 (c) and from 2n=2x=40 (d). Bar=100 µm (a-b) and 50 µm (c-d).

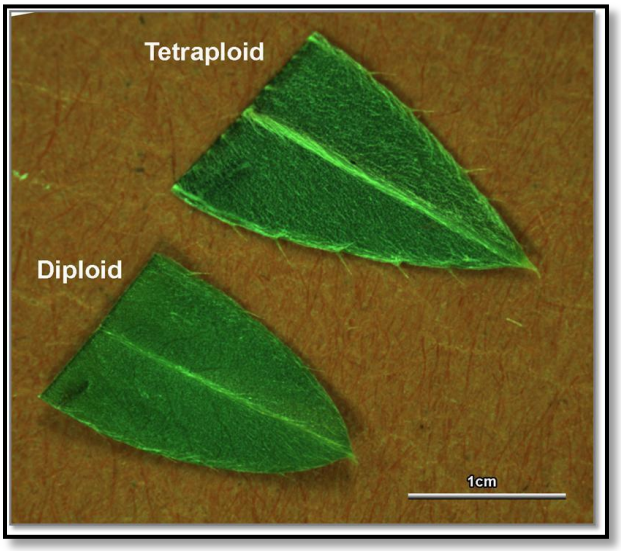

Figure 4-5. Abaxial surface of upper leaf from acclimatized diploid and tetraploid plants showing increased density of trichomes on the tetraploid.

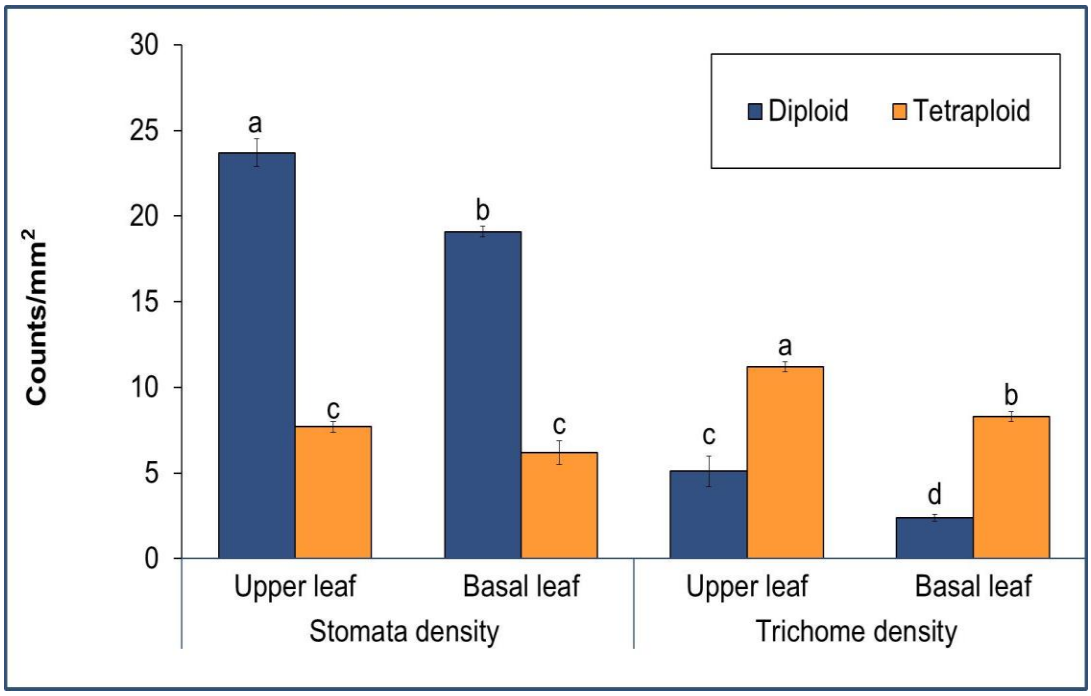

Figure 4-6. Leaf characteristics of diploid and tetraploid *A. paraguariensis*. Different letters on top of each bar within each parameter indicate a significant difference according to Tukey's Honestly Significant Difference Test (P = 0.05). Error bars represent ± SE.

