GERMPLASM CHARACTERIZATION OF *Arachis pintoi* Krap. and Greg. (*LEGUMINOSAE*)

By

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To my wife, Aline Varandas, who bravely decided to follow me in pursuing this dream.

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GERMPLASM CHARACTERIZATION OF *Arachis pintoi* Krap. and Greg.
(*Leguminosae*)

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Arachis pintoi Krap. and Greg. is a herbaceous, perennial legume, exclusively native to Brazil. It is considered a multiple use legume, being grown for forage; ground cover in fruits orchards, forest, and low tillage systems; erosion control; and ornamental purposes. Although several cultivars have been released in different countries, little is known about the genetic diversity of the germplasm stored at genebanks. Our objective was to characterize and evaluate the genetic diversity of the germplasm of 35 accessions of *Arachis pintoi* at molecular, morphological, and agronomic levels. *A. pintoi* accessions were used to study the genetic diversity at the molecular level using RAPD markers. Concurrently, two tissue culture protocols were evaluated for their organogenesis ability, and capacity to generate somaclonal variation. From the original 18 primers tested, amplifications were obtained with eight, which amplified 100 polymorphic bands. Average genetic distance was estimated as 0.36, indicating that a large amount of genetic diversity exits among the accessions. The accessions were grouped by their genetic

similarities into four distinct groups. Callus induction was achieved on two different Murashige and Skoog basal protocols, and shoot regeneration was achieved for several accessions on both media. Regenerated plants recovered in both protocols presented no differences in their RAPD band profile. Morphological characterization using data from stems, leaves, flowers, pegs, pods, and seeds demonstrated that the germplasm presented great morphological variability. Principal component analysis was able to discriminate the accessions in terms of three dimensions, and Cluster analysis differentiated four distinct groups. Average dry matter yield in 2003 was 4.35 Mg ha^{-1}, and ranged from zero to 9.10 Mg ha^{-1}. Average crude protein was 180 g kg^{-1} of DM and ranged from 130 to 220 g kg^{-1}. The average value of *in vitro* organic matter digestion was 670 g kg^{-1} of DM and ranged from 600 to 730 g kg^{-1}. Some accessions produced high seed yields reaching values above 1.00 Mg ha^{-1}. Average seed production was 0.32
Mg ha^{-1} in 2003, and 0.43 Mg ha^{-1} in 2004. *A. pintoi* germplasm presented high levels of resistance to the nematode species *Meloidogyne arenaria*, *M. javanica*, and *M. incognita*.

CHAPTER 1
INTRODUCTION

Biodiversity can be defined as the total variation found within all living organisms and their habitats. It can be accessed at three different levels: communities (environment), species, and genes. When accessing biodiversity at the species level, we are interested in observing differences among individuals or populations of that particular species. This can be referred to as the genetic diversity of the species. Thus, we can consider genetic diversity as a form of biodiversity.

Genetic diversity is associated with the degree of differentiation among individuals in a population at their genetic material level. The genetic material corresponds to the DNA, genic or cytoplasmic, called the genotype. Expression of genes contained in the DNA is the result of interaction between the environment and the genotype, which is called the phenotype. Genetic diversity is important because it will enable evolution and adaptation of the species to an ever changing environment. Thus it is essential for the long-term survival of a species.

Since very early in the history of the world, humans have exploited the genetic diversity of plants, primarily as sources of food, and then to improve their land races and cultivars. With the advance of modern agriculture, plant breeders around the world began to collect genetic diversity of the most important food crops and to store these materials at national research institutes and local institutions. By the late 1960s, the Food and Agriculture Organization (FAO) of the United Nations (UN) was concerned about the problem of conserving plant genetic diversity, and a series of conferences were arranged.

As the result of these conferences, in 1974 the International Board for Plant Genetic Resources (IBPGR) was established. Coordinated actions were then made to increase collection efforts and to increase the creation of germplasm banks at national and global levels. As a consequence of these efforts, about 1300 *ex-situ* genebanks were created. Today, they together conserve an estimated 6.1 million accessions (Hawkes et al. 2000).

Although actions to increase conservation of genetic diversity were very successful, the use and characterization of these resources were not. In 1996, FAO held the Fourth International Technical Conference on Plant Genetic resources in Leipzig/Germany. During this event, a global plan for the conservation and sustainable use of plant genetic resources for food and agriculture was prepared and formally adopted by more than 150 countries. Among other issues, a call was made for expanding characterization and evaluation, and for increasing the number of core collections to facilitate the use of the stored germplasm (FAO, 1996).

Information obtained in studies of germplasm can be used for accession classification, analysis of genetic diversity, and studies of the genetic divergence. Studies of genetic diversity are important because they are a tool for genetic improvement allowing the efficient use of the available germplasm of a species.

The genus *Arachis* has great importance at the world level. *Arachis hypogaea* L. (peanut), the species with greatest importance in the genus, is cultivated commercially in more than 80 countries, supplying food with high protein levels and oil of excellent quality. According to FAO, world production of peanut is about 23 million metric tons annually.

Because of the importance of the peanut as a food crop, great importance is given to the whole genus, and thousands of accessions of the common peanut and its wild relatives have been collected and stored in genebanks, especially those located at ICRISAT, USDA-NPGS and EMBRAPA.

Although *A. hypogaea* is the most important species of the genus, some of its wild relatives also have agronomic potential. Some of these species are used as ground cover or forage crops.

According to Kerridge (1994), the major research priorities in the genetics of species with potential as forage crops in the genus *Arachis* are:

- More thorough studies of germplasm in the sections Caulorhizae and Rhizomatosae.
- Rigorous characterization of genetic variability at the molecular, physiological, and agronomic levels.
- Development of molecular markers for use in genotype identification and studies of breeding behavior.
- Quantification of genetic variation, inheritance of important traits, and identification of sources of traits of agronomic interest.
- Survey of accessible sources of genetic resistance for disease and insect pest resistance, and survey of diseases in natural populations.

Arachis pintoi is a perennial species of the section Caulorhizae, and it is considered by many as the most promising of the wild species of the genus. It is a multiple use legume, which has been used predominantly as a forage crop, with released cultivars in several countries.

The importance of the genus, the agronomic potential presented by *Arachis pintoi*, and the lack of information about its germplasm (stored at the USDA-NPGS) were the primary justification for this research.

The hypothesis, objective, and goals of this work are presented below.

Hypotheses

Genetic diversity is limited in the *Arachis pintoi* germplasm stored at the USDA-NPGS germplasm bank.

Objectives

To characterize the germplasm of *Arachis pintoi* at molecular, morphological, and agronomic levels.

Goals

- To quantify the genetic variability of accessions of *Arachis pintoi* at the molecular, morphological, and agronomic levels.
- To supply basic information to the breeding programs that are using the germplasm to develop new improved cultivars.

CHAPTER 2
LITERATURE REVIEW

Plant Genetic Resources Characterization

Economic exploration of plants is essential for maintenance of a wide base of their genetic resources, because the same ones compose the vital patrimony of the species. The most important way to increase a species' productivity is to know the variability in its existing germplasm. Incorporating new organisms into the group of useful plants, by direct use or through programs of genetic improvement, requires use of available genetic resources. These resources are collected to be used in breeding programs and not just to be conserved (Paterniani, 1988).

According to Smith and Linington (1997) the cost to collect a single germplasm accession in its country of origin and incorporate that accession into a local genebank is about US $870. In addition, the Keystone Center (1991) estimated the cost to maintain an individual germplasm accession is US $50 per year. The value of the material itself and the aggregate costs to acquire and maintain the tremendous amounts of genetic diversity stored in the genebanks around the world are enough to justify germplasm characterization and evaluation.

After the IBPGR was established in 1974, great attention was given to collecting germplasm of the most important food crops. This was particularly important because of the changes brought about by the green revolution to producers everywhere. Land races were rapidly being replaced by improved cultivars and the risk of losing these genetic resources was great. Furthermore, the population explosion and the expansion of

agricultural lands to supply the needs of expanding urban populations also increased the risk of genetic erosion in some ecosystems. As a result of these efforts, FAO (1998) estimated that 6.2 million accessions of 80 different crop species were stored in 1,320 genebanks and related facilities in 131 countries. These numbers show that today, germplasm conservation is well recognized issue, and is deemed important by most of the world community.

However, plant genetic resource conservation is much broader than just collection and storage. Characterization and evaluation should be an integral part of the general scheme of genetic resource conservation, but because of the great effort made to increase the number of accessions stored in the genebanks, these activities often have been relegated to a second level. This was not intentional, but reflects the size that germplasm collections have reached and the cost of complete evaluation. For instance, according to Holbrook et al. (1993), the number of common peanut genotypes stored in the USDA-NPGS genebank is about 7,500. However, the most recent data from the Germplasm Resources Information Network (GRIN) account for 9,232 accessions.

Germplasm characterization consists of studies of eco-geographic and demographic adaptation (Martins, 1984), and according to Solbrig (1980) involves mostly the parameters of the vital cycle of the organism, genetic and physiological studies, plant pathology, and yield evaluation, among other studies. Characterization often also involves taxonomic confirmation and should produce an easy and quick way to differentiate the germplasm, using highly heritable and visible traits (Hawkes et al., 2000). Breeding programs should begin only after appropriate germplasm characterization (Cameron, 1983).

Improved knowledge of the available germplasm provides essential information for its more intense use. Characterization and evaluation of available resources will allow the establishment of nuclear collections (core collections) that, by definition, embrace the maximum genetic diversity contained in cultivated species and in the related wild species, with the minimum possible number of accessions (Frankel and Brown, 1984).

Another positive result of characterization is the detection of duplications, which is a serious problem especially when we have in mind the size of the collections and the resources needed to maintain them. Plucknett et al. (1987) estimated that as few as 35% of the accessions in world collections are actually distinct.

In summary, characterization is the best way to understand the variability contained in a germplasm collection and to increase use of the germplasm by plant breeders. It is also important in monitoring the genetic stability of the germplasm storage processes. Characterization of germplasm can be based on molecular, biochemical, morphological, and agronomic features.

The use of biochemical and molecular characterization can be a more precise way to discriminate among accessions in germplasm collections. This is especially true in species where the application of phenotypic morphological descriptors is delayed by very slow growth or delay in reaching the reproductive stage, when many markers tend to be evidenced (Valls, 1988).

During the past 25 years, many improvements in molecular biology techniques have allowed the direct application of these methods in studies of genetic characterization. According to Karp et al. (1997), the advent of the polymerase chain reaction (PCR) permitted the development of several molecular technologies that can be

used with great success in detecting, characterizing and evaluating genetic diversity. Several different types of molecular markers were generated after the advent of the PCR, and they differ among themselves in the way they resolve genetic differences; in the type of information that they generate; in the taxonomic levels where they should be applied; and finally in costs, labor requirement, and training.

Although an extensive number of molecular markers are available, DNA-based markers have greatly overtaken markers based on proteins or enzymes, because the latter can be influenced by the environment.

According to Ford-Lloyd (2001), DNA-based markers can be classified into three different categories:

- **Non-PCR-based methods**: Restriction fragment length polymorphism analysis (RFLP) and variable number of tandem repeats (VNTRs) are some examples of this category.
- **Arbitrary (or semi-arbitrary) primed techniques**: This is a PCR-based category that uses random primers during the PCR reaction. The most well known and widely used of these methods is the "Random Amplified Polymorphic DNA" (RAPD).
- **Site-targeted PCR**: In this class, primers that amplify specific regions of the DNA are used during the PCR reaction. Examples are single nucleotide polymorphism (SNP's), microsatellite repeats (VNTR's), and many others.

Karp et al. (1997) also indicated that DNA-based markers can be useful in defining an accession identity; defining the degree of similarity among individuals of an accession or a group of accessions; and defining the presence of a particular allele or nucleotide sequence in an individual, population, or taxon. Among all DNA-based markers, RAPD have been extremely popular with plant genetic scientists. Reiter (2001) revealed that in a recent bibliographic search more than 2,000 publications were listed where RAPDs markers had been used.

According to Reiter, the popularity of this marker arises from the fact that the method is very simple and the cost required for its application is low.

RAPDs are the product of PCR reaction where a single, short, sequence-arbitrary primer (oligonucleotide – 10mer usually) is used. Amplification happens by setting a low annealing temperature (35-37ºC typical) in the thermal-cycling program. Amplification products are separated by size in an electrophoresis agarose gel. A DNA fragment pattern of low complexity is observed after ethidium bromide staining. Fragment patterns of individuals of the same species are almost identical (with some exceptions where one band is present in one individual and absent in another). These fragment polymorphisms are heritable and can be classified as a new category of genetic marker (Williams et al., 1990).

Most RAPD bands are amplification polymorphisms due a nucleotide base change at one of the priming sites or an insertion/deletion event within the amplified region. DNA amplification from heterozygous individuals at RAPD marker loci are normally identical to the homozygous parent. Thus, RAPD markers are typically dominant markers. Dominant markers are less informative than co-dominant markers for genetic mapping in some types of segregating populations (Williams et al., 1990).

When working with several accessions amplified using the same 10mer primer, similar DNA fragment patterns may be observed. However, it is not possible to know if all fragments of the same size class have the same DNA sequence (and thus are allelic). Bands from related germplasm lines are likely to be allelic. However, as genetic distance increases so does the probability of non-allelism. Fragment allelism is an important

concern when RAPDs are used for population genetic studies, varietal fingerprinting, and some forms of marker-assisted selection (Reiter, 2001).

Another criticism regarding RAPD is incomplete pattern reproducibility (Westman and Kresovich, 1997). Any sequence-arbitrary amplification method will be considerably less robust than conventional PCR for the following reasons:

- Multiple amplicons are present competing for available enzyme and substrate
- Low-stringent thermal-cycling permits mismatch annealing between primer and template

To overcome these problems, it is important to create optimal and consistent amplification conditions, and to optimize the quantity and quality of reaction components (DNA, MgCl, Taq polymerase, etc).

Characterization of genetic variability can use conventional methods of evaluation complemented by biochemical and molecular methods (Hulli, 1987; Marcon, 1988). It is recognized that morphological data, despite its importance, is not always easily understood at the level of genes (Simpson and Withers, 1986). The phenotypic expression of botanical and agronomic characteristics, which in general have polygenic inheritance, is the result of genotype x environment interactions (phenotypic plasticity). On the other hand, simple verification of the existence of molecular markers does not imply knowledge of their expression in the phenotype. Thus, phenotypic characterization of accessions should still be used in germplasm evaluation. Thus morphological and molecular methods present different advantages, and the complementation between them can produce benefits that would not be obtained in separate analyses (Hulli, 1987).

Morphological characterization is the simplest way to gather information about germplasm; and before molecular marker techniques became popular and accessible, it

was the primary way for plant genetic scientists to improve their knowledge about the resources available to them.

The term morphological characterization refers to a broad subject that involves taxonomy, botany, genetics and other disciplines. Sometimes, the first step in characterizing a germplasm accession involves taxonomic confirmation. After this step, highly heritable and visible botanical features are used to prepare a list of descriptors that will be applied to the plants. Usually, qualitative traits that show little environmental influence are preferred, but sometimes quantitative traits are also used. Previous knowledge about the morphology and phenology of the species is required (Hawkes et al., 2000).

The importance of morphological features was demonstrated by Mendel's work. Mendel used simple morphological traits and studied their inheritance in hybrids produced from distinct homozygous parents. The outcome of his work was the demonstration that traits are transferred from parents to offspring by factors later called genes. The discipline of genetics (and a new world) was opened by a work based on morphological traits. Nowadays, Mendel's work and morphological traits are still used to understand the mechanism of inheritance and genetics of traits in many different species.