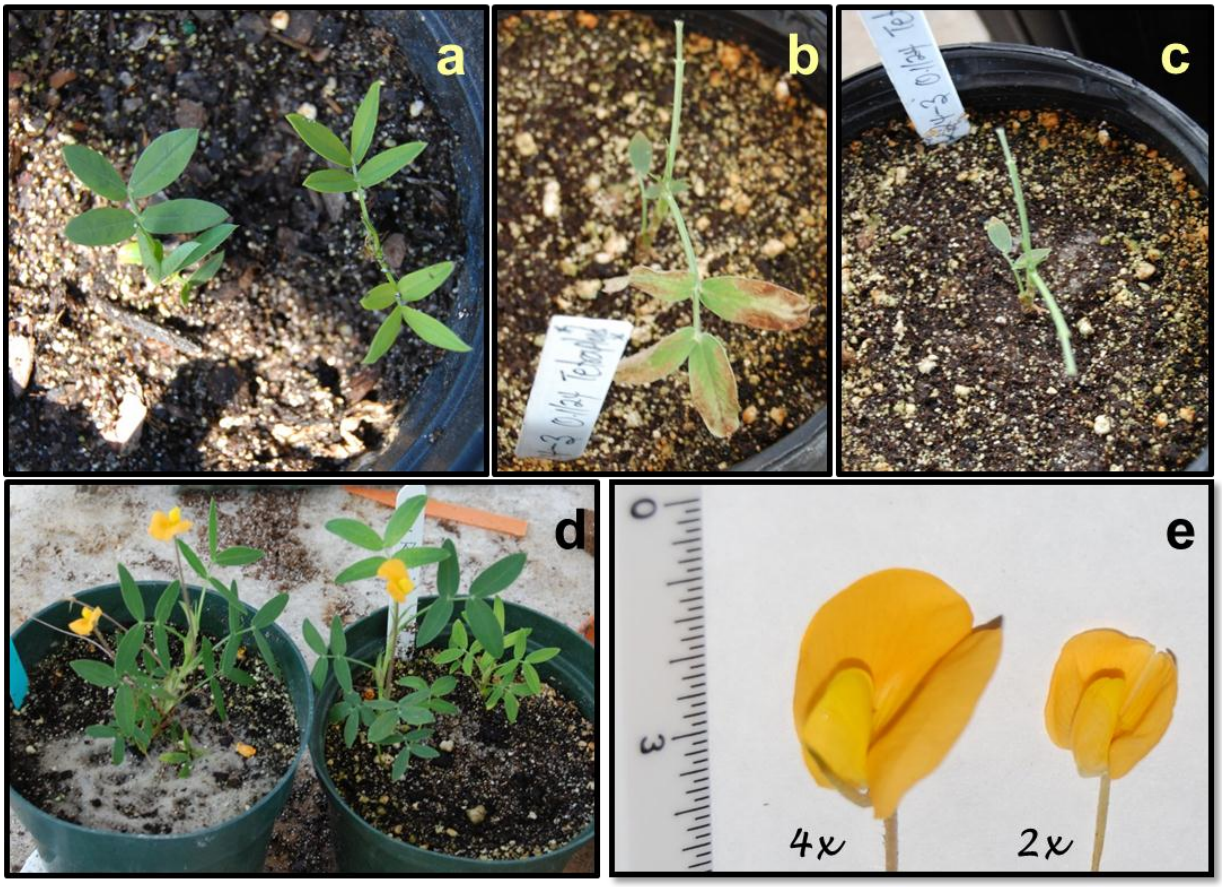

Figure 4-7. *Ex vitro* performance of diploid and induced tetraploid *A. paraguariensis*. (a) tetraploid plants 2 weeks after acclimatization before the occurrence of heat stress; (b) a tetraploid plant severely affected by heat stress; (c) defoliation of tetraploid plant 5 days after the heat stress; (d) diploid plants recovered and remain healthy after heat stress; (e) flowers from induced tetraploid (4x) and diploid (2x) plants of *A. paraguariensis*

Table 4-1. The induction of polyploidy from different explants of *A. paraguariensis.*

Explant source	No. of explants treated	No. of plantlets produced	No of tetraploids (%)	No. of mixoploid (%)
Quartered-seed	160	408	75 (18)a	0 (0)
Shoot-tip	160	25	3 (12)b	0 (0)
Callus	160	19	0 (0)c	5 (26)

Values in parentheses represent percentage of induced tetraploid or mixoploid. Values with identical letters indicate no significant difference at α = 0.05 using a factorial logistic regression model.

CHAPTER 5
SEED PRODUCTION IN *A. glabrata* AND TISSUE CULTURE REGENERATION FROM THE SEED-DERIVED EXPLANTS

The rhizoma perennial peanut (*Arachis glabrata* Benth.) (RPP) is an important forage and ornamental crop in the southern US. Its introduction into the US dates back to the 1930s, but today over 150 accessions have been introduced mostly from Brazil, Paraguay and Argentina (USDA–ARS National Genetic Resources Program, 2010). Although 'Florigraze' and 'Arbrook' are the two most successful cultivars, the recent release of cv. ,'UF Tito', and cv. 'UF Peace' based on increased yield, persistence, and tolerance to virus diseases was part of the strategy to address potential genetic vulnerability in this species (Garay et al., 2004; Quesenberry et al., 2010b).

Numerous field observations and previous research efforts have shown that most accessions of RPP produce very few seeds (Venuto et al., 1997; Niles, 1989). The seeds produced often do not germinate well, and the resultant seedlings often fail to survive due to lack of vigor (Venuto, 1997). Therefore, large scale field establishment is usually carried out by vegetative propagation using rhizomes. Apart from the fact that establishment from rhizomes is slow and prone to stand loss from drought, their purchase, transportation and sprigging can be expensive when compared to other forage crops. Niles and Quesenberry (1992) reported that poor pollen germinability was not the reason for low seed production in RPP, but the two main constraints were desiccated pollen and high rate of embryo abortion as a result of competition in dense canopies. Furthermore, Williams (1994) attributed the low sexual reproductive effort to the need for partitioning energy resources between rhizome and seed reproduction. Even though these hypotheses were put forward to explain the reproductive behavior of RPP, the factors that regulate every stage involved in viable seed production remain

largely unknown. This lack of significant seed production coupled with prevalent hybridization barriers, and very low frequency of tissue culture regeneration observed *in vitro* have limited genetic improvement of RPP to conventional breeding efforts through germplasm evaluation and capturing of occasional favorable mutations (Williams et al., 2004).