Morphological descriptors must be universal and simple to score. The universality of the descriptor will allow plant breeders and researchers working with plant genetic resources to exchange information about germplasm accessions generated in different places. To achieve this goal the International Plant Genetic Resources Institute (IPGRI), in collaboration with crop specialists, published guides to descriptor selection and also descriptor lists for more than 100 different species, including the most important food

crops and some wild relatives of the same (Hawkes et al., 2000). The IPGRI (www.ipgri.cgiar.org) lists species with published descriptors, and these descriptors lists can ultimately be used to generate germplasm catalogues and to build computational databases of different species that can be accessed by the World Wide Web, contributing to increased use of plant genetic resources.

Another level of germplasm characterization is agronomic research. This type of research measures useful traits related to agronomic performance. These usually include quantitative traits related to yield, disease resistance, and environmental stress tolerance are measured. Although it seems insignificant, some authors make a distinction between agronomic characterization and evaluation. Hawkes et al. (2000) suggested that evaluation involves traits important for crop improvement, which may often be affected by their interaction with the environment. They also suggested that one big difference between characterization and evaluation is that the first should be carried out by the germplasm curator and the latter by plant breeders and plant pathologists working outside the genebank.

Therefore, it is important to exercise caution when using agronomic data generated in different places at different times. The use of standard checks will permit a more extensive comparison of the germplasm, but will never replace local evaluations, especially when a more detailed analysis is needed (Ford-Lloyd and Jackson, 1986).

The Genus *Arachis*

The genus *Arachis* has great importance at the world level. *Arachis hypogaea* L., the most important species in the genus, is cultivated commercially in more than 80 countries, supplying food with high protein levels and oil of excellent quality. According to FAO, world production of peanut reaches about 23 million tons annually and assumes

great agronomic importance in developing countries. These countries are responsible for 80% of the world production with 67% being produced in the semi-arid tropics (Singh and Singh, 1992).

The genus *Arachis*, with diploid (2n=2x=20) and tetraploid (2n=4x=40) species, belongs to the Fabaceae family, Papilionoideae subfamily, Stylosanthinae subtribe, Aeschynomeneae tribe. It possesses herbaceous, annual and perennial species, leaves with 3 or 4 leaflets; papilionaceous corolla; tubular hypanthium, sessile ovaries and underground fruits (Gregory et al., 1980; Krapovickas and Gregory, 1994).

The genus has natural occurrence exclusively in South America, extending from east of the Andes Mountains, south of Amazonia, north of the Planície Platina and northwest of Argentina, until the northeast of Brazil. It is believed that the genus originated in the Amambai Mountain, near the border of Mato Grosso do Sul-Brazil and Paraguay, from where it was dispersed over an area of 4000 km in diameter (Krapovickas and Gregory, 1994).

Krapovickas and Gregory (1994) describe 69 species, with 39 exclusively from Brazil. Valls and Simpson (1994), however, assert that the genus has about 80 species.

The genus can be divided into nine groups or sections; one of them with two series:

- Section Arachis
- Section Caulorrhizae
- Section Erectoides
- Section Extranervosae
- Section Heteranthae
- Section Procumbentes
- Section Rhizomatosae
    - Series Prorhizomatosae
    - Series Rhizomatosae
- Section Trierectoides
- Section Triseminatae

The importance of *A. hypogaea* as a major food crop generated a need for a better understanding of the wild species of the genus, because of the potential to use them in breeding programs of the common peanut. Valls and Simpson (1994) reviewed the two different proposed gene pools of the genus (Table 2-1). Some degree of cross-ability is present among some species, and this could represent a chance to move genes from one species to others. Examples of introgression of genes from wild species to the common peanut are presented by Simpson and Starr (2001), Starr et al. (1990), and Mallikarjuna and Sastri (2002). Cultivar "Spancross" (Starr et al., 1990), generated by the crossing of *A. hypogaea* and *A. monticola*; and cultivar "COAN" (Simpson and Starr, 2001), a runner market type derived from a backcross introgression pathway involving a complex interspecific amphiploid hybrid (*A. cardenasii* x *A. diogoi*) using cv. "Florunner" (Norden et al., 1969) as the recurrent parent are real examples of the potential of the wild species in breeding programs of the common peanut.

Parallel to the interest in improvement of cultivated peanut, the acknowledgment of the forage potential of some wild species of the genus brought a general interest in these species. This interest generated an effort to recollect most of the available genetic diversity. According to Valls and Pizarro (1994) more than 30 expeditions were conducted between 1981 and 1993, which greatly impacted the number of accessions of perennial *Arachis* species stored in germplasm banks.

The Forage Potential of the Genus *Arachis*

Many species of the genus *Arachis* produce forage high in quantity and quality, comparable or superior to species of other tropical legumes used commercially as forage crops (Valls and Simpson, 1994). Species of the genus Arachis, belonging to the sections Rhizomatosae, Arachis, Erectoides, Procumbentes, Caulorrhizae and Triseminatae have

been evaluated for forage in Australia since 1950. Many of those species have presented great potential as forage crops, mainly because of persistence under grazing (Cook et al., 1994).

Table 2-1. Proposed gene pools of the genus *Arachis* based on *Arachis hypogaea* breeding perspective

Gene Pools	Wynne and Halward (1989)	Singh et al. (1990)
Primary	• Cultivated varieties of *A. hypogaea* • Landraces of *A. hypogaea*• • Breeding lines derived from the above	• *Arachis hypogaea* and *A. monticola*
Secondary	• Arachis monticola • Other wild tetraploid forms in section *Arachis* (as yet uncollected)	• Diploids in section *Arachis* cross-compatible with *A. hypogaea*
Tertiary	• Diploids in section *Arachis* cross-compatible with *A. hypogaea*	• Species in all sections
Quaternary	• Diploid and tetraploid species of other sections of the genus	

The forage importance of the common peanut (*A. hypogaea*) has been long recognized, especially because of the great nutritional value of its stem and leaves (Cook and Crosthwaite, 1994). Although long recognized, breeding programs neglected this fact and selections based on the forage potential were not emphasized, a point that seems to have changed in recent years during which evaluation of the forage production of *A. hypogaea* has been conducted. Gorbet et al. (1994) reported dry matter yields (DMY) of 9 Mg ha^{-1} in a 140 days growth period in research done in Florida (USA). Crude protein values ranged from 140 to 200 g kg^{-1} and IVOMD ranged from 600 to 720 g kg^{-1}.

Also in the southeastern USA, Muir (2002) studied the forage production and nutritive value of eight warm-season legumes in north-central Texas under two dairy manure compost rates, and two harvest regimes during 2 years. He reported that *Arachis*

hypogaea produced over 2.5 Mg ha^{-1} yr^{-1} of high quality forage and was one of the most productive. He concluded that although not highly productive under dryland conditions, groundnut can contribute with both forage and seeds for livestock and wildlife.

Pizarro et al. (1996a) evaluated 34 germplasm lines of *A. hypogaea* in a clay oxisol in Brazil, and reported DMY that varied from 0.4 to 2 Mg ha^{-1} in a 180 growth days period. In Nigeria, Larbi et al. (1999) investigated variation in forage and seed yields, crude protein (CP), neutral detergent fiber (NDF), *in situ* degradation of dry matter (DM), and nitrogen (N) of 38 late-maturing groundnut cultivars. Average yield of forage was 4550 Mg ha^{-1} and seed production was 1.25 Mg ha^{-1}. Crude protein, NDF, and DM degradation of leaf and stem varied among cultivars. Forage and seed yields were significantly correlated (r = 0.53), but seed yield was poorly correlated with forage quality indices examined. They suggested that plant breeders could select genotypes with higher seed and forage yield, and better forage quality.

Also in Nigeria, a 3-yr study was carried out with 11 peanut varieties to select superior lines for use in crop livestock systems. Crude protein (CP) concentration ranged from 148 to 216 g kg^{-1}, with seven varieties recording forage yields above 5 Mg ha^{-1}. Mean seed yield (over 3 yr) varied significantly from 0.73 to 1.68 Mg ha^{-1} (Omokanye et al., 2001).

Moreover, other species of the genus have also shown great potential to be used as a forage crop, principally because they have great persistence under grazing. Cook and Crosthwaite (1994) reported that stands of some species of the genus have been grazed for more than 30 yr. In Brazil where the genus originated, native populations have been grazed and utilized by producers for many years. Cameron et al. (1989) argued that wild

species of the genus *Arachis* are excellent alternatives to improve tropical and subtropical pastures, particularly due to this strong persistence under grazing. Kretschmer and Wilson (1988) pointed to superior forage and seed production, and adaptation to wetlands as some of the important characteristics of an *Arachis* genotype from section *Procumbentes* (Pantanal peanut) evaluated by them and later described as *A. kretschmeri* by Krapovickas and Gregory (1994).

Among the several species of the genus that can be utilized as forage crops and ultimately will impact the quality of the pastures, we can point to *Arachis glabrata* Benth., *A. pintoi* Krap. & Greg.; and *A. repens* Handro as the ones with the highest potential (Grof, 1985; Valls, 1992).

Cultivars "Florigraze" (Prine et al., 1986) and "Arbrook" (Prine et al., 1990) of *Arachis glabrata* were released by the University of Florida, and they are used in an area of approximately 10,000 ha throughout the southeastern USA. These cultivars are primarily used to produce hay with high nutritional quality that is consumed by the dairy and beef cattle and by racing horses. Although *A. glabrata* has shown excellent forage characteristics, the fact that it produces few seeds, and consequently that its propagation is exclusively vegetative, causes establishment difficulties. This has prevented its utilization over larger areas (French et al., 1994).

According to some authors, however, the species with the most forage potential is *A. pintoi*, a endemic species in Brazil. *Arachis pintoi* produces high dry herbage yield with excellent quality, as well as high seed production (Valls and Simpson, 1994). The geographical distribution of the species spreads over an area that is part of the states of

Goiás, Bahia, and Minas Gerais, and extends to the Atlantic coast of Brazil, where the original accession of *Arachis pintoi* was collected (Figure 2-1).

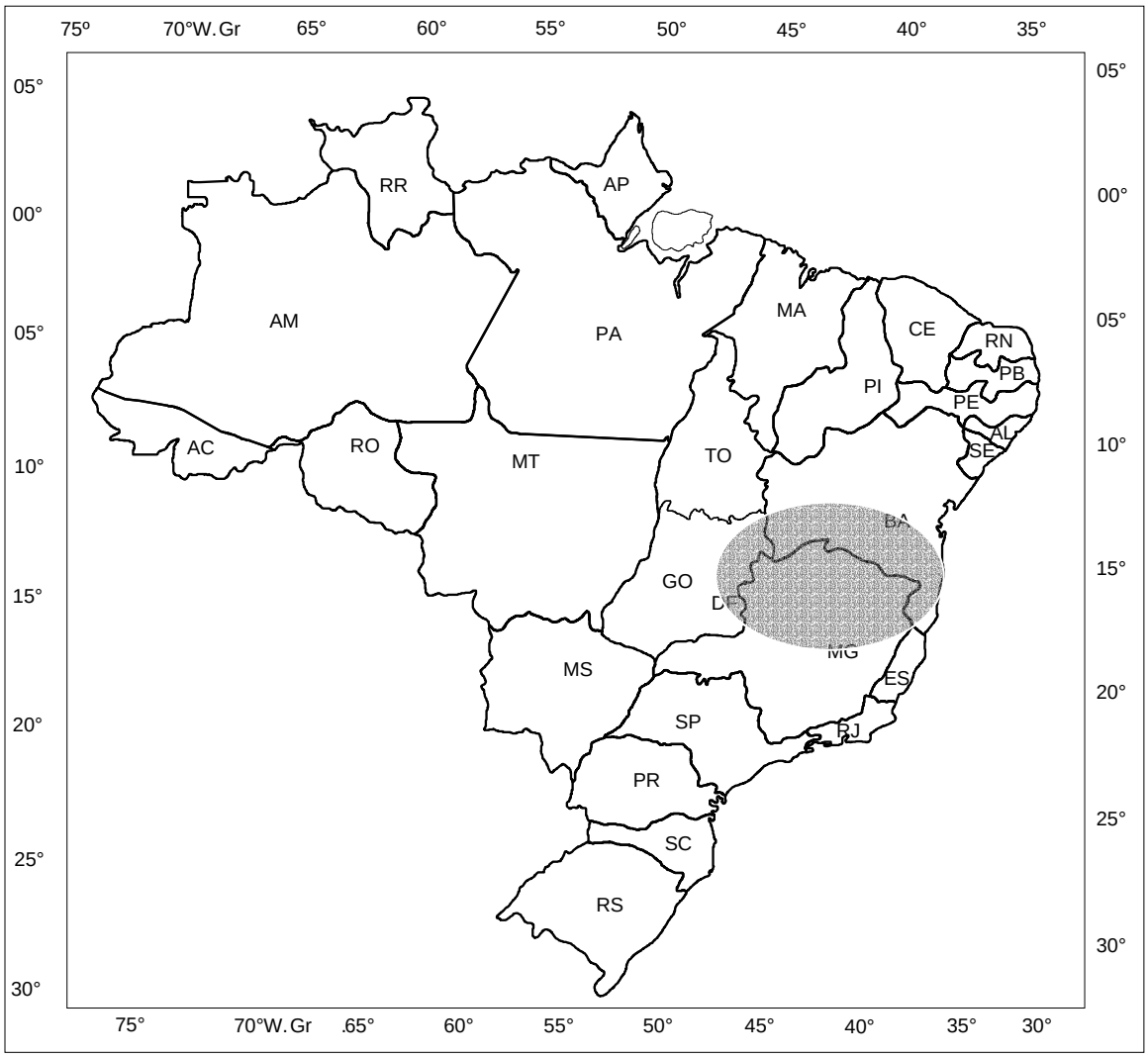

Figure 2-1. Area of natural occurrence of Arachis pintoi (Valls, J.F.M. and E.A. Pizarro. 1994. Collection of wild *Arachis* germplasm. p. 19-27. *In* P.C. Kerridge and B. Hardy (ed.). Biology and Agronomy of Forage *Arachis*, CIAT, Cali, Colombia).

Arachis pintoi

Botanical characteristics

A. pintoi is a herbaceous, perennial legume, with a low stoloniferous growth habit. Growth heights range from 20 to 40 cm, with axonomorfus roots, without enlargements (Figure 2-2). The first branch is erect with attached low branches, rooting at the nodes,

and stems are cylindrical, angular, and hollow (Krapovickas and Gregory, 1994). The leaves are alternate, compound, with four obovate leaflets (50 mm length x 32 mm width). Stipules have the basal portion attached to the petiole, and measure 10-15 mm length x 3 mm length with the free portion measuring 10-12 mm length x 2.5 mm length in the base (Krapovickas and Gregory, 1994).

A. pintoi shows indeterminate and continuous flowering. The inflorescence is axillary, in very short spikes, with four to five flowers, covered by the joined portion of the stipules. The flowers are sessile, protected by two bracts. The hypanthium is well developed, and can reach 10 cm in length, with silky hair. The calyx is bilabiate, with silky hairs. The corollas are yellow in the typical condition. Standard petals are 11 mm long x 13 mm wide, with yellow nerve and keel petals of 6-7 mm of length. Four oblong anthers and four spherical anthers are typically present. The species is considered normally self-pollinated (Krapovickas and Gregory, 1994). The flowers can be yellow, orange, cream and white (Valls, 1992).

The small fruit of *A. pintoi* are located underground in an articulated legume form, with each articulation classified as an indehiscent capsule, which usually contains a single seed (Cook et al., 1990). The pericarp is flat and resistant, covered with fine hairs that retain the soil. It presents two distinct segments, each one with a seed (Krapovickas and Gregory,1994). The number of chromosomes is 2n=2x=20 (Fernandez and Krapovickas, 1994).

Agronomic characteristics

A. pintoi grows well in tropical areas from sea level to heights of 1800 m, with 1500 to 3500 mm of annual rainfall (Rincón et al., 1992; Valls and Simpson, 1994).

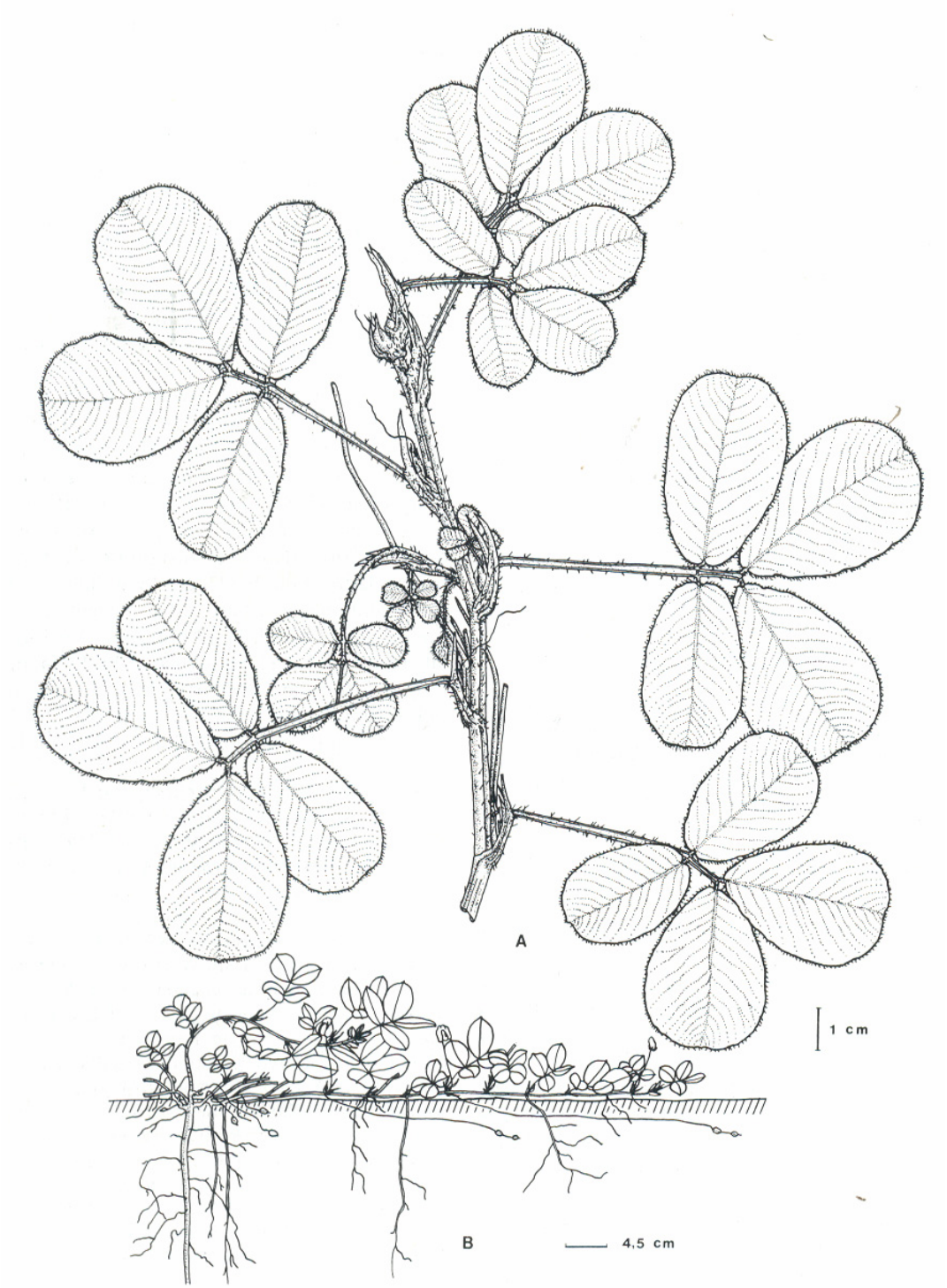

Figure 2-2. Arachis pintoi plant characteristics (Krapovickas, A. and W.C. Gregory. 1994. Taxonomy of the genus *Arachis* (Leguminosae). Bonplandia 8: 1-186)

According to Rao and Kerridge (1994), *A. pintoi* CIAT 17434 (PI 338447) exhibits good adaptation to acid soils, with optimal growth occurring at pH 5.4. It is also tolerant to

high soil Al concentrations (70%), and requires soil organic matter higher than 3% for normal growth. *A. pintoi* (PI 338447) grows in a variety of soil textures ranging from sandy to clay, although optimum growth occurs in sandy soils (Argel and Pizarro, 1992).

A. pintoi (PI 338447) is able to absorb P in soils with very low concentrations of this element, or in situations when relatively insoluble forms are applied. It has low response to N, Cu, Mo, Fe, S, and K (Rao and Kerridge, 1994). It is adapted to poorly-drained soils, but also grows well in well-drained soils having long periods without precipitation (Pizarro and Rincón, 1994).

Under defoliation, *A. pintoi* (PI 338447) has a good initial regrowth and high light interception capacity, with forage DMY, estimated between 30 and 40 d, of 0.08 Mg ha^{-1} day^{-1} (Fisher and Cruz, 1994). *A. pintoi* (PI 338447) grows better under shade than in full sun conditions. Plants under full sun presented lower leaf area and less above-ground biomass than counterparts maintained under 30, 50, and 70% shaded conditions. The below-ground biomass was not different in the same circumstances. This characteristic provides *A. pintoi* with the ability to be used as a ground cover in coffee (*Coffea Arabica* L.) and fruit groves (CIAT, 1991).

Arachis pintoi (PI 338447) nodulated with native *Bradyrhizobium* strains in Colombia, although the nodules were not active. Inoculation with selected strains was more efficient and demonstrated superior plant growth. Small doses of N fertilizer (50 kg ha^{-1}) increased the initial infection and sped the nodulation process. Rates of N fixation of *A. pintoi* ranged from 0.07 to 0.20 Mg ha^{-1} yr^{-1} (Thomas,1994).

Pizarro and Rincón (1994), reported that *A. pintoi* CIAT 17434 (PI 338447), 18748 (PI 604858), 18749 (PI 604859) and 18750 (PI 604815), growing in a subtropical

environment in Pelotas-RS, Brazil, were resistant to severe frosting. The authors stated that, although the plants presented freezing burning symptoms that affected their development, regrowth of herbage was achieved after the first rainfall.

Arachis pintoi germplasm characterization

In 1991, *Arachis pintoi* had a very narrow genetic base, approximately 30 accessions. Starting from that date, intense collection of materials of the species was initiated by various projects. The germplasm base was enlarged to more than 150 accessions (Valls et al., 1994).

The large number of accessions today available implies in a need for discrimination among them, because they can present different agronomic performance. Knowledge of the genetic variability of *A. pintoi* will be obtained through an appropriate characterization of the accessions. Characterization and evaluation of the wide range of *A. pintoi* germplasm should be carried out according to the priorities and strategies for handling the genetic resources of *Arachis* (Valls, 1988).

A. pintoi has been spread world wide by the international germplasm exchange network that consists of international centers under the International Plant Genetic Resources Institute (IPGRI) and the national germplasm centers. Despite this world wide distribution of *A. pintoi*, much of the evaluation research was conducted using only the original accession (PI 338447). As a result of these works, *A. pintoi* was released as a commercial cultivar in 9 countries, including Australia, USA, Costa Rica, Honduras, Colombia and recently in Brazil. Most of these released cultivars however represent accession PI 338447. Recently new cultivars have been released which originate from accessions other than PI 338447.

According to Pizarro and Carvalho (1996), CIAT distributed about 1.2 Mg of seeds of *A. pintoi* accession (PI 338447) to Europe, Africa, Asia, Southeast Asia and North, Central and South America. A total of 61 countries were supplied with seeds.

Even when more than one accession has been evaluated at a location, much of this characterization of germplasm has been with small numbers of accessions and based primarily on agronomic evaluation in different locations, with great emphasis on herbage production. Most of these works were conducted in South America, Central America, and Australia.

In South America, under the coordination of the International Center for Tropical Agriculture (CIAT), the International Tropical Pastures Evaluation Network (RIEPT) evaluated *A. pintoi* accession CIAT 17434 (PI 338447) in several places of Colombia, inside the savanna ecosystem. In that network, this accession presented poor adaptation when compared with other legumes. The DMY accumulated in 12 wk of regrowth, using the methodology of RIEPT (Toledo, 1982), varied from 0 to 0.47 Mg ha^{-1} during the rainy season, and from 0 to 0.25 Mg ha^{-1} in the dry season (Pizarro and Rincón, 1994).

In Brazil, Valentim (1994) evaluated the adaptation and forage production of accessions BRA-013251 (PI 338447) and BRA-015121 (PI 604858) in Rio Branco-AC (North). The results showed excellent adaptation of the accessions to the environmental conditions in Acre state. The average DMY of the accessions during 16 wk of growth in the rainy period of 1992 was 4.6 Mg ha^{-1} and 5.3 Mg ha^{-1} during the period of minimum precipitation. In 1993, the average production was of 6.1 Mg ha^{-1} in the rainy season and 4.2 Mg ha^{-1} in the period of minimum rainfall. In mixed pastures with *Paspalum* spp. in humid areas of low fertility in Campo Grande-MS, *A. pintoi* accession BRA-015598 (PI

604815) yielded 0.6 to 1.3 Mg ha^{-1} in the dry season and 0.2 to 0.5 Mg ha^{-1} during the rainy season (Fernandes et al., 1992).

Pizarro et al. (1996b) evaluating forage legumes mixed with *Brachiaria decumbens* Stapf. in Uberlândia-MG, obtained, with the accessions BRA-013251 (PI 338447), 015121 (PI 604858), 015598 (PI 604815) and 031143, dry matter production of 2.2, 0.6, 2.3 and 4.4 Mg ha^{-1}, respectively, in the period of maximum precipitation during 1994/1995. Data obtained in Planaltina-DF by EMBRAPA-CPAC, starting from 9 accessions in a lowland area, showed great variability of forage production of *A. pintoi* in those conditions. The total DMY varied from 5 to 13 Mg ha^{-1} in the first year of evaluation and from 3 to 11 Mg ha^{-1} in the second year (Pizarro et al., 1992; Pizarro et al., 1993).

In Veracruz, Mexico, *A. pintoi* CIAT 17434 (PI 338447), showed average DMY of 2.0, 1.2 and 0.8 Mg ha^{-1} in three cuttings of 12-wk regrowth during the rainy season, in two years of evaluation (Valles et al., 1992). In Guápiles, Costa Rica, the accessions CIAT 17434 (PI 338447), 18744 (PI 476132), 18747 (PI 497574), 18748 (PI 604858) and18751 of *A. pintoi* presented DMY of 4.1, 4.9, 4.0, 3.8 and 3.7 Mg ha^{-1}, respectively, in 2-yr of evaluation (Argel, 1994). In the USA, Kretschmer and co-workers have evaluated a number of seed-propagated accessions of wild species of *Arachis* for use on the poorly drained soils of South Florida and identified an *A. pintoi* accession (IRFL 4222) that was persistent under grazing (French et al., 1994).

In Australia, only three accessions were initially available for evaluation, CPI 58113 (PI 338447), CPI 28273 and CPI 93472, with the last two being considered mostly the same. CPI 58113 was evaluated in several locations and presented great adaptation

and persistence under grazing. Subsequently it was released as cultivar "Amarillo" in 1987, and since then it has gained increasing acceptance with producers as a forage crop (Cook et al., 1994). These authors reported that in 1991, 9 Mg of seed were produced and commercialized.

In seven locations in Central and West Africa, *A. pintoi* CIAT 17434 (PI 338447) showed production that varied from 0.6 to 3.2 Mg ha^{-1} in the rainy season and 0.1 to 2.0 Mg ha^{-1} during the dry season, confirming its wide adaptation (Stur & Ndikumana, 1994). The same authors reported yields ranging from 0.5 to 4.5 Mg ha^{-1} yr^{-1} for Amarillo, in evaluations conducted at three locations in the dry areas of Fiji.

Although the majority of the characterization work conducted with the germplasm of *A. pintoi* has been agronomically based, some examples of germplasm characterization at other levels can be found in the literature. Monçato (1997), working with approximately 45 accessions of *A. pintoi*, applied a series of morphological descriptors to describe the variability of this germplasm. The accessions showed great variability in morphological traits. Oliveira et al. (1999) demonstrated the morphological variability and inheritance of flower color. The yellow flower is dominant over the orange flowers.

Mass et al. (1993) used 60 morphological descriptors to characterize and demonstrate the variability of eight accessions. The accessions were organized into two groups representing the plant types, one homogeneous, and the other divided into four distinct subgroups. These results pointed to a lack of continuous patterns of variation in morphology and highlighted the need for further germplasm collection.

Paganella and Valls (2002) applied a list of 12 descriptors to evaluate seven cultivars and 13 accessions of *A. pintoi*. The objective of this work was to review the

origin of the cultivars and check inconsistencies in the literature about the germplasm accessions that gave rise to the commercial cultivars. The results confirm that five of seven cultivars derived from the original or first accession of the species collected (PI 338447).

Perennial *Arachis* germplasm has also been evaluated at the molecular DNA level. Genetic variation within and among accessions of the genus *Arachis* representing sections Extranervosae, Caulorrhizae, Heteranthae, and Triseminatae was evaluated using RFLP and RAPD markers. RAPD markers revealed a higher level of genetic diversity than RFLP markers, both within and among the species (Galgaro et al., 1998).

Gimenes et al. (2000) working with sixty-four accessions of section Caulorrhizae utilized RAPD analysis to characterize the genetic variation and the phylogenetic relationships. A total of 104 fragments of DNA of different sizes were generated, and 97 were polymorphic in the accessions tested. Despite the large number of polymorphic fragments detected, the mean number of unique genotypes detected by each RAPD primer was low. However, when data from all primers were considered together, all accessions were uniquely fingerprinted.

Genetic characterizations of accessions of *A. pintoi* using isoenzymes, RFLP and RAPD molecular markers were also done by Valente et al. (1998), Bertozo and Valls (1996), Bertozo & Valls (1997a), Bertozo & Valls (1997b), and Carvalho et al. (1998) with a small set of germplasm accessions, different from those used in this research.

CHAPTER 3
MOLECULAR CHARACTERIZATION OF *Arachis pintoi* GERMPLASM

Introduction

Until the late 1960s most genetics studies were associated with genes that controlled morphological features, which were in general easily identified. These features appropriately named morphological markers and were very important to the understanding of gene action and expression, and for the construction of the first genetic linkage maps. However, the low number of morphological markers linked to important agronomic traits made their use inefficient for the purpose of genetic improvement. Further, morphological markers were generally available for few species, which were used as model systems for genetic studies. For the great majority of crop species and their wild relatives few or no morphological markers were available (Ferreira and Grattapaglia, 1998).

By definition, a molecular marker is every single molecular phenotype expressed by a particular gene. The nucleotide sequence and function of the marker could be known or unknown. A molecular marker can only be considered a genetic marker after its mendelian segregation is observed in segregating populations (Ferreira and Grattapaglia, 1998).

Currently, several molecular biology techniques are available for the study of genetic variability at the DNA level. These techniques provide for the possibility of identification of innumerable molecular markers that theoretically could cover the whole genome of a species. These markers can be used for many different applications

including fingerprinting, genetic mapping, phylogenetic relationships, genetic diversity studies, and plant breeding.