Biotechnology approaches such as genetic transformation and somatic hybridization provide an alternative avenue for overcoming hybridization barriers through direct introduction of genes across taxa. However, an efficient cell and tissue culture regeneration procedure is usually a major requirement for these procedures. The few papers that have investigated tissue culture regeneration of RPP from leaf derived explants have reported very low frequency and a long duration of regeneration. For instance, McKently et al. (1991) and Vidoz et al. (2004) reported low conversion rates of 10% of meristems to shoots and 6% of embryos to shoots, respectively. Consequently, it is evident that an efficient tissue culture regeneration system for RPP is still lacking. Seed derived explants have been a suitable starting material for high frequency *in vitro* regeneration of the cultivated peanut (*A. hypogaea* L.) (Sharma and Anjaiah, 2000), and several other wild *Arachis* species (Srinivasan et al., 2010) that were previously considered recalcitrant. This appears to be because seeds possess the precursor-cells for self-renewing, totipotent cells that are needed to generate whole plants. Thus, the use of seed-derived explants can be an appropriate strategy for enhancing tissue culture regeneration. The objectives of this study were: (1) to evaluate the seed production potential of two RPP cultivars and, (2) to assess the quality of

seeds that were produced. A third objective was to develop an improved tissue culture regeneration procedure for RPP using seed-derived explants.

Materials and Methods

Seed Production

An area of approximately 0.34 ha each at the UF Agronomy Department Forage Research Unit near Hague, Florida was planted to the RPP cvs. 'UF Tito' and 'UF Peace' by vegetative propagation using rhizomes in February 2009. Rhizome planting material was distributed in shallow furrows approximately 1.8 m apart and covered with soil. Elimination of perennial grasses and broadleaved weeds prior to planting was achieved chemically while other cultural practices for RPP according to IFAS recommendation (Ferrell, J. and B. Sellers, 2009) were followed. Monthly visual evaluations were made to assess plant survival, vegetative growth, and onset of sexual reproduction. Data on reproductive and vegetative characteristics were collected at 8- and 20-mo after planting which was approximately 2 months after the onset of peg initiation each year. Within each plot, an area of 1.0 m x 0.5 m was randomly selected and dug with a shovel to loosen the soil around the plants. The plant material was then hand-lifted, and inverted with the pods and rhizome pointed upwards. Pods were removed, washed and air dried in a cool, dry area. For each sampled area, the canopy/rhizome spread, plant height, number of opened flowers, number of pegs, pedicel length and number of harvested seeds were recorded while the weights of dry seeds were determined one month after harvest.

Seed Quality Evaluation

Four different but complementary germination tests were conducted in a completely randomized design consisting of 3 replicates of 10 seeds each. For each of

the experiments, cultivated peanut (AT VC-2) was used as a control treatment. All seeds were stored for about 10 months at 5 C and were surface sterilized with 1% mercuric chloride to eliminate fungi contamination. For filter paper germination, the seeds were placed on moist filter paper inside a plastic bag and then incubated at 26±1 ^{o}C. The *in vitro* study involved pretreatment of the seeds with 2.2 mgL^{-1} TDZ for 24 h before placement in Phytatry^{TM} culture vessels containing semisolid MS basal medium. Culture vessels were wrapped with parafilm® and placed in a growth chamber at 27 ^{o}C and 12 h photoperiod.

Germination in sand medium was tested *in vivo* by planting seeds directly into a tray containing sand in a laboratory growth room at 26±1 ^{o}C. After 3-wk, germinated seeds were grouped as normal or abnormal while non-germinated seeds were classified as fresh or dead. The germination percentage was calculated based on normal seedlings according to the method of ISTA (2010). Prior to the tetrazolium test, seeds were physiologically prepared by soaking in a beaker of water for 18 hours at 27 C. For each seed, the seed coat was removed and the cotyledons were separated with the embryonic axis still remaining attached to one of them. Each pair of cotyledon from the same seed were covered with 1% tetrazolium solution in a 6.0 x 1.5 cm petri dish and allowed to stain for 8 hours at 30 C. Seed by seed analyses were performed based on staining patterns.

Tissue Culture Regeneration

To test if the tissue culture conditions developed for *A. paraguariensis* using seed derived explants could be modified to achieve high frequency of *in vitro* regeneration of RPP. The two media formulations that were successful for shoot induction of *A. paraguariensis* in previous experiments were tested. Prior to culturing, seeds were

surface-sterilized by rinsing in 70% ethanol for 1min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10min. Thorough washing with sterile-distilled water was performed before seeds were soaked in sterile water for 4 h. Quartered-seed explants were derived after the removal of seed coat by aseptically dissecting each seed into four equal longitudinal pieces while the embryonic axis explants were surgically removed from a different set of seeds. Each explant was carefully implanted on semi-solid MS medium containing 3% (w/v) sucrose (Sigma # S5390), Gamborg B5 vitamins (Sigma # G1019), 0.8% (w/v) agar (Sigma # A7921), 4.4 mgL^{-1} TDZ and 2.2 mgL^{-1} BAP or 2ip. Three MS media formulations containing one of either 4.4 mgL^{-1} TDZ or 2.2 mgL^{-1} BAP or 2.2 mgL^{-1} 2ip and a fourth medium (MS0) that lacked growth regulator were used as control treatments.

All media were solidified with 0.8% (w/v) agar and adjusted to pH 5.8 before autoclaving for 20 min at 121 °C and 1.1 kg cm^{-2}. Approximately 20 ml of each media formulation was poured into 100 × 25 mm petri dishes. Explants were implanted on the different media inside petri-dishes with cut ends embedded in the medium. The incomplete 2 (cultivar) x 2 (explants) x 2 (media) factorial experiment in a completely randomized design with sub-sampling was repeated twice. Each petri dish contained four explants to constitute a subsample while 2 dishes were evaluated per explant per cultivar for each replication. Culture incubation was at 26±1ºC under continuous lighting provided by white cool fluorescent lamps with 60 $\mu Em^{-2}s^{-1}$ light intensity.