Among all DNA-based markers, Random Amplified Polymorphic DNA (RAPD) has been the most popular with plant geneticists. Reiter (2001) revealed that in a recent bibliographic search there were more than 2,000 publications were in which RAPD markers had been used. According to the same author, the popularity of this marker arises from the fact that the method is very simple and the cost required for its application is low. RAPD markers have also been the marker of choice in several genetic studies with the common peanut (*Arachis hypogaea L.*) and its wild relatives.

Hilu and Stalker (1995) used RAPD markers to access genetic relationships between the common peanut and wild species of the section Arachis of the genus. These authors reported 132 polymorphic bands that were useful for separating species and accessions, and for evaluation of the genetic diversity presented by the germplasm.

Chang et al. (1999) accessed the genetic diversity of *A. hypogaea* cultivars released in Taiwan using RAPD markers. They were successful in estimating genetic distances among varieties and grouping them in accordance with their genetic similarities. The mean genetic distance among cultivars was 0.411, and they were classified into six groups.

RAPD markers have also been used to characterize wild species of *Arachis*. Nobile et al. (2004) studied the genetic variation within and among species of the section Rhizomatosaea of *Arachis* using RAPD markers. These authors reported that by using 110 polymorphic RAPD bands they were able to describe the genetic diversity and draw a dendogram displaying the similarities among accessions and species of this section.

Another very important technique in genetic resources research is tissue culture. The use of *in vitro* techniques is particularly critical to species with vegetative propagation and recalcitrant seeds. Other potential applications of tissue culture techniques are: micropropagation, long-term storage, germplasm exchange, embryo culture of interspecific hybrids, induction of mutations, production of synthetic seeds, and genetic transformation. Another advantage of plants originated *in vitro* is that they are generally free of pathogens and diseases.

Although *in vitro* techniques are useful for genetic resources conservation, attention must be given to the genetic stability of the systems used in this process. Tissue culture-induced genetic variation or somaclonal variation is defined as the variation that arises during the period of dedifferentiated cell proliferation that takes place between the explant culture and recovering of regenerated plants. Such genetic variation has been observed among regenerants of several species and they usually present mutations that include: chromosome breakage, changes in ploidy number, single base changes, changes in copy numbers of repeated sequences, increased transposon mutagenegis, sister chromatid exchange and alteration in DNA methylation (Hawkes et al., 2000).

Because *in vitro* techniques have a wide spectrum of applications to genetic resources conservation and plant breeding, the development of protocols optimized to a particular species should have high priority. Several factors may affect the efficiency of the process and should be considered when research is conducted in this field. Genotypes, source and age of explant, hormone concentration in the medium, and day length are some factors that affect the success of regeneration of plants from callus tissue culture (Flick et al., 1983).

Tissue culture regeneration protocols for *A. pintoi* were proposed by Rey et al. (2000) based on a single genotype, and Ngo and Quesenberry (2000) based on a small number of accessions. In terms of genetic resources it is very critical to evaluate the efficiency of protocols in terms of plant regeneration among different genotypes and also the preservation of the genetic characteristics of the germplasm regenerated or stored with these protocols. Additionally, in terms of plant breeding, it is important to assess the genetic diversity of the germplasm with respect to their plant regeneration abilities. It is also important to estimate the capacity of the protocols to generate somaclonal variations, which ultimately could generate mutations and gene diversity.

The objectives of this research were:

- To characterize the *Arachis pintoi* germplasm accessions stored at the USDA-NPGS germplasm banks using RAPD markers.
- To evaluate the organogenic regeneration ability of these *A. pintoi* accessions with two tissue culture protocols.
- To study the variation in RAPD band profile of plants regenerated compared to the parent plants.

Materials and Methods

Accessions of *Arachis pintoi* stored in the Southern Regional PI Station of the National Plant Germplasm System (NPGS) located at Griffin-GA were transferred to the University of Florida in 2001 and 2002 (Appendix A) A subset composed of 35 accessions was used to study the genetic diversity of this collection at the molecular level using RAPD markers.

In addition to the RAPD characterization of the parent plants, 25 out of 35 accessions were selected randomly and evaluated for their organogenesis ability using two tissue culture protocols. Extent of generated somaclonal variation was accessed by

comparing the RAPD band profile of the regenerated tissue culture plants to the parent plants.

Parent Plants

DNA was extracted from leaves of single plants stored at the Agronomy Department greenhouse using a modified CTAB protocol first proposed by Rogers and Bendich (1985). In this protocol, 0.1 g of ground tissue was mixed with 400 µl of 2x CTAB extraction buffer in a 2.5 ml eppendorf tube and incubated in a 65ºC water bath for 60 min. After that 400 µl of a chloroform:isoamyl alcohol (24:1) was added and the mixture was centrifuged for 5 min. The supernatant was then transferred to a new tube where 1/10 volume of 10% CTAB was combined, and 400 µl of a chloroform:isoamyl alcohol (24:1) was added again. The solution was centrifuged once more, and the supernatant was transferred to a new tube where an equal volume of CTAB precipitation buffer was added. The mixture was then centrifuged, the supernatant was removed and the DNA pellet was rehydrated in 100 µl of high salt TE buffer resting in a water bath. Ten minutes latter the DNA was reprecipitated with 0.6 volumes of isopropanol and centrifuged for 15 min at 10,000 rpm. Finally the pellet was washed with 80% ethanol, dried, and resuspended in 50 µl of DDW (Appendix B).

DNA concentrations were analyzed by measuring absorbance at 260 nm, and DNA quality was determined by spectrophotometer readings of the ratio of 260/280 nm. Working solutions were prepared by diluting the DNA stock solutions with DDW and standardized to concentrations of 25 *n*g of DNA per µl.

Eighteen primers of ten nucleotides length from the Operon Technologies kit (Table 3-1) were used to amplify regions of genomic DNA under thermocycling conditions proposed by Gimenes et al. (2000). Thermocycling conditions were: 40 Cycles

of 92ºC/1 min, 35ºC/1 min, and 72ºC/2 min. Twenty-five µl of PCR reaction mixtures were prepared by adding 17.3 µl ddH2O, 2.5 µl PCR Buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM mg $MgCl_2$, and 0.1% gelatin), 1.0 µl MgCl, 1.0 µl of 2.5mM dNTP solution, 1.0 µl Primer, 0.2 µl *Taq* polymerase (5 units/µl), and 2.0 µl DNA solution.

Amplification products were separated by electrophoresis on 1.5% agarose gels, and the 1kb DNA ladder (Promega Coorporation) was used as a standard. Banding patterns were visualized by staining the gels in ethidium bromide solutions and viewing under UV radiation. Images were captured with Quantity One quantification software version 4.1.1 from BIO-RAD Laboratories and then bands were scored.

For each combination of accession and primer, five PCR reactions were prepared. Only bands present in at least three out of five gels were considered. RAPD bands were scored as presence (1) or absence (0) of homolog bands for all accessions and a phenotypic binary matrix was produced. This matrix was used to perform genetic analysis using the software POPGENE version 1.32 (http://www.ualberta.ca/~fyeh/index.htm). Allele frequency, number of polymorphic loci, Nei's genetic distance (Nei, 1972), Nei's genetic diversity index (Nei, 1973), and Shannon-Weaver's genetic diversity index (Shannon and Weaver, 1949) were the parameters calculated. Genetic distance (D= -ln I) was later used as a criterion for differentiation among accessions to prepare a cluster analysis.

Tissue Culture Regenerated Plants

Two protocols were used to access their organogenesis ability, and capacity to generate somaclonal variation. Protocol 1 was proposed by Rey et al. (2000) and Protocol 2 proposed by Ngo and Quesenberry (2000).

Table 3-1. List of Operon Technologies* primers used to amplify *Arachis pintoi* DNA regions

Primer number	Nucleotide sequence
A4	5'-AATCGGGCTG-3'
A10	5'-GTGATCGCAG-3'
A12	5'-TCGGCGATAG-3'
A15	5'-TTCCGAACCC-3'
B4	5'-GGACTGGAGT-3'
B5	5'-TGCGCCCTTC-3'
B10	5'-CTGCTGGGAC-3'
B16	5'-TTTGCCCGGA-3'
C2	5'-GTGAGGCGTC-3'
C4	5'-CCGCATCTAC-3'
D4	5'-AATCGGGCTG-3'
D13	5'-GGGGTGACGA-3'
E4	5'-GTGACATGCC-3'
E5	5'-TCAGGGAGGT-3'
E8	5'-AATCGGGCTG-3'
E15	5'-ACGCACAACC-3'
G5	5'-CTGAGACGGA-3'
G15	5'-ACTGGGACTC-3'

* Operon Technologies, Alameda, CA, USA

To compare these two protocols, callus rating and weight and number of regenerated plants were used. The experiment was conducted using a completely randomized design with 50 treatments and 3 replications. Quantity of callus produced was rated based on a 1 to 5 scale, where 1 = no callus growth, and 5 = largest amount of callus (Figure 3-1). In addition, callus weight was measured with a digital scale placed in the laminar flow hood to prevent contamination.

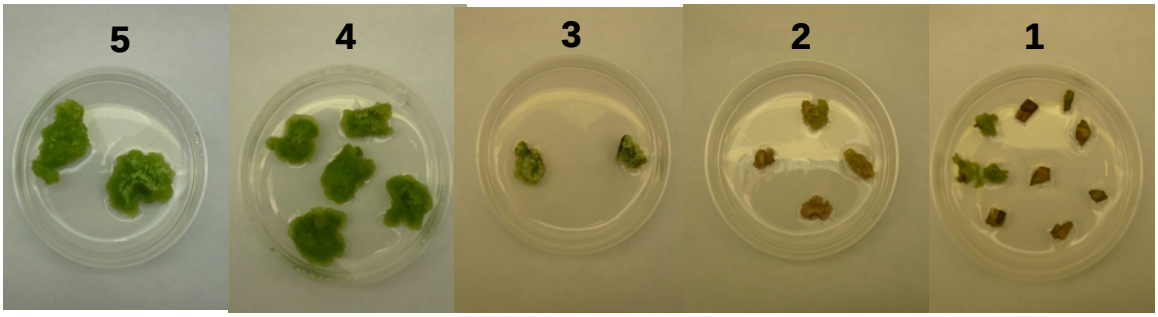

Figure 3-1. Rating scale applied to callus pieces of *Arachis pintoi* explants incubated in two different protocols.

Leaflet pieces from adult plants were the tissue source for callus initiation. Leaflets were surface sterilized by immersion in 70% ethanol for 1 to 2 min, followed by immersion in a solution of commercial bleach (0.9% sodium hypochlorite, final concentration) plus one drop of Tween® for 1 to 2 min. Leaflets were then washed three times with autoclaved distilled water. Circles of approximately 19.4 mm² area of the median portion of the laminae were cut and placed with the abaxial side down on the media in 60 mm x 15 mm petri dishes (Figure 3-2).

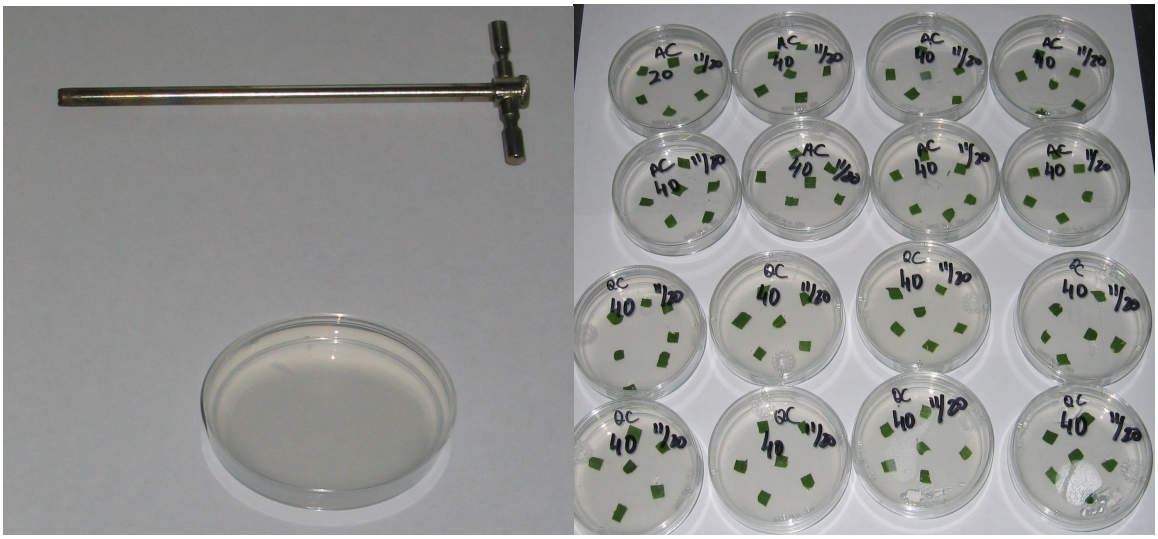

Figure 3-2. Leaflet cutter and callus induction dishes of Protocol 1 and 2 of *Arachis pintoi* accessions.

Protocol 1

The callus induction medium consisted of major and minor salts, as well as vitamins according to Murashige and Skoog (1962), with 3% sucrose, 0.7% agar, and 10 mg L^{-1} NAA (1-naphthaleneacetic acid) + 1 mg L^{-1} BA (6-benzylaminopurine). The pH of the medium was adjusted to 5.7 with 0.1 N KOH or HCl prior the addition of agar. To induce shoots, small pieces of callus (30 mg fresh wt.) were transferred to fresh medium composed of MS basal salts and vitamins+1g L^{-1} BA. For root induction, the regenerated

shoots were transferred to a medium composed of MS basal salts and vitamins plus 0.01mg L^{-1} NAA (Table 3-2).

Plantlets resulting from rooted shoots were rinsed gently under running tap water to remove adhering cultured media and immediately planted in pots containing potting mix. These plants were acclimatized in a humidity box and then placed in the greenhouse.

Protocol 2

The MS basal salts and vitamins medium (Murashige and Skoog, 1962) was the base for all media used in this protocol. For callus induction the MQC medium, which is a modification of the MS medium proposed by Wofford et al. (1992) for tropical legumes, was the medium of choice. In this medium a 2:1 auxin/cytokinin ratio was employed, with a final concentration of 2 mg L^{-1} of IAA (indole-3-acetic acid) and 1 mg L^{-1} of kinetin. The pH was adjusted to 5.8 prior to autoclaving.

For shoot development, medium IBA which was composed of MS basal salts and vitamins plus 0.1 mg L^{-1} of IBA (3-Indol butyric acid) was employed. For root induction, the regenerated shoots were transferred to medium MS basal salts and vitamins with growth regulators.

Plantlets resulting from rooted shoots were rinsed gently under running tap water to remove adhering cultured media and immediately planted in pots containing potting mix. These plants were acclimatized and then placed in the greenhouse.

Table 3-2. Tissue culture protocols used to regenerate *Arachis pintoi* plants

Protocol	Callus induction Media	Shoot development Media	Root induction Media
1	MS†	MS+1g L^{-1} BA	MS+0.01mg L^{-1} NAA
2	MQC††	MS+0.1mg L^{-1} IBA	MSNH†††

† MS + 3% sucrose, 0.7% agar, and 10 mg L^{-1} NAA + 1 mg L^{-1} BA
†† MS + 3% sucrose, 0.7% agar, and 2 mg L^{-1} of IAA and 1 mg L^{-1} of kinetin
††† MS basal salts and vitamins and no hormones

DNA was extracted from regenerated plants in accordance with the protocol described earlier. PCR reactions and band separation and scoring were also identical to that used for the parent plants. RAPD band profile of the regenerated tissue culture plants was compared to the parent plants.