Daily observations for greening, swelling, and formation of callus or globular structures on explants were recorded for each subsample unit. When shoots appeared, they were transferred to into Phytatray™ (Sigma) culture vessels containing 50 ml of

MS medium without growth regulator and incubation continued under same conditions except that the photoperiod was reduced to 12 h. After two weeks, elongated shoots were rooted on MS medium that was supplemented with 2 mgL^{-1} of NAA. When 2 to 3 roots have been formed, plantlets were transplanted into jiffy pellets and acclimatized for two weeks inside a humidity chamber. Shoots were evaluated based on their *in vitro* vigor, rooting ability and *ex vitro* survival. To enhance the frequency of plantlet regeneration and eliminate browning of explants due to oxidation of phenolic compounds, another experiment was carried out to determine if supplementation of the culture media with activated charcoal would improve the frequency of shoot formation. Hence, supplementation of the nutrient medium with different concentrations of activated charcoal (0.5 and 1.0 gL^{-1}) was incorporated into the same procedure used in the first experiment.

Statistical Analysis

All data were analyzed according to MIXED procedure (SAS, 2011) for ANOVA. Means obtained from field data were separated by Duncan's Multiple Range Test (P = 0.05) while the means obtained from the seed viability tests and *in vitro* tissue culture experiments were separated based on Turkey's honest significant difference (HSD) at the 5% levels. Percent germination data were first subjected to arcsine transformation before analysis and then back-transformed for presentation in tabular form.

Results

Seed Production

It was evident from the observations on vegetative and reproductive growth of the two RPP cultivars between 2009 and 2010 that an increase in vegetative growth appeared to lead to a decrease in seed production (Figure 5-1). Observations from seed

yield and seed weight of the two cultivars during 2009 and 2010 harvest seasons are presented in Fig. 5-2. Seed yield of 'UF Tito' in 2009 (404 ± 57 $Kgha^{-1}$) was higher ($P<0.05$) than that of 'UF peace' (165 ± 52 $Kgha^{-1}$). However, these yields declined approximately 65% (145$kgha^{-1}$) for cv. 'UF Peace' and 100% (nil) for cv. 'UF Tito' from the preceding year. Interestingly, highest 100-seed weight (28.8 ± 2.5) was recorded for cv. 'UF Tito' during the first year while seed weight was not determined in the second year due to no seed being harvested in the sampled areas. In addition, the more extensive rhizome mat present made harvesting of seeds more difficult and laborious during the second year than in the previous year when the seeds were still firmly attached to their pedicels and in open soil not yet filled with rhizomes (Fig. 5b.).

Seed Quality Evaluation

Differences were evident in the color, size and weight of seeds obtained from both cultivars as well as the pod characteristics (Fig. 5a). Pods of cv. 'UF Tito' appeared more reticulated, harder and darker than those of cv. 'UF Peace'. Results from the four different viability tests are summarized in Table 1. Germination rates for all the viability tests ranged from 7-73% for cv. 'UF Peace', 10-44% for cv. 'UF Tito' and 53-98% for the cultivated peanut (control). From the ANOVA, considerable variation existed among cultivars for percentage seed germination *in vitro* (44-73%). Significant difference in germination rates between tests also existed for each of the cultivars. Obviously, germination was highest *in vitro* and lowest in sand for all the cultivars while the. standard germination test on filter paper gave values intermediate between the *in vitro* media germination and the *in vivo* sand germination However, a few of the seeds that germinated *in vitro* formed multiple shoots on the MS medium, but there was no significant callus formation. Rupture of RPP seed coats and emergence of radicle on

sand and on moist filter began after two weeks when the germinated peanut (control) plants were already at the two leaf stage. On the other hand, germination of seeds on MS nutrient medium occurred as early as three days after planting irrespective of the cultivar.

Tissue Culture Regeneration

While the embryonic axis failed to respond to any medium treatment *in vitro,* callus formation and plantlet regeneration from quartered-seed explant occurred approximately 6 wk after culture initiation (Fig 5c-e). Production of phenolic compounds reduced the frequency of regeneration in medium containing a combination of TDZ and BAP but regeneration of shoots was enhanced when BAP was replaced with 2ip (Figure 5-3f-g). An experiment was therefore carried out to determine if supplementation of the culture media with activated charcoal will improve the frequency of shoot formation especially in medium containing BAP. Even though browning of explants decreased when activated charcoal was added to the medium, shoot formation was not achieved. Dark green callus was formed which regenerated into individual leaves without buds. The effect of combinations of different cytokinins on callus formation and shoot regeneration from quartered-seed explant of RPP is presented in Table 5-2. The ANOVA revealed highest number of shoots per explant (4.38 ± 0.47) for cv. ‘UF Peace’ cultured on MS medium containing TDZ and 2ip while cv. ‘UF Tito’ gave lowest result (0.54 ± 0.49) on medium containing TDZ and BAP. With 3.00 ± 0.26 acclimatized plantlets per explant ccultured, the overall performance of cv. ‘UF Peace’ undoubtedly surpassed that of cv. ‘UF Tito’. The four negative control treatments failed to produce callus or shoots thus indicating that a combination of TDZ and BAP or 2ip is required for callus induction and shoot regeneration. Acclimatization of rooted shoots was achieved

within two weeks in a humidity chamber but some of the plantlets failed to survive due poor root development. Nevertheless, acclimatized plantlets (Fig. 5h) thrived well after transplanting into pots containing sand in the greenhouse and flowering was obseved after 2 months.