Result and Discussions

Parent Plants

Of the 18 primers evaluated, eight were able to generate reproducible and reliable bands. Primers A4, B4, B5, C2, D4, D13, E4, and G5 amplified 100 different bands. The number and size of amplified bands for each primer is shown in Table 3-3.

Table 3-3. List of Operon primers, number and size of amplified bands, and number of bands accession-specific of *Arachis pintoi* germplasm genomic DNA

Primer number	Number of amplified bands	Size of amplified bands (bp)	Number of bands accession-specific
A4	15	250, 550, 670, 750, 870, 1000, 1100, 1350, 1500, 1700, 1900, 2100, 2500, 3000, 3100	0
B4	12	700, 850, 900, 1000, 1200, 1400, 1600, 1750, 2000, 2300, 2700, 3000	1
B5	11	500,750, 1000, 1100, 1250, 1400, 1700, 2000, 2500, 3000, 3500	3
C2	15	465, 520, 600, 700, 750, 850, 1000, 1300, 1500, 1750, 2000, 2500, 2750, 3000, 3500	2
D4	08	580, 650, 750, 910, 1200, 1350, 1500, 2000	1
D13	16	370, 500, 650, 750, 850, 1000, 1200, 1400, 1500, 1700, 2000, 2100, 2300, 2500, 2800, 3310	2
E4	10	600, 900, 1000, 1100, 1250, 1500, 1800, 2100, 2500, 2800	1
G5	13	350, 450, 500, 650, 750, 850, 1000, 1100, 1250, 1350, 1750, 2000, 2500, 2750	1

The average number of amplified bands per primer was 12.5, with primer D4 amplifying eight fragments and primer D13 amplifying 16 fragments. The size of these 100 fragments ranged from 250 bp to 3500 bp. From the 100 bands amplified, 98

presented polymorphism with the only two exceptions being Primer C2-1000 bp and primer E4-1250 bp. The average presence of bands per accession was 32, ranging from 20 to 44 bands.

The observed number of amplified bands was significantly variable for each primer and accession analyzed. Primer A4 amplified 15 different bands with average bands per accession of 5.4 and a range of 2 to 10 bands. Primer B4 amplified 12 fragments with average bands per accession of 4.3 and a range of 1 to 10. Primer B5 amplified 11 bands with an average band per accession of 3.5 and range of 1 to 7. Primer C2 amplified 15 bands and average bands per accession of 5.2 and a range of 1 to 10. Primer D4 amplified only 8 bands and the average bands per accession was 1.7 with a range of 1 to 4. Primer D13 displayed 16 bands with average bands per accession of 5.2 and a range of 2 to 11. Primer E4 showed 10 different fragments with an average of 4 bands per accession and a range of 2 to 8. Finally, primer G5 presented 13 bands with average bands per accession of 3 and a range of 1 to 7 bands.

Ten bands were unique to an individual germplasm accession. Accession PI 604856 was discriminated by band C2-465bp, accession PI 604858 by band E4-600bp, accession PI 604810 by band C2-520bp, accession PI 604799 band B5-1250bp, PI 604809 by band B5-1100bp, accession PI 604807 by band G5-1750bp, accession CIAT 22256 by D4-1200bp, accession CIAT 22159 by band D13-3310bp, CIAT 22152 by band B4-700bp, accession CIAT 22265 by band D13-2800bp, and finally accession CIAT 22260 by band B5-500bp. Table 3-4 summarize the results obtained with RAPD markers among the 35 germplasm accessions of *A. pintoi* analyzed in this research. Figure 3-3 displays stained eletrophoresis gels of primer A4 and 24 accessions.

Table 3-4. Characteristics of RAPD patterns of the 35 *Arachis pintoi* germplasm accessions

Total number of screened primers	18
Number of polymorphic primers	08
Total number of bands amplified	100
Size of the amplified bands	250 bp – 3500 bp
Minimum and maximum number of bands per primer	08 (D4) – 16 (D13)
Average bands per primer	12.5
Total number of polymorphic bands	98
Total number of monomorphic bands	02
Average number of bands per accession	32
Minimum and maximum number of bands per accession	20 – 44
Number of accession-specific bands	10

According to Nei (1973), when a large number of loci are examined to evaluate the genetic variability of a population, the amount of variation is measured by the proportion of polymorphic loci and average heterozygosity per locus. Also according to Nei a locus is called polymorphic when the frequency of the most common allele (x_i) is equal to or less than 0.95, in cases where the sample size is smaller than 50.

In Table 3-5, information about the gene frequency of each allele at every RAPD locus is presented. Great variability in gene frequency was observed for each different RAPD locus. Using the criterion described above to characterize polymorphic loci we can observe that 11 loci presented the frequency of the most common allele higher than 0.95, and then were classified as monomorphic. Therefore, we can conclude that 89 out of 100 loci, or 89% of the RAPD loci, was the proportion of polymorphic loci. Although the proportion of polymorphic loci is a good estimation of genetic variability, a more precise and appropriate measure of gene diversity is obtained by the use of the gene diversity statistics (Nei, 1987).

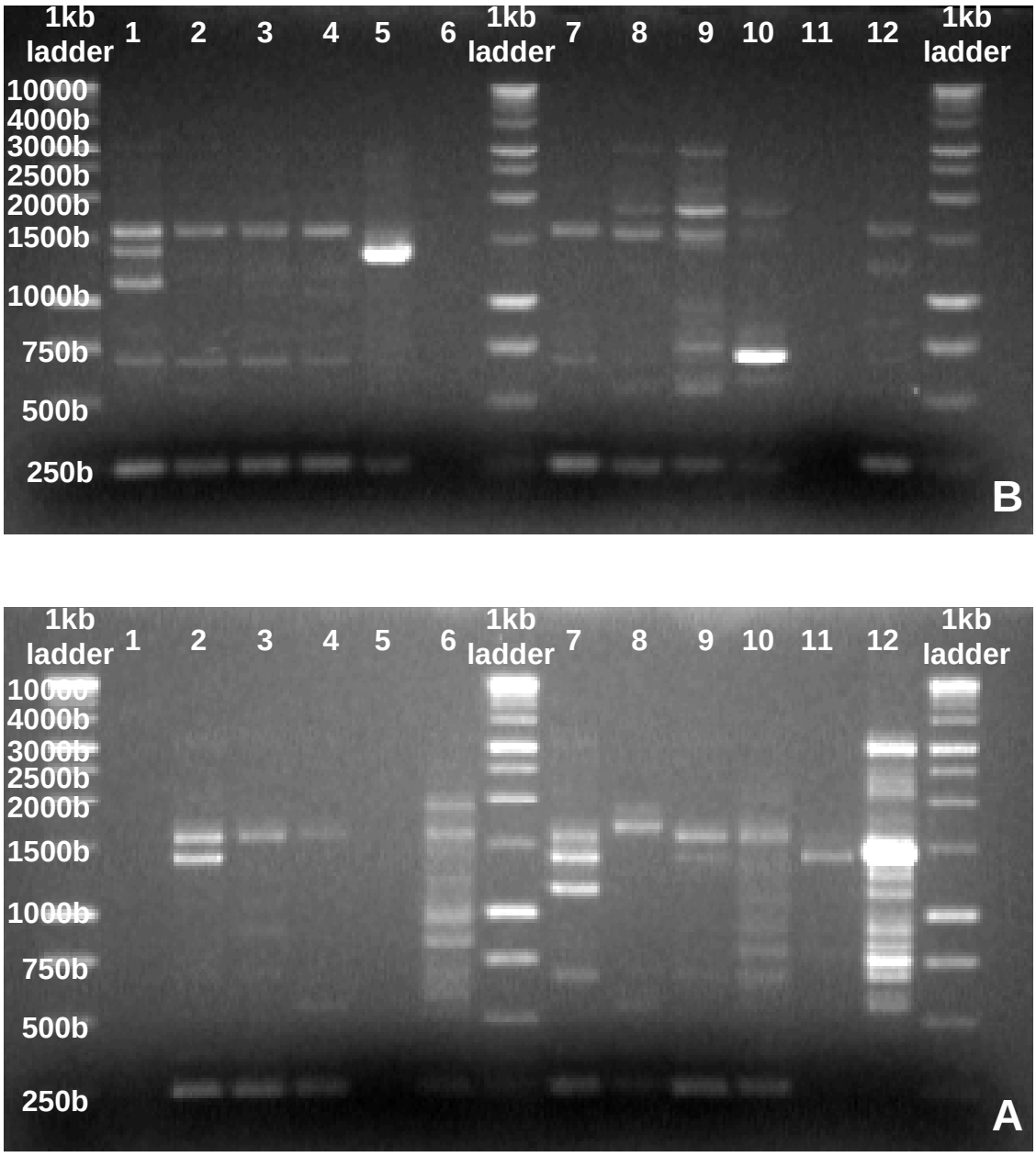

Figure 3-3. RAPD band profile of 24 accessions of *Arachis pintoi* amplified by primer A4 (Operon Technologies).

A. lane 1-PI604856, 2-PI604857, 3-PI604858, 4-PI604798, 5-PI604803, 6-PI604805, 7-PI604812, 8-PI604810, 9-PI604811,10-PI604799, 11-PI604800, 12-PI604809.

B. lane 1-PI604817, 2-PI604815, 3-PI604814, 4-PI497541, 5-PI604813, 6-PI604801, 7-PI604804, 8-PI604808, 9-PI604807, 10-PI476132, 11-PI497574, 12-PI604859

Table 3-5. Gene frequency of the 100 RAPD locus of *A. pintoi* germplasm accessions

Allele	Loci/Gene Frequency						
	A4-250	A4-550	A4-670	A4-750	A4-870	A4-1000	A4-1100
0	0.4722	0.8611	0.6667	0.7222	0.5278	0.8056	0.5833
1	0.5278	0.1389	0.3333	0.2778	0.4722	0.1944	0.4167
	A4-1350	A4-1500	A4-1700	A4-1900	A4-2100	A4-2500	A4-3000
0	0.3333	0.3889	0.6111	0.6667	0.8611	0.6667	0.7222
1	0.6667	0.6111	0.3889	0.3333	0.1389	0.3333	0.2778
	A4-3100	B4-700	B4-850	B4-900	B4-1000	B4-1200	B4-1400
0	0.6667	0.0278	0.6944	0.8889	0.7500	0.5000	0.6944
1	0.3333	0.9722	0.3056	0.1111	0.2500	0.5000	0.3056
	B4-1600	B4-1750	B4-2000	B4-2300	B4-2700	B4-3000	B5-500
0	0.8333	0.8889	0.3889	0.7500	0.5833	0.7500	0.9722
1	0.1667	0.1111	0.6111	0.2500	0.4167	0.2500	0.0278
	B5-750	B5-1000	B5-1100	B5-1250	B5-1400	B5-1700	B5-2000
0	0.9444	0.5556	0.9722	0.0278	0.9444	0.8056	0.6667
1	0.0556	0.4444	0.0278	0.9722	0.0556	0.1944	0.3333
	B5-2500	B5-3000	B5-3500	C2-465	C2-520	C2-600	C2-700
0	0.2500	0.4444	0.9444	0.9722	0.9722	0.8333	0.8056
1	0.7500	0.5556	0.0556	0.0278	0.0278	0.1667	0.1944
	C2-750	C2-850	C2-1000	C2-1300	C2-1500	C2-1750	C2-2000
0	0.9444	0.4167	0.0000	0.4444	0.2778	0.6389	0.7500
1	0.0556	0.5833	1.0000	0.5556	0.7222	0.3611	0.2500
	C2-2500	C2-2750	C2-3000	C2-3500	D4-580	D4-650	D4-750
0	0.6944	0.7222	0.5000	0.8611	0.8889	0.9444	0.8333
1	0.3056	0.2778	0.5000	0.1389	0.1111	0.0556	0.1667
	D4-910	D4-1200	D4-1350	D4-1500	D4-2000	D13-370	D13-500
0	0.0833	0.9722	0.9444	0.9444	0.6944	0.7222	0.0833
1	0.9167	0.0278	0.0556	0.0556	0.3056	0.2778	0.9167
	D13-650	D13-750	D13-850	D13-1000	D13-1200	D13-1400	D13-1500
0	0.2500	0.3611	0.7500	0.6667	0.2778	0.8056	0.8333
1	0.7500	0.6389	0.2500	0.3333	0.7222	0.1944	0.1667
	D13-1700	D13-2000	D13-2100	D13-2300	D13-2500	D13-2800	D13-3310
0	0.5556	0.8056	0.9167	0.9167	0.9444	0.9722	0.9722
1	0.4444	0.1944	0.0833	0.0833	0.0556	0.0278	0.0278
	E4-600	E4-900	E4-1000	E4-1100	E4-1250	E4-1500	E4-1800
0	0.9722	0.9444	0.8889	0.0833	0.0000	0.2222	0.7500
1	0.0278	0.0556	0.1111	0.9167	1.0000	0.7778	0.2500
	E4-2100	E4-2500	E4-2800	G5-350	G5-450	G5-500	G5-650
0	0.5278	0.6944	0.9444	0.9444	0.7778	0.8889	0.6944
1	0.4722	0.3056	0.0556	0.0556	0.2222	0.1111	0.3056
	G5-750	G5-850	G5-1000	G5-1100	G5-1250	G5-1350	G5-1750
0	0.7778	0.8611	0.6944	0.6667	0.2778	0.7500	0.9722
1	0.2222	0.1389	0.3056	0.3333	0.7222	0.2500	0.0278
	G5-2500	G5-2750					
0	0.9167	0.8056					
1	0.0833	0.1944					

Genetic diversity was estimated by Nei's gene diversity and by the Shannon-Weaver diversity index (Shannon and Weaver, 1949). Nei's gene diversity is defined as $h = 1-\Sigma x_i^2$, where x_i is the frequency of i^{th} allele.

Additionally, Shannon-Wiever's diversity index is defined as: $H = -\Sigma$ (pi log pi)/log pi, where I = 1 to n, and p is the proportion of the total genotypes made up to the i^{th} genotype. In both indices values close to one indicate high genetic diversity.

Values of h and H among the 100 RAPD loci were extremely variable; with some loci presenting numbers close to one and others small numbers close to zero (Table 3-6). In general, H (Shannon-Wiever index) values were higher than the ones presented by h (Nei index). To estimate the genetic diversity of the whole set of germplasm, the average of both indices was calculated and was named total genetic diversity. Total h was estimated as 0.29 ± 0.16, and total H was estimated as 0.45 ± 0.20. Both values can be considered high, which demonstrates the great genetic variability contained in this set of germplasm.

Another measure of genetic diversity is obtained by the genetic distance (D) statistic. According to Nei (1972) genetic distance is calculated using the following formula: $D = -\log_e I$, where $I = Jxy/(JxJy)^{1/2}$, Jxy is the number of bands in common among accessions x and y, and Jx and Jy is the number of bands of accessions x and y, respectively. I values 1 when two accessions or populations have identical gene frequencies over all loci examined, and zero when they share no alleles. Here genetic distances were calculated between every pair of accessions and then the average genetic distance was estimated as 0.36. This value also indicates that a great genetic diversity exits among the germplasm evaluated in this research.

Table 3-6. Nei's gene diversity and by Shannon-Weaver diversity index of RAPD loci

Index	Locus/Genetic Diversity						
	A4-250	A4-550	A4-670	A4-750	A4-870	A4-1000	A4-1100
h	0.50	0.24	0.44	0.40	0.50	0.31	0.49
H	0.69	0.40	0.64	0.59	0.69	0.49	0.68
	A4-1350	A4-1500	A4-1700	A4-1900	A4-2100	A4-2500	A4-3000
h	0.44	0.48	0.48	0.44	0.24	0.44	0.40
H	0.64	0.67	0.67	0.64	0.40	0.64	0.59
	A4-3100	B4-700	B4-850	B4-900	B4-1000	B4-1200	B4-1400
h	0.44	0.05	0.42	0.20	0.38	0.50	0.42
H	0.64	0.13	0.62	0.35	0.56	0.69	0.62
	B4-1600	B4-1750	B4-2000	B4-2300	B4-2700	B4-3000	B5-500
h	0.28	0.20	0.48	0.38	0.49	0.38	0.05
H	0.45	0.35	0.67	0.56	0.68	0.56	0.13
	B5-750	B5-1000	B5-1100	B5-1250	B5-1400	B5-1700	B5-2000
h	0.10	0.49	0.05	0.05	0.10	0.31	0.44
H	0.21	0.69	0.13	0.13	0.21	0.49	0.64
	B5-2500	B5-3000	B5-3500	C2-465	C2-520	C2-600	C2-700
h	0.38	0.49	0.10	0.05	0.05	0.28	0.31
H	0.56	0.69	0.21	0.13	0.13	0.45	0.49
	C2-750	C2-850	C2-1000	C2-1300	C2-1500	C2-1750	C2-2000
h	0.10	0.49	0.00	0.49	0.40	0.46	0.38
H	0.21	0.68	0.00	0.69	0.59	0.65	0.56
	C2-2500	C2-2750	C2-3000	C2-3500	D4-580	D4-650	D4-750
h	0.42	0.40	0.50	0.24	0.20	0.10	0.28
H	0.62	0.59	0.69	0.40	0.35	0.21	0.45
	D4-910	D4-1200	D4-1350	D4-1500	D4-2000	D13-370	D13-500
h	0.15	0.05	0.10	0.10	0.42	0.40	0.15
H	0.29	0.13	0.21	0.21	0.62	0.59	0.29
	D13-650	D13-750	D13-850	D13-1000	D13-1200	D13-1400	D13-1500
h	0.38	0.46	0.38	0.44	0.40	0.31	0.28
H	0.56	0.65	0.56	0.64	0.59	0.49	0.45
	D13-1700	D13-2000	D13-2100	D13-2300	D13-2500	D13-2800	D13-3310
h	0.49	0.31	0.15	0.15	0.10	0.05	0.05
H	0.69	0.49	0.29	0.29	0.21	0.13	0.13
	E4-600	E4-900	E4-1000	E4-1100	E4-1250	E4-1500	E4-1800
h	0.05	0.10	0.20	0.15	0.00	0.35	0.38
H	0.13	0.21	0.35	0.29	0.00	0.53	0.56
	E4-2100	E4-2500	E4-2800	G5-350	G5-450	G5-500	G5-650
h	0.50	0.42	0.10	0.10	0.35	0.20	0.42
H	0.69	0.62	0.21	0.21	0.53	0.35	0.62
	G5-750	G5-850	G5-1000	G5-1100	G5-1250	G5-1350	G5-1750
h	0.35	0.24	0.42	0.44	0.40	0.38	0.05
H	0.53	0.40	0.62	0.64	0.59	0.56	0.13
	G5-2500	G5-2750					
h	0.15	0.31					
H	0.29	0.49					

Results obtained in this research are equivalent to thoses reported in the literature for *A. pintoi* with a different set of germplasm accessions and primers. Gimenes et al. (2000) used RAPD markers to evaluate the genetic variation of the *A. pintoi* Brazilian germplasm collection and obtained a total of 104 different bands resolved by ten primers. Average number of bands per primer was 10.4, ranging from 7 to 15. The proportion of polymorphic loci was 90%, and accessions were grouped in 3 different groups based on their genetic distances.

Bertozo et al. (1997), also working with a small germplasm collection of *A. pintoi,* reported 220 amplified bands resolved by 22 primers. Average number of bands per primer was 7.5, ranging from three to 14 bands. The proportion of polymorphic loci observed was 48.5%. The greatest genetic variability was observed within accessions (0.53), while genetic variability among accessions was reported as 0.39.

Nobile et al. (2004) working with germplasm of *A. glabrata* (58), *A. burkartii* (12), *A. nitida* (10), *A. pseudovillosa* (2), and *A. lignosa* (1) stated that 110 polymorphic RAPD bands were resolved for 10 different primers. They also presented average genetic distances of 0.30, 0.38, and 0.38, respectively to *A. glabrata*, *A. nitida*, and *A. burkartii*.

Hilu and Stalker (1995) worked with 26 accessions of wild species and domesticated peanut, and reported that 10 primers resolved 132 RAPD bands. The most variation was observed among accessions of *A. cardenasii* and *A. glandulifera*, and the least was observed in *A. hypogaea* and *A. monticola*.

Findings in this research were also compared to results obtained with RAPD markers to other species. Renu (2003) stated that 147 bands were determined by 15 primers when 47 germplasm lines of *Pisum sativum* L. were used to access the genetic

variability of the species. The proportion of polymorphic loci obtained was 87%. The author concluded that RAPD markers are quick, easy to use and refractory to many environmental influences, which makes the technique a very important complement to traditional methods of germplasm characterization.

Lowe et al. (2003) studied 56 germplasm accessions of *Pennisetum purpureum* using 67 RAPD bands. They concluded that the genetic diversity across all accessions was high based on the Shannon-Weaver diversity index, which they estimated as 0.31.

The next step in this research was the grouping of the accessions based on their genetic distances. In Figure 3-4 the dendogram constructed for the 35 *A. pintoi* accessions is presented. Four groups were formed according to this dendogram. Group 1 was formed by accessions PI 604798, 604801, 604804, 604805, 604808, 604809, 604814, 604815, 604817, 604856, and 604857. Group 2 was formed by accessions PI 497541, 604800, 604812, 604858, 604859, and CIAT 18745, 20826, 22150, 22152, 22256, 22260, and 22265. Group 3 was formed by accessions PI 476132, 497574, 604803, 604807, 604810, 604811, 604813, and CIAT 22159, 22234, 22271. Finally, accession PI 604799 was grouped by itself in group 4.

Tissue Culture Regenerated Plants

Callus induction was achieved by both protocols from most of the *A. pintoi* accessions evaluated. Callus quantity ratings ranged from 1 to 5, with the most of the accessions having a mean rating of ≥ 3 on at least one protocol.

There were highly significant ($P \leq 0.001$) effects of protocols, accessions, and the interaction of protocols by accessions (Table 3-7). Protocol 1 was superior to Protocol 2 for both variables related with callus growth.

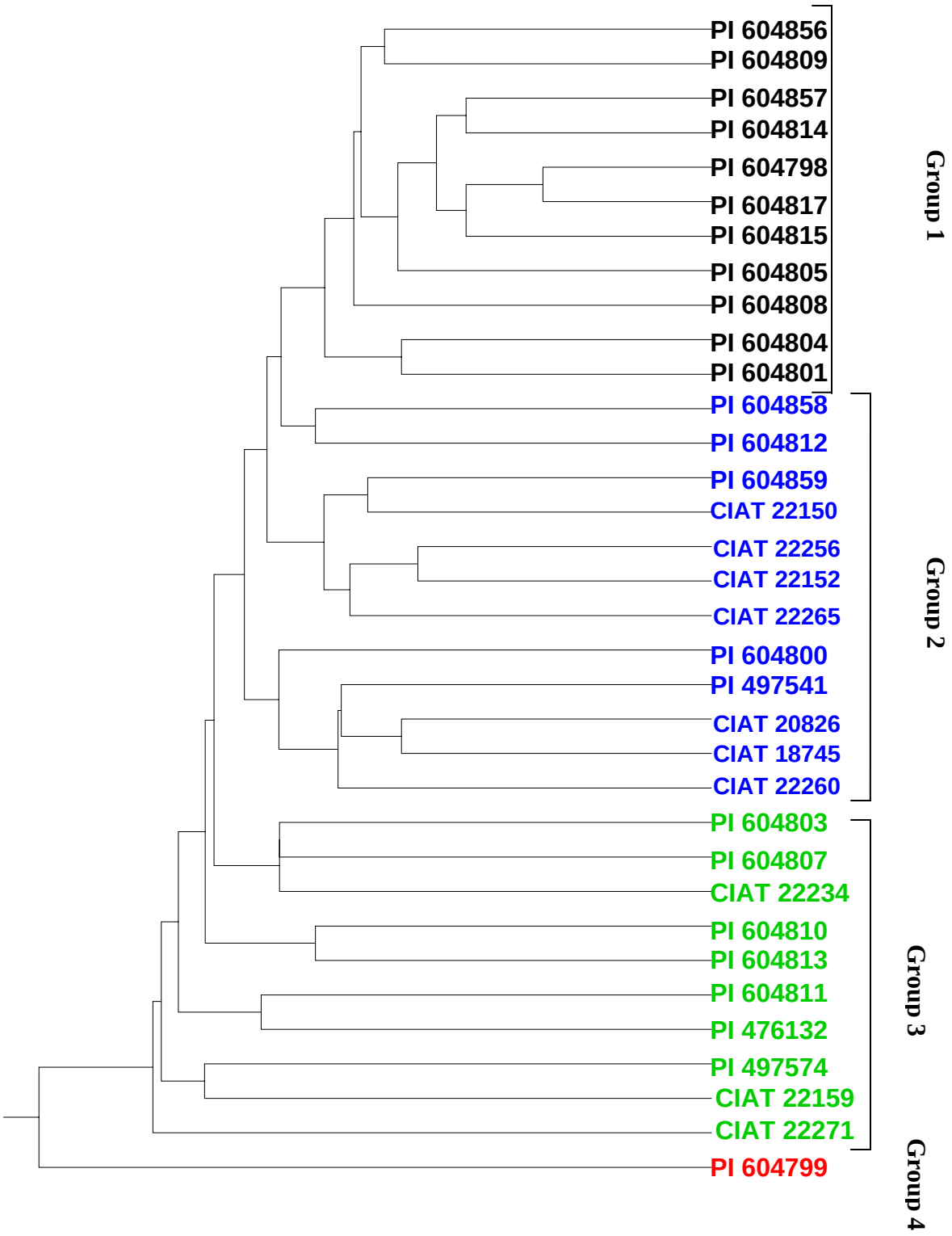

Figure 3-4. Dendogram illustrating the genetic relationships among 35 *A. pintoi* accessions based on Nei's genetic distance (Nei, 1972) obtained by 100 RAPD markers resolved by 8 random primers and generated by the UPGMA method.

Mean values of callus quantity rating and callus weight for both protocols are presented in Table 3-8. Differences in callus growth among protocols can be seen in Figure 3-5, where dishes of accessions PI 604812 are presented.

Table 3-7. Analysis of variance table of callus rating and weight produced from *Arachis pintoi* leaf discs incubated on two different tissue culture media

Source	df	Callus rating		Callus weight	
		MS	Pr>F	MS	Pr>F
Protocol	1	194.384	0.0001	4.585	0.0001
Accession	24	1.762	0.0001	0.059	0.0001
Protocol*Accession	24	1.006	0.0001	0.033	0.0001
Error	240	0.287		0.007	
Total	289				

Table 3-8. Callus rating and weight of *Arachis pintoi* tissue culture incubated in two different protocols

Protocol	Callus rating	Callus weight (g)
1	3.10 a*	0.333 a
2	1.46 b	0.078 b

* Means followed by the same letter in the same column were not different by Duncan's test ($p<0.05$).

Because the interaction of protocols by accessions was significant, average values of callus rating and weight were presented by protocol. In Table 3-9 callus quantity rating and weight of accessions incubated in Protocol 1 are presented. As expected, great variability for these two variables was displayed among the accessions. Average callus quantity rating was 3.11 and average callus weight 0.333 g. Accessions PI 604800, 604815, 604858, 604798, 604812, 604805, 604799, and CIAT 22234 were the ones with superior values for callus quantity rating and callus weight. Average callus quantity rating and weight were significantly lower for Protocol 2 than Protocol 1 (Table 3-8). Overall average callus quantity rating was 1.46 and average callus weight was 0.078 g. Accessions PI 497541 and 604857 did not produce any callus growth with Protocol 2. Accessions, PI 604799, 604804, 604809, 604814, 604815, 604858 and CIAT 22256 had

the highest values on Protocol 2, but only PI 604799, 604815, and 604858 were in the highest callus quantity group on both protocols (Table 3-10). Callus ratings and weight were highly correlated (r = 0.98) when both protocols were analyzed, which validates the callus rating scale used in this research.

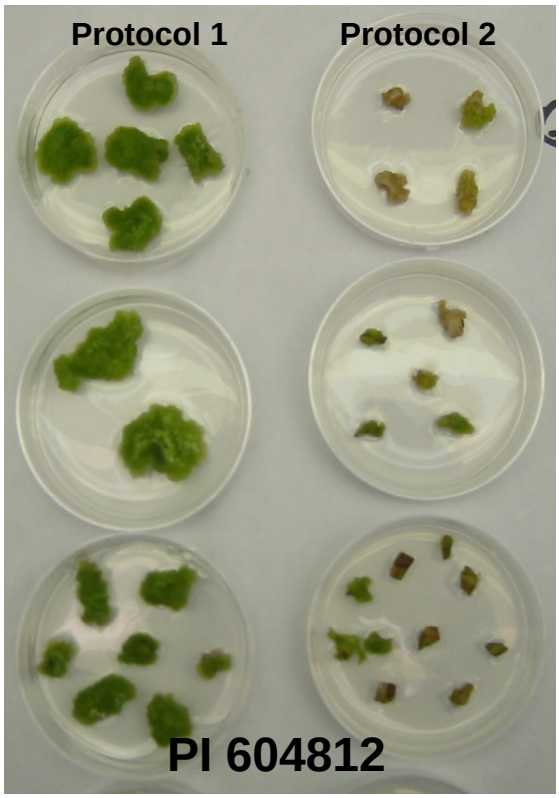

Figure 3-5. Callus growth of *A. pintoi* PI 604812 on two different tissue culture media.

Shoot regeneration was achieved for several accessions on both media. However, shoot development was variable for each accession and medium, with no structures indicative of somatic embryogenesis being detected. It seemed that callus quantity was not correlated with shoot regeneration. In Protocol 1 shoot regeneration was obtained from accessions PI 604856, 604857, 604805, 604811, 604809, 604814, 604818, and CIAT 22234, 20826, 22152, and 22265.

Table 3-9. Callus rating and callus weight of *Arachis pintoi* callus induced with Protocol 1

Accession	Protocol 1	
CIAT / PI number	Callus rating	Callus weight (g)
20826	3.00 cdef*	0.316 def
22152	2.17 gh	0.120 h
22234	3.50 abcde	0.430 abcd
22256	2.20 fgh	0.183 fgh
22265	3.00 cdef	0.264 efgh
22271	3.00 cdef	0.320 def
497541	3.00 cdef	0.325 def
604798	3.83 abc	0.425 abcd
604799	3.50 abcde	0.521 ab
604800	4.25 a	0.427 abcd
604803	3.33 bcde	0.281 defg
604804	3.17 cde	0.390 bcde
604805	3.50 abcde	0.559 a
604809	3.17 cde	0.327 def
604810	3.00 cdef	0.236 efgh
604811	2.17 gh	0.203 fgh
604812	3.67 abcd	0.427 abcd
604813	3.00 cdef	0.341 cdef
604814	2.83 defg	0.265 efgh
604815	4.00 ab	0.485 abc
604817	3.00 cdef	0.281 defg
604818	2.67 efg	0.234 efgh
604856	3.00 cdef	0.327 def
604857	1.83 h	0.146 hg
604858	4.00 ab	0.501 ab

*Means followed by the same letter in the column were not different by Duncan's test ($p<0.05$).