Discussion

The objectives of this study were to evaluate the seed producing potential and seed quality of 2 RPP cultivars, and to determine optimal tissue culture regeneration conditions for RPP using seed-derived explants. Increased vegetative growth resulted in the suppression of sexual reproduction in the two cultivars evaluated. This is in agreement with the general hypothesis that plant resource allocation to a specific biological function is traded off against investment in other function(s). The presence of this trade-off between sexual (flower and seed) and asexual (rhizome) reproduction is a strong indication that that both functions are dependent on the same resource pool. Williams (1994) observed that RPP flower production increased when rhizome production was moderately restricted but attempts to increase seed production through monitoring of the defoliation frequency was unsuccessful. In addition, excessive defoliation was found to be detrimental to flower and seed production. In a recent finding, Narbona and Dirzo (2010) reported that defoliation affects male but not female reproductive performance of *C. suberosus* because monoecious plants can allocate resources separately to male and female functions more easily than hermaphrodites. While it is expected that manipulation of resource allocation through experimental defoliation should result in an increased seed production in a hermaphroditic plant such as RPP, the factors that regulate partitioning of these resources are still largely unknown. Additionally, there is need for proper understanding of how certain

compensatory mechanisms influence resource uptake in such a way that the reproductive trade-offs become unnoticeable. The wide difference in seed production potential of the two cultivars could be considered a genotype effect. Although results of seed yield were based on data obtained for two consecutive years, the results are still preliminary due to inability to evaluate the cultivars under a suitable experimental design. The field plots which were initially established as a source of foundation rhizome stock were planted in several rows without blocks. Even though proper randomization was performed during sub-sampling of plots, it was impossible to adequately eliminate experimental errors through blocking of confounding factors. Nevertheless, production of a substantial amount of seed in the two RPP cultivars was truly a novel and exciting discovery. Pursuing this further, there is need to conduct a more extensive evaluation of RPP germplasm to identify and carry out selection for adapted cultivars with high seed yields.

Observations from the germination tests defeated the common expectation that larger seeds should yield higher germination percentages. While Venuto et al. (1997) detected considerable variation in seed germination and seedling vigor among five genotypes of RPP, results from the present study did not show differences in the frequency of seed germination among these two RPP cultivars except on MS medium under *in vitro* condition. However, the germination percent for both RPP cultivars were significantly lower in comparison to the cultivated peanut. The experiments involving seed germination in sand was terminated after 45 days while the filter paper germination test lasted for 30 days. Because most of the non-germinated seeds remained fresh until the end of the study, the low germination recorded for the RPP

could possibly be due to either seed dormancy or seed persistence in the soil, or a combination of both factors. Besides that, insufficient levels of calcium in the soil has been found to be connected to seed abortion and poor seed germination in cultivated peanut (*A. hypogaea*), but addition of supplementary calcium in combination with irrigation was found to greatly improve the seed maturity and germination of some peanut cultivars. (Tillman and Stalker, 2009). In this study, the amount of calcium in the soil was not determined and supplementary calcium was not applied. When seeds were germinated on MS nutrient medium, multiple shoot buds were formed even though no growth regulator was present in the medium. This may be as a result of the 24 h pretreatment with TDZ. The roles of TDZ in breaking of dormancy in seeds and in promoting direct embryogenesis from explants have been reported in many species including *A. hypogaea* (Gill and Saxena, 1992).

The frequency of shoot regeneration obtained when both TDZ and 2ip were included in the tissue culture media for regenerating explants exceeds previous reports (McKently et al., 1991; Vidoz et al., 2004). Cotyledon seed sections were highly suitable as an explant source for high frequency tissue culture regeneration of RPP. Moreover, they were easy to disinfect, store, and can be made available for deriving explants all year long. Inhibition of shoot regeneration when BAP was present in the medium led to brown discoloration of explants as a result of excessive production of polyphenol. This has been reported in tissue cultures of *Arachis* species (Li et al., 1993; Medina-Bolivar et al., 2007). Common methods of alleviating this problem include increasing the number of subcultures and adding activated charcoal to the tissue culture medium. Activated charcoal can reduce or prevent discoloration by rendering polyphenol oxidase

and peroxidase inactive. On the contrary, cytokinins such as TDZ, BAP and 2ip may have great adsorption affinity for activated charcoal thus reducing their effect on cultured explants.

Concluding Remarks

This preliminary study on seed production demonstrated that increased allocation of resources towards vegetative growth in RPP led to obvious reduction in sexual reproduction. Therefore, it is possible to obtain a substantial amount of viable seeds during the first year of establishment when the below ground vegetative growth is still minimal. Afterwards, the seed yield rapidly declined due to increased rhizome and canopy spread. The observation of considerable variation in seed production potential of cv. ‘UF Tito’ and cv. ‘UF Peace’ calls for intensive breeding and selection for cultivars that are good producers of high quality seeds. The seeds could be utilized for rapid stand establishment as well as an easy source of explants for high frequency tissue culture regeneration. However, due to the competition between rhizomes and seeds, any seed production for propagation might be restricted to an initial year after planting.

The discovery of substantial amount of seed production in cultivars ‘UF Peace’ and ‘UF Tito’ has the potential of changing the current approach to genetic improvement and breeding of RPP. For example, breeding programs for RPP may be able to utilize methods such as mass selection, backcross breeding and pedigree selection in the near future. In final consideration, the propagation, breeding, conservation and germplasm dissemination of RPP should be greatly enhanced.