For Protocol 2, which yielded lower callus quantity, shoot regeneration was attained from 15 accessions: PI 604856, 604805, 604799, 604804, 604818, 604809, 604810, 604800, 604813, 604857, and CIAT 22256, 22234, 20826, 22152, and 22265. Figure 3-6 illustrates shoot regeneration of accession PI 604813 subcultured in Protocol 1 shoot media induction (MS+1g L^{-1} BA).

Developed shoots were transferred to root induction medium and then rooted plants were placed in pellets in a growth chamber for acclimatization and subsequently

transferred to pots in the greenhouse. Figure 3-7 presents a picture of every stage of the process used to tissue culture and regenerate *A. pintoi* accessions in this research.

Table 3-10. Callus rating and callus weight of *Arachis pintoi* callus induced with Protocol 2

Accession	Protocol 2	
PI/CIAT number	Callus rating	Callus weight (g)
20826	1.50 bcd	0.057 cde
22152	1.17 cd	0.065 cde
22234	1.50 bcd	0.090 bcd
22256	1.80 abc	0.057 cde
22265	1.67 bcd	0.096 bcd
22271	1.67 bcd	0.080 cde
497541	1.00 d	0.000 e
604798	1.50 bcd	0.072 cde
604799	2.00 ab	0.173 a
604800	1.67 bcd	0.103 bc
604803	1.00 d	0.050 cde
604804	1.83 abc	0.057 cde
604805	1.17 cd	0.041 de
604809	2.00 ab	0.105 bc
604810	1.00 d	0.027 e
604811	1.17 cd	0.044 de
604812	1.33 bcd	0.094 bcd
604813	1.17 cd	0.032 e
604814	2.33 a	0.142 ab
604815	1.83 abc	0.084 cde
604817	1.17 cd	0.058 cde
604818	1.50 bcd	0.055 cde
604856	1.50 bcd	0.077 cde
604857	1.00 d	0.000 e
604858	1.83 abc	0.143 ab

*Means followed by the same letter in the column were not different by Duncan's test ($p<0.05$).

Although, shoot regeneration was achieved for several accessions as stated before, shoot development and root induction were not achieved in a reliable or repeatable way. Root induction was very difficult to attain, and invariably many shoots died during this process. The addition of 1 g L^{-1} of actived charcoal in the root medium was tested to evaluate its effect on root formation. However, little or no effect was achieved.

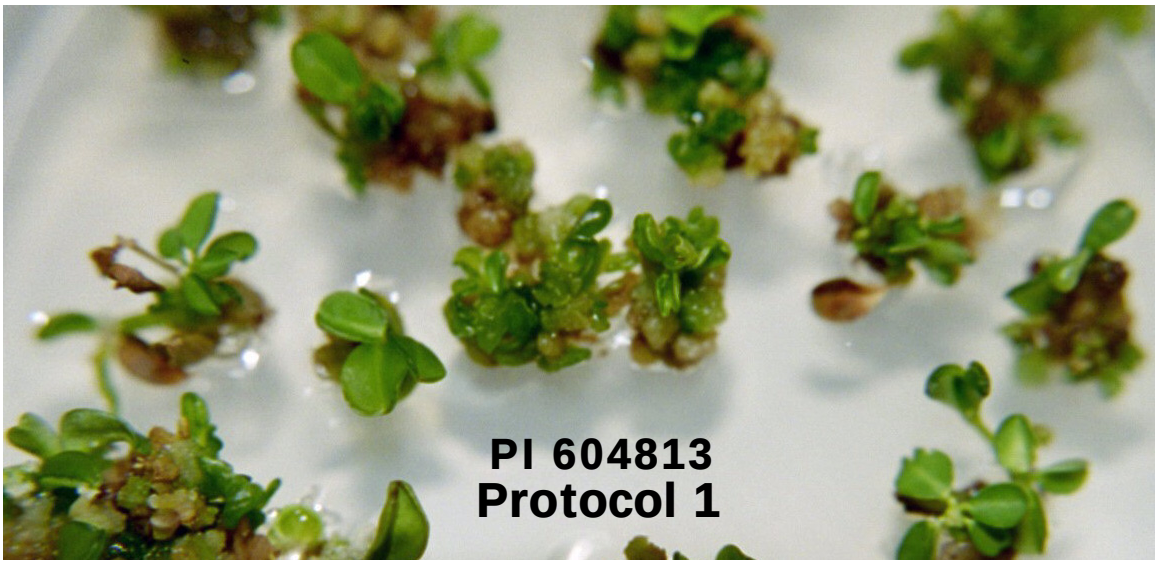

Figure 3-6. Shoot regeneration of *A. pintoi* accession PI 604812 subculture in medium MS+1g L^{-1} BA (Protocol 1).

Several reports in the literature account for the developmental problems that face organogenic tissue culture protocols of *Leguminosae* species. Flick et al. (1983) suggested that plant regeneration has been very difficult to achieve among legumes, and they continue, stating that in cases where it is attained, low frequency of regeneration is often present. The same authors stated that forage legumes have been more difficult to induce and form roots than seed legumes.

Figure 3-7. Representation of the different steps in the process used to tissue culture *A. pintoi*.

Arachis species are among the legume species that have problems with shoot development and root formation. Chengalrayan et al. (1995) obtained 33% average shoot rooting in MS hormone free medium, and 52% of shoot rooting when NAA in concentrations of 0.01, 0.05, and 0.5 mg L^{-1} was added to the medium with *A.hypogaea* cultivar J.L. 24. However this result was specific for this particular cultivar and in general *A. hypogaea* is recalcitrant in tissue culture.

Akasaka et al. (2000) were successful in inducing bud formation and shoot development from leaf segments of *A. hypogaea* cv. Chico. The percentage of conversion of buds to shoots was relatively high (34.7%). However, the highest frequency of shoot regeneration was 14.3%, suggesting that some buds failed to grow into normal shoots and plants.

Burtnick and Mroginski (1985) studying methods to regenerate plants from leaf explants of *A. pintoi* cultured on Murashige and Skoog (MS) nutrient medium containing different combinations of NAA (0.1-2 mg L^{-1}) and BA (0.1-3 mg L^{-1}) reported that callus induction was nearly 100%. However calluses were usually friable and produced no shoots or roots.

The same problems described above are also reported for other wild species of the genus *Arachis*. McKently et al. (1990) were able to regenerate plants of *A. glabrata* using MS medium supplemented with 3 to 5 mg L^{-1} of BAP. They reported, however that only 10% of shoot meristems continued growth and development into whole plants.

Due to the problems reported above, only 16 regenerated plants were obatined by both protocols. Table 3-11 presents the number of regenerated plants of *A. pintoi*

accessions from protocols 1 and 2. All plants were phenotypically normal and uniform in their appearance, with no somaclonal variation visually observed.

Table 3-11. Number of regenerated plants of *Arachis pintoi* accessions cultured on two different protocols

Accession	Protocol	
PI/CIAT number	1	2
	---------- no. pls ----------	
20826	0	2
22234	1	0
22265	1	0
604799	0	1
604805	0	1
604809	4	0
604810	0	1
604811	3	0
604856	0	1
604857	1	0
Total	10	6

Differences in callus induction and growth, shoot regeneration and development, root formation, and plant regeneration were observed among accessions. Callus induction and growth was observed in all 25 accessions in Protocol 1, but only in 23 in Protocol 2. Shoot induction was observed in 11 accessions in Protocol 1 and 15 in Protocol 2. Both protocols were able to produce regenerated plants of five accessions. Although differences in callus quantity rating and weight among protocols were observed earlier in the tissue culture process, we can conclude that based on shoot development and plant regeneration both protocols were equivalent. Additional research to study the shoot development and rooting problems observed in this work should be carried out to secure an efficient tissue culture protocol to regenerate *A. pintoi* plants.

Leaf tissue of the 16 regenerated plants was collected, ground, and used to extract DNA using the protocol described earlier. No differences in RAPD band profiles were detected between the original source plants and the tissue culture regenerated plants.

Although just a few plants were regenerated and analyzed, these results indicate that both protocols could be suitable for *in vitro* plant genetic conservation of *A. pintoi* accessions since no variation in RAPD band profile was observed. Further tests on the regenerated plants must be conducted to assure that no somaclonal variation occurred during the tissue culture process.The application of *in vitro* genetic conservation has several advantages, which include small space required to store large number of accessions, low costs compared to growing and organizing plants annually in the field, and maintaining large living collections of fruit trees (Hawkes et al., 2000).

Summary and Conclusions

Thirty-five *A. pintoi* accessions stored at the Southern Regional PI Station of the National Plant Germplasm System (NPGS) located at Griffin, GA were used to study genetic diversity at the molecular level using RAPD markers. Concurrently, two tissue culture protocols, proposed by Rey et al. (2000) and Ngo and Quesenberry (2000), were evaluated for their organogenesis ability, and capacity to generate somaclonal variation.

From the original 18 primers tested, amplifications were obtained in just eight of them. Primers A4, B4, B5, C2, D4, D13, E4, and G5 amplified 100 different bands. The average number of amplified bands per primer was 12.5, with primer D4 amplifying eight fragments and primer D13 amplifying 16 fragments. The size of these 100 fragments ranged from 250 bp to 3500 bp. From the 100 bands amplified, 98 presented polymorphism with the only two exceptions being Primer C2-1000 bp and primer E4-1250 bp. The average presence of bands per accession was 32, ranging from 20 to 44 bands. Ten bands were able to discriminate individual germplasm accessions. The proportion of polymorphic RAPD loci was 89%. Genetic diversity of the whole set of germplasm was estimated by Nei's gene diversity (h) and by Shannon-Weaver diversity

index (H). The average h was0.29 ± 0.16, and average H was 0.45 ± 0.20. Average genetic distance was estimated as 0.36, and indicated that a great genetic diversity exits among the germplasm evaluated in this research. Genetic distances were used to prepare a dendogram for the 35 *A. pintoi* accessions, which separated them in four distinct groups.

Callus induction was achieved on two different M.S. basal protocols after 28 d of incubation. Analysis of variance demonstrated that Protocol 1 was superior to Protocol 2 for both variables related with callus growth. Callus rating and weight values of Protocol 1 confirmed the great variability for these two variables among the accessions. Average callus rating was 3.11 and average callus weight 0.333 g. Accessions PI 604800, 604815, 604858, 604798, 604812, 604805, 604799, and CIAT 22234 showed the highest values. In Protocol 2, average callus ratings and weight were significantly lower than the values obtained with Protocol 1. Average callus rating was 1.49 and average callus weight was 0.072 g. Accessions PI 497541 and 604857 did not produce callus growth. Accessions PI 604799, 604804, 604809, 604814, 604815, 604858, and CIAT 22256 were the ones with highest values. Shoot regeneration was achieved for several accessions on both media with no structures indicative of somatic embryogenesis being detected. Callus growth was not correlated with shoot regeneration. In Protocol 1 shoot regeneration was obtained from 15 accessions, whereas in Protocol 2, shoot regeneration was attained from 18 accessions, but only PI 604856, 604818, 604809, and CIAT 20826 and 22234 regenerated shoots on both protocols. Root induction was very difficult to obtain, and invariably many shoots died during this process. At the end, just 16 regenerated plants were recovered between the two protocols.

Although differences in callus ratings and weight among protocols were observed earlier in the tissue culture process, we conclude that based on shoot development and plant regeneration both protocols were similar. RAPD band profiles of regenerated tissue culture plants were similar to their parent plants. However, we should point out that the number of regenerated plants was too small to conclusively affirm that genetic stability will be maintained by these two protocols. Further investigations should be conduced to definitely confirm our findings.

CHAPTER 4
MORPHOLOGICAL CHARACTERIZATION OF *Arachis pintoi* GERMPLASM

Introduction

Morphological characterization is used to assess and understand the genetic variability of germplasm collections. When working with a wild species of a genus of interest, this usually is the chance to first gather basic knowledge about it.

Polymorphic, highly heritable morphological traits were originally used in early scientific investigations of genetic diversity; such as the ones performed by Mendel and DeVries .

In general, morphological studies did not involve sophisticated equipment or laborious procedures, and these monogenic or oligogenic morphological traits were simple, rapid, and inexpensive to score (Hawkes et al., 2000).

The information generated from this type of morphological characterization research can be used to identify individual accessions based on a set of particular phenotype traits. Such data can also be useful to estimate genetic diversity of a germplasm collection, which will possibly impact the decision to enlarge the gene pool by further collection trips. Additionally, this activity can generate information about the genetic divergence among the accessions, which can be used to group accessions. Such grouping of the germplasm accessions can be used to assemble core collections, especially important in large germplasm banks.

Several statistical techniques may be used to assess genetic divergence. However, when a large number of accessions are present, multivariate analysis is most appropriate.

The advantage of this technique is the ability to discriminate among accessions considering multiple variables at the same time. Among the multivariate techniques the most used in genetic studies are Principal Component and Cluster Analysis (Hawkes et al., 2000).

Principal components analysis (PCA) has been widely used in genetic resource related research. The technique can be used with several objectives:

- Quantification of genetic divergence among germplasm accessions
- Selection of divergent parental genotypes to hybridize
- Variable reduction in sets of data with great number of parameters
- Variable exclusion based on its contribution to the total variance
- Calculation of a similarity index for the purpose of grouping accessions

The goal of this research was to morphologically characterize the *A. pintoi* germplasm accessions stored at the USDA-NPGS germplasm bank and to cluster the accessions based on similarity indices.

Materials and Methods

Accessions of *A. pintoi* stored in the Southern Regional Plant Introduction Station of the National Plant Germplasm System (NPGS) located at Griffin, Georgia were transferred to the University of Florida in 2001 and 2002. A list of these accessions with information related to the respective PI numbers and sites of collection is presented in Appendix A.

Morphological characterization of the above accessions was accomplished by evaluating each individual accession for a list of morphological descriptors selected from the IBPGR/ICRISAT list of morphological descriptors for *Arachis* (1990 and1992). Data from leaves, stems, flowers, pegs, pods, and seeds were collected from plants of each

accession. The list of morphological descriptors evaluated is presented in more detail in Table 4.1.