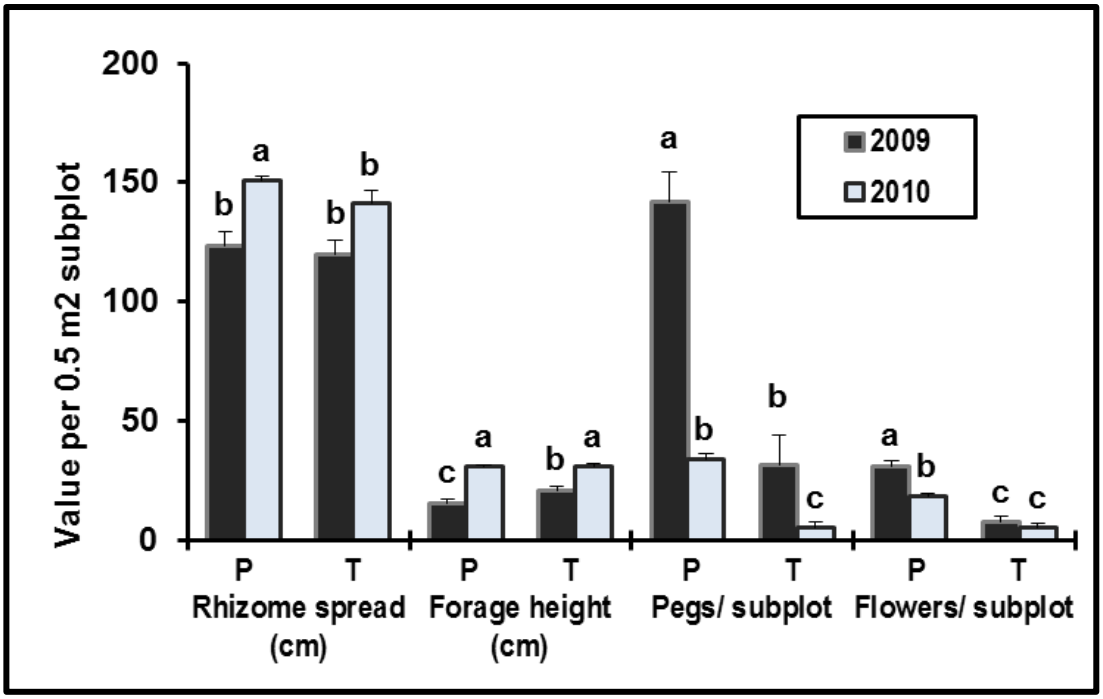

Figure 5-1. Vegetative and reproductive growth of 2 rhizoma perennial peanut cultivars from 2009 to 2010. P= cv. 'UF Peace' while T= cv. 'UF Tito'. Different letters on top of each bar for the same parameter indicate a significant difference according Duncan's Multiple Range Test (P = 0.05). Error bars =SE.

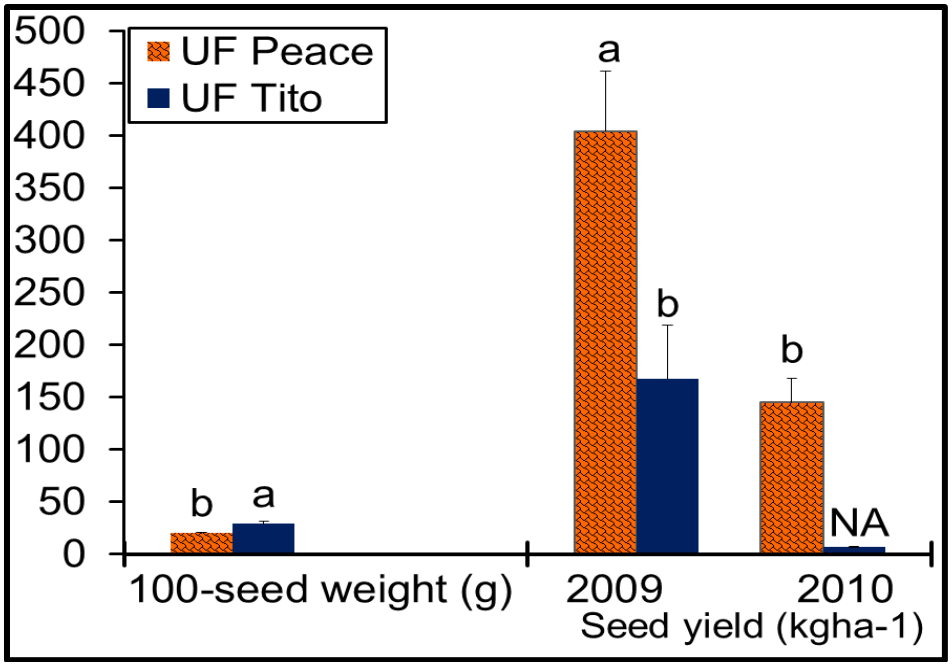

Figure 5-2. Seed weight and yield of RPP in fall 2009 and 2010. Different letters on top of each bar for the same parameter indicate a significant difference according to Duncan's Multiple Range Test (P = 0.05). Error bars =SE.

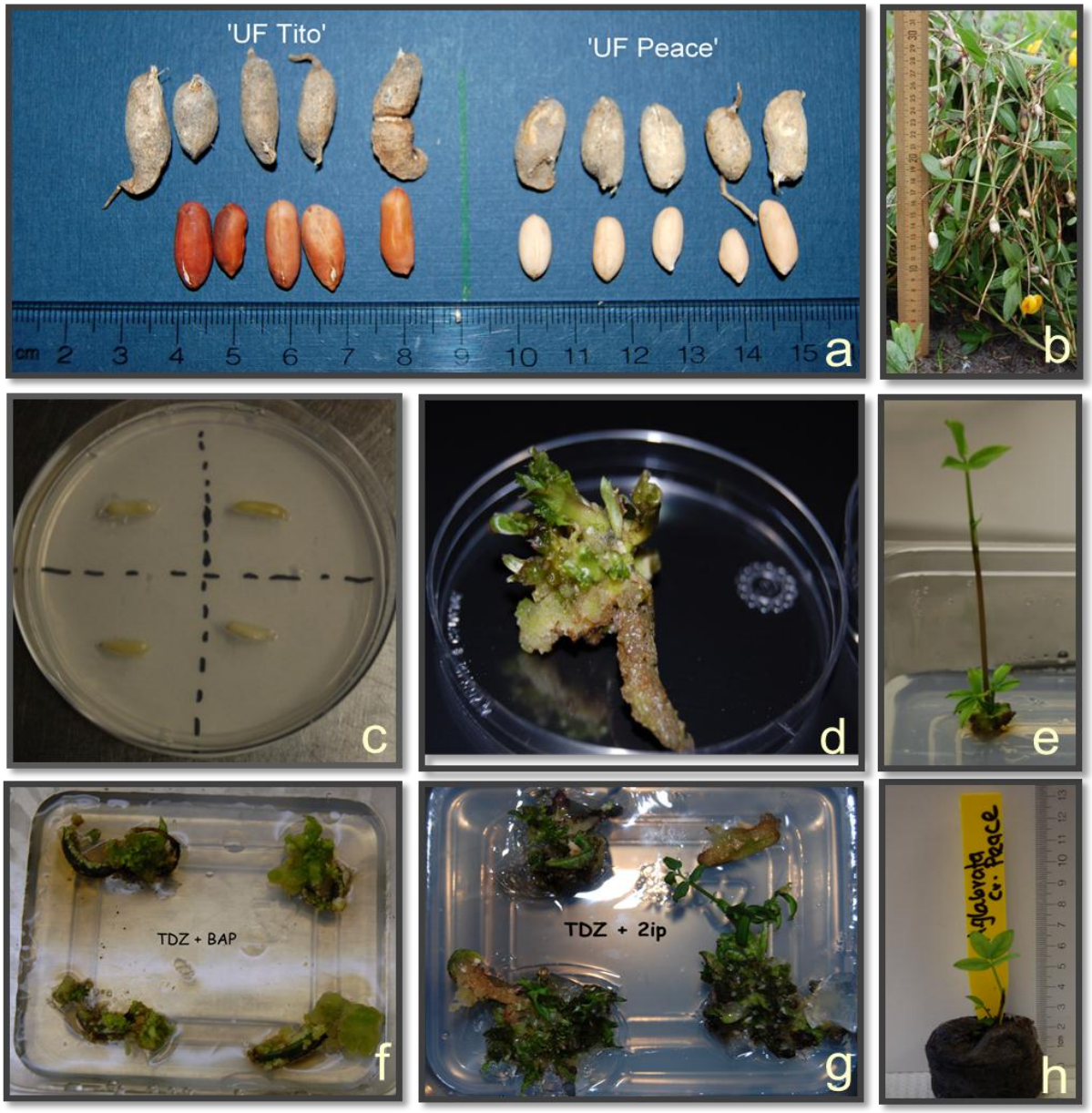

Figure 5-3. Seed production and tissue culture regeneration from seed-derived explant of RPP. (a) variation in pod and seed characteristics of cv. ‘UF Peace’ and ‘UF Tito’;(b) mature pods still firmly attached to pedicels during 2009 harvest season; (c) quartered-seed explants on MS medium containing 4.4 mgL^{-1} TDZ and 2.2 mgL^{-1} 2ip; (d) embryogenic callus mass on a quartered-seed explant after 2 wk in culture; (e) elongated shoot of cv. Peace on MS medium without growth regulator at 4 wk after culture initiation; (f) brownish discoloration of explants due to production of polyphenols in MS medium containing BAP; (g) regeneration without much interference of polyphenols when BAP was substituted with 2ip in the culture medium; (h) acclimatized plantlet of cv. ‘UF Peace’ at 6 wk after culture initiation.

Table 5-1. Percent germination and viability of rhizoma perennial peanut obtained from four different tests.

		Germination/ viability (%)			
Species	Cultivar	*In vitro*	Warm	Sand	Tetrazolium
A. glabrata	'UF Peace'	*73aB	25bB	7cB	30bB
	'UF Tito'	44aC	18bB	10bB	26abB
A. hypogaea	AT VC-2 (control)	98aA	85aA	53bA	77aA

* Percentages in the same row for the same cultivar followed by the same lower case letter are not significantly different at P = 0.05 when subjected to Tukey's honestly significant difference (HSD) test. Percentages in the same column for the same parameter followed by the same upper case letter are not significantly different at P= 0.05 when subjected to HSD test.

Table 5-2. Effect of combination of different cytokinins on callus formation and shoot regeneration from quartered-seed explant of rhizoma perennial peanut.

Cultivar	Cytokinin	Callus rating (1-5)	Callus weight (g)	Shoots/ explant	Acclimatized plant/explant
UF Peace	TDZ + BAP	1.68 (0.12)b	4.27 (0.40)a	2.77 (0.47)b	0.42 (0.26)b
	TDZ + 2ip	2.68 (0.13)a	2.76 (0.39)ab	4.38 (0.47)a	3.00 (0.26)a
	MS0/TDZ/BAP / 2ip (Control)	NA	NA	NA	NA
UF Tito	TDZ + BAP	1.71 (0.12)b	3.27 (0.40)ab	0.54 (0.49)c	0.31 (0.27)b
	TDZ + 2ip	1.96 (0.13)b	3.79 (0.41)a	0.85 (0.49)c	0.88 (0.27)b
	MS0/TDZ/BAP /2ip (Control)	NA	NA	NA	NA

Each value represents the mean (SE) of 2 replicates. Means followed by the same letter(s) are not significantly different at P=0.05 when subjected to Tukey's honestly significant difference (HSD) test. NA = data not available due to lack of callus/shoot formation.

APPENDIX
PROCEDURE FOR PLASTIC (GLYCOL METHACRYLATE–BASED) EMBEDDING

Paraformaldehyde stock solution. A stock solution of paraformaldehyde (16%) is prepared by adding the appropriate weight of paraformaldehyde into a beaker containing boiling distilled water in which a few drops of 1 N KOH have been added. The solution is stirred continuously with heat to dissolve the powder. After approximately 5 minutes, the solution should be clear with a few undissolved particles. The final volume of the solution is then adjusted. The solution should then be filtered and stored in a tightly capped glass bottle.

Fixative. To prepare 100 ml of fixative, 50 ml of 0.1 M phosphate buffer is mixed with 10 ml of each paraformaldehyde and glutaraldehyde stock solutions and 30 ml of distilled water. Using the above procedure, the final concentration of the fixative is a 1.6 % paraformaldeyde, 2.5% glutaraldehyde in 0.05 M phosphate buffer. The preparation of the paraformaldehyde solution and fixative solution were carried out in the fume hood.

Explant/ tissue preparation. The explants and organogenic/ embryogenic tissues are collected from culture vessels and the appropriate part is carefully excised and trimmed to the desired orientation with a sharp double edge razor blade.

Fixing and vacuuming. The tissues are fixed at room temperature for 1-2 h prior to a vacuuming step. After vacuuming, the fixative is replaced and the vials are transferred to a refrigerator and left overnight at 4ºC. The total fixation time should be no more than 24 h.

Dehydration. After fixation, the specimen is dehydrated with methyl cellosolve followed by two changes of absolute ethanol. Dehydration should take place at 4ºC to

minimize extraction of macromolecules from cells. After the completion of dehydration, if the specimens are not processed immediately, they can be stored in the freezer and used at a later date.

Infiltration. To prepare the infiltration solution, one packet of activator (benzoyl peroxide powder, moistened with 20% H2O, supplied in packets of 0.5 gm) should be dissolved in 50 ml of the basic resin. The infiltration solution should be kept at 4 ºC but bottle should be allowed to warm up to room temperature prior to its use in order to prevent condensation of water vapor from the air. Infiltration should be carried out gradually with a mixture of absolute ethyl alcohol and infiltration solution in a ratio of 2:1, 1:1, 1:2 before transferring to the pure infiltration solution. The duration of infiltration depends on the size and the density of the specimens.

Embedding. Plastic molding cups are used for the embedding of specimens. For embedding, the embryos/tisssues are poured together with the infiltration solution into one or more wells. Once the embryos are in place, the embedding solution is prepared. This is done by mixing 15 ml of infiltration solution with 1 ml of the hardener. This solution should be used immediately because polymerization begins as soon as it is prepared. The embryos are rinsed briefly with the embedding solution by adding a small quantity of the embedding solution into each well and then removed immediately. After rinsing, the embedding solution is added so that it fills the wells close to their rim. Working quickly, the orientation of the embryos within the wells should be checked using a stereomicroscope if necessary, prior to the addition of the round plastic specimen adapter. Once the embryos are properly arranged within the well, a plastic block holder is placed gently on top of each well to exclude air from the surface of the

mold as oxygen interferes with the polymerization process. The entire tray is then left on the bench for at least two hours, by which time the embedding solution should be polymerized.

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BIOGRAPHICAL SKETCH

Born to a nuclear family in Nigeria, West Africa, Olubunmi Aina (Bunmi) has two older brothers. Her father was a high school principal, while her mother was more involved in mixed farming than her teaching job. As a result, she developed an interest in caring for plants early in childhood. After a BS in agriculture from Ladoke University, Bunmi worked as a member of the National Staff at the International Institute of Tropical Agriculture (IITA) Ibadan, and later as a consultant to the International Livestock Research Institute (ILRI). Following this further, she earned two master's degrees; one was in agronomy from the University of Ibadan, and the other was in organic food chain management from the University of Hohenheim, Germany.

A strong interest in creating new things was a major reason why she chose to pursue doctoral studies in plant breeding and genetics. Her decision to start a doctoral program at The University of Florida in Spring of 2008 was due to an interest in research projects that faculty members in Agronomy Department were focusing on. Her current research on *in vitro* and *in vivo* evaluation of wild peanut germplasm is aimed at contributing towards sustainability. To facilitate the exploration of her research questions, she has utilized *in vitro* methods for doubling the chromosome number of a wild peanut species and determined how this approach could be used to remove the barriers existing in gene transfer between the wild species and the cultivated peanut.

Curious by nature, Bunmi enjoyed the fact that her research work allowed her to discover, think and inquire. As a research assistant in the Agronomy Department, she gained hands-on experiences both in the lab and on the field. A unique accomplishment in this capacity was identifying the correct timing in abundant seed production in rhizoma peanut. This novel and exciting discovery has the potential of changing the

current approach to the genetic improvement and breeding of this forage crop. Bunmi has earned a Doctor of Philosophy in agronomy from The University of Florida in 2011.