Table 4-1. Morphological descriptors applied to *Arachis pintoi* accessions

Morphological descriptor	Descriptor code	# of structures measured	Unit	Equipment used
Flower/inflorescence	FPI	10	number	Visual evaluation
Flower standard width	FSW	10	mm	Caliper
Flower standard length	FSL	10	mm	Caliper
Flower standard color	FSC	10	Color scale	Visual evaluation
Flower standard crescent	FScr	10	Present/absent	Dissect scope
Flower wing width	FWW	10	mm	Caliper
Flower wing length	FWL	10	mm	Caliper
Flower keel length	FKL	10	mm	Caliper
Flower hypanthium length	FHL	10	mm	Caliper
Flower hypanthium width	FHW	10	mm	Caliper
Flower hypanthium color	FHC	10	Present/absent	Dissect scope
Flower hypanthium hairiness	FHH	10	Present/absent	Dissect scope
Flower pollen size	FPSi	10	mm	Caliper
Flower pollen shape	FPS	10	IBPGR scale	Microscope
Stem internode length	SIL	50	mm	Caliper
Stem internode diameter	SID	50	mm	Caliper
Stem color	SC	50	Present/absent	Dissect scope
Stem hairiness	SH	10	Pres/abs/abun	Dissect scope
Stem bristles	SGH	10	Present/absent	Dissect scope
Leaflet shape	LS	10	Shape scale	Visual evaluation
Leaflet hairiness sup. surface	LHU	10	Present/absent	Dissect scope
Leaflet hairiness margin	LHM	10	Present/absent	Dissect scope
Leaflet hairiness inf. surface	LHL	10	Present/absent	Dissect scope
Leaflet bristles sup. surface	LGHU	10	Present/absent	Dissect scope
Leaflet bristles margin	LGHM	10	Present/absent	Dissect scope
Leaflet bristles inf. surface	LGHL	10	Present/absent	Dissect scope
Leaflet length	LL	10	mm	Caliper
Leaflet width	LW	10	mm	Caliper
Leaflet length/Leaf width	LLLW	10	mm	Caliper
Leaf Petiole length	LPL	10	mm	Caliper
Peg length	PegL	10	mm	Caliper
Peg width	PegW	10	mm	Caliper
Peg color	PegC	10	Present/absent	Visual evaluation
Peg hairiness	PegH	10	Present/absent	Visual evaluation
Pod weight	PodWe	10	grams	Scale
Pod length	PodL	10	mm	Caliper
Pod width	PodW	10	mm	Caliper
Pod beak	PodB	10	IBPGR scale	Dissect scope
Pod reticulation	PodR	10	IBPGR scale	Dissect scope
Seed width	SW	10	mm	Caliper
Seed length	SL	10	mm	Caliper
Seed weight	SWe	10	grams	Scale
Seed color	Scolor	10	ISCC-NBS color chart	Dissect scope
Pod weight/Seed weight	PodweSwe	10	%	-

Two plots of four plants (2 m x 2 m) were established at the Forage Research Unit of the Agronomy Department of the University of Florida in Gainesville-FL. Six random plants of each accession were selected from these plots and used as plant-part sources.

For each genotype, 10 stems per plant were collected, the terminal part of each stem (1^{st} 3 internodes) was discarded and then five internodes were evaluated on each stem. For the other part categories, 10 pieces were collected and evaluated. Continuous variables were measured with a 15 cm electronic digital caliper (Chicago Brand Industrial Inc., Fremont, CA), and categorical variables were scored under the dissecting microscope using the standards proposed by IBPGR/ICRISAT (1990 and 1992).

The data were tabulated in a Microsoft Excel spreadsheet and the mean, standard deviation, and range were calculated for quantitative descriptors, and the mode was determined for qualitative ones.

Genetic variability among the accessions with respect to the morphological descriptors was examined by calculating Simpson's (1949) and Shannon-Weaver's (1949) diversity indices. These indices give a measure of phenotypic diversity and range from zero to one, where one represents great genetic diversity and zero the opposite or no genetic diversity. The indices correspond to the probability that two individuals randomly selected from a group of populations will have the same morphological feature. The formula to calculate both indices is presented below.

Shannon-Wiever Diversity index: $H = -SUM (pi \log pi)/\log pi$

Simpson Diversity index: $D = 1-SUM (p_i^2)$

where i= 1 to n, and p is the proportion of the total morphotypes made up of the i^{th} morphotype.

The data matrix was then analyzed with SAS software (SAS institute, 1989). Phenotypic correlations among morphological descriptors were computed with the PROC CORR procedure. After that, qualitative characteristics were transformed with PROC PRINQUAL, and a principal component analysis was performed with the procedure PROC PRINCOMP. Finally, a cluster analysis using the "Complete linkage method" was prepared. Means of quantitative traits of each group were compared by using the Newman-Keuls procedure (SAS institute, 1989).

Result and Discussions

Great morphological variability was observed among the accessions for all descriptors, exceptions being, pollen size and shape, and bristles on the superior and inferior leaf surface, which showed no polymorphism. In Table 4-2, the mean/mode, standard deviation, variance and range of each morphological descriptor are listed.

Flowers arise from inflorescences located at reproductive buds under the lea axils. According to Conagin (1959), each inflorescence produces one to nine flowers, which will bloom in a sequence, usually with a 1-2 d interval. Numbers observed in this study revealed accessions with a mean of two flowers per inflorescence and a maximum of four flowers per inflorescence. Although important, these differences do not seem to have great impact in terms of seed production.

Arachis pintoi flowers are typical of papilionacea legumes possessing five petals. They displayed one standard petal, two wing petals, and a keel that is actually formed by the fusion of two petals. The other flower structures are the calyx and the hypanthium.

Table 4-2. Morphological characteristics of *Arachis pintoi* germplasm accessions

Morphological Descriptor	Mean/Mode	Standard Deviation	Variance	Range
FPI	3.14	0.33	0.11	2.00 – 3.80
FSW	15.31	1.63	2.67	11.99 – 18.85
FSL	11.58	1.10	1.22	9.84 – 14.45
FSC	Yellow	-	-	-
FScr	Present	-	-	Present/Absent
FWW	6.27	0.69	0.47	4.47 – 7.95
FWL	8.07	0.69	0.48	6.92 – 9.80
FKL	5.09	0.23	0.05	4.45 – 5.55
FHL	70.66	12.58	158.28	52.39 -104.79
FHW	0.97	0.10	0.01	0.77 – 1.12
FHC	Absent	-	-	Present/Absent
FHH	Present	-	-	Present/Absent
FPSi	4.00	0.00	0.00	-
FPS	Round	-	-	-
SIL	29.47	7.43	55.27	18.23 – 52.23
SID	2.95	0.84	0.71	2.05 – 7.15
SC	Absent	-	-	Present/Absent
SH	Present	-	-	Present/Absent
SGH	Absent	-	-	Present/Absent
LS	Obovate	-	-	-
LHU	Absent	-	-	Present/Absent
LHM	Absent	-	-	Present/Absent
LHL	Present	-	-	Present/Absent
LGHU	Absent	-	-	Present/Absent
LGHM	Present	-	-	Present/Absent
LGHL	Absent	-	-	Present/Absent
LL	24.89	4.07	16.60	15.79 – 32.03
LW	15.14	2.79	7.80	9.45 – 20.78
LLLW	1.66	0.21	0.04	1.29 – 2.06
LPL	22.26	6.40	40.91	11.67 – 39.33
PegL	11.90	3.36	11.28	6.30 – 21.40
PegW	0.94	0.14	0.02	0.57 – 1.23
PegC	Present	-	-	Present/Absent
PegH	Present	-	-	Present/Absent
PodWe	0.13	0.07	0.01	0.07 – 0.25
PodL	9.33	4.52	20.40	7.55 – 14.25
PodW	4.70	2.22	4.91	4.53 – 6.91
PodB	Moderate	-	-	-
PodR	Slight	-	-	-
SW	3.89	1.86	3.45	3.55 - 5.46
SL	7.13	3.50	12.22	5.71 - 10.51
SWe	0.10	0.06	0.001	0.04 - 0.18
Scolor	Orange yellowish	-	-	-
PodweSwe	75.47	6.71	44.99	59.71 – 87.07

Most of the accessions (91%) have yellow flowers, with the 9% exception showing a lighter shade of yellow classified as lemon yellow (Figure 4.1). This was the case of accessions PI 604814, PI 604818, and CIAT 20826. Standard color is considered a very good genetic marker, and so it could be used in genetic studies with *A. pintoi*.

Our results in terms of standard petals color are different from the ones reported by Maass et al. (1993) who performed morphological characterization of eight accessions of *A. pintoi.* They reported that lemon yellow was the standard petal color displayed by all eight germplasm lines. Qualitative traits such standard petals color are subject to individual interpretation and that is probably why their results are very different them the ones reported in this research.

Also different are the results of Upadhyaya (2003) who worked with the ICRISAT core collection (1704 accessions) of *Arachis hypogaea.* He stated that 97% of the germplasm in that collection had orange standard petals. White, lemon yellow, yellow, and dark orange were not observed in any of his accessions.

Differences in standard petal length and width, wing length and width, keel length, and hypanthium length and width were also observed. Those features are directly related to overall flower size. Figure 4.2 illustrates the large differences displayed by the germplasm in relation to this group of characteristics. Also, differences in hypanthium color were found and can be observed in Figure 4.2. The mode for this characteristic was absence of color, but some accessions presented a distinct purple color, which is probably associated with the presence of anthocyanin. One of the reported functions of anthocyanin is to attract insects: since it absorbs UV radiation that is very attractive to them (Mann, 1987).

Arachis pintoi stems were hollow with reproductive and vegetative nodes occurring along the stem length. Vegetative nodes have the ability to root which allows the species to be vegetatively propagated, a desirable agronomic characteristic.

Figure 4-1. Flower standard colors of *Arachis pintoi* germplasm.

Figure 4-2. Flower sizes and hypanthium colors displayed by *Arachis pintoi* germplasm.

Large differences were observed in relation to stem internode length and diameter, and stem color. The average internode length was 29 mm, ranging from 18 to 52 mm. Average internode diameter was 3 mm, varying from 2 to 7 mm. As in the case of hypanthium color, it seems that stem internode color is determined by the presence of

anthocyanin. However, here more variants in color were present than in the former feature, although scoring was just made in terms of presence and absence. Absence was the mode for this characteristic with 57% of the accessions showing no purple coloration, which is translated as a light green color. Among those that displayed stem coloration, yellowish, pinkish, light and deep purple were observed (Figure 4-3).

A. pintoi leaves are compound with four leaflets. Usually the basal leaflets were smaller than the distal, and exhibited an elliptic shape. As observed in flower and stem features, large differences were also present in leaves.

Figure 4-3. Stem characteristics of Arachis pintoi germplasm.

When examining leaflet characteristics, the features related to overall leaf size (leaflet length and width and petiole length) and shape were the ones that showed the most variability. Average leaflet length was 25 mm with a range of 16 to 32 mm. Leaflet width varied from 9 to 21 mm, with an average value of 15 mm. Due to leaflet length and width variation, an array of leaflet sizes arise (Figure 4-4). In terms of leaflet shape ten different types were present with obovate shape being the mode, this agreed with the findings of

Maass et al. (1993). However, Upadhyaya (2003) reported different results among the 1704 accessions of the ICRISAT *A. hypogaea* core collection, which had elliptic leaf shape without exceptions.

Similar to others species of the genus, *A. pintoi* also possesses geocarpic fruits. Flowers self-pollinate and then lose their petals as the fertilized ovary begins to enlarge. The budding ovary or "peg" grows under the ground, away from the plant, forming a small stem which contains the ovary and the embryo in its tip. The embryo turns horizontal to the soil surface and begins to mature inside a pod. In the cultivated species (*A. hypogaea*), two to four or more seeds may be formed in each pod. However, in *A. pintoi*, single seed pods were exclusively observed.

Figure 4-4. Leaflet characteristics of *Arachis pintoi* germplasm.

Pegs, pods, and seeds exhibited an array of variability among the accessions. One of the differences is the fact that accessions PI 604804, 604813, 604817, and CIAT 22152, 22159, 22234 did not produce any of the above plant structures under the conditions of these experiments. Additionally, differences were also present in the

accessions that produced these structures. Average peg length was 12 mm, ranging from 6 to 21 mm. With respect to the other peg features, average peg width was 0.90 mm, and presence of peg color and hairiness were the mode.

Pod size and weight showed great divergence among the germplasm. These characteristics were most impacted by pod length and width. Large pod size and weight were reflected in large seed size and weight. Figure 4-5 illustrates the differences in seed size among the genotypes. Average seed weight was 0.12 g, ranging from 0.04 to 0.18 g. Also, the average ratio of seed weight/pod weight was 0.75, ranging from 0.59 to 0.87.

Figure 4-5. Seed characteristics of *Arachis pintoi* germplasm.

Phenotypic correlations were calculated among morphological descriptors, and Pearson's correlation coefficient and significance test were also calculated (Appendix C). Pollen size and shape (FPSi and FPS) and bristles on the superior and inferior leaf surface (LGHU and LGHL) were not included because they did not show any variability. In addition, leaf length/leaf width ratio (LLLW) and pod weight/seed weight ratio (PodweSwe) were left out of the correlation matrix because they were derived from two other variables. Accessions PI 604804, 604813, 604817, and CIAT 22152, 22159, 22234 were excluded because they did not produce any pegs, pods, or seeds. In summary, 35

morphological descriptors and 28 germplasm accessions were used to calculate correlation coefficients.

Therefore, with 26 degrees of freedom (df = N-2 or 28-2), any correlation coefficient with an absolute value greater than 0.361 had a *P* value of ≤ 0.05. From 595 correlations computed, 96 were higher than 0.361. These 96 significant correlations could be divided into eight different groups (Table 4-3).

Table 4-3. Correlations among morphological descriptors of *Arachis pintoi* germplasm

Group	Group Description
1	Correlations between flower features dimensions
2	Correlations between leaflet features dimensions
3	Correlations between flower features dimensions and leaflet dimensions
4	Correlations between stem diameter and leaflet dimensions
5	Correlations between flower features dimensions and pod and seed dimensions
6	Correlations between stem diameter and pod and seed dimensions
7	Correlations between leaflet features dimensions and pod and seed dimensions
8	Correlations between pod features dimensions and seed dimensions

Correlations in Groups 1, 2, and 8 are not very meaningful and could be explained by the fact that part proportions should be maintained within each plant organ or structure. So, flowers with large standards length will also have large standard width, wing length and width, and keel length. For the same reason we should expect that pods with large measures will also have large seed if an adequate development had occurred, because the seeds are enclosed by the pod.

Correlations in Groups 3 and 4 would have agronomic importance if leaflet size is translated into higher vegetative mass production. Likewise leaf/stem ratio could be important assuming leaves have higher nutritive values than stem. These are very important features in forage species. These correlations suggest that flower size and stem diameter could be included as selection indices in *A. pintoi* forage selection and breeding

programs. The same idea could be applied to Groups 5, 6, and 7, where stem, flower, and leaflet size appear to impact pod and seed size.

Although 96 correlation coefficients were considered significant ($r = 0.361$). Skinner et al. (1999) suggested that only correlation coefficients with absolute values higher than 0.71 should be considered biologically meaningful. They explain that only in these situations is more than 50% of the variance of one trait is predicted by the other.

Reexamining the correlation coefficient table (Appendix C) under this new criterion only 29 correlation coefficients out of 595 would be considered biologically meaningful. In this situation only Groups 1, 2, 7, and 8 remain in place. As stated before, Groups 1, 2, and 8, do not have great biological importance, since they could be explained by the fact that part proportions should be maintained within each plant organ or structure. Therefore, only correlations relative to Group 7 may have meaningful biological importance. Examining this information more carefully, we can observe that meaningful correlations were found between leaf length (LL) and Pod weight (Podwe); leaf length (LL) and pod width (PodW); leaf length (LL) and seed weight (Swe); leaf length (LL) and seed width (SW); and finally leaf length (LL) and seed length (SL). Thus, leaf length could be used as a selection criterion in programs where increased seed size/weight is one of the objectives.

One of the biggest problems with forage legumes species is slow establishment, which sometimes is associated with small seed size. Having a large seed could represent more stored reserves and higher seedling vigor, which would reduce the establishment time. These suggestions based on the above correlations should be verified in future research to evaluate the use of these characteristics as selection indices.

To support this hypothesis we could examine the findings of Skinner et al. (1999) who worked with the Australian annual *Medicago* collection (20997 accessions) measuring 27 traits. They reported that the traits seeds per gram and winter and spring herbage yield were correlated (0.42 and 0.29). They also stated that seeds per gram and seedling vigor were correlated (0.44). Upadhyaya (2003) also found correlations between 100 seed weight and yield (0.32) in the ICRISAT *Arachis hypogaea* core collection.

Although we can affirm that phenotypic genetic diversity was observed among the accessions by examining the information contained in Appendix B and Table 4.2 there is a need to quantify this diversity. To achieve this goal we should make use of a genetic diversity index. Two of the most used diversity indices are Shannon-Weaver and Simpson indices. These indices are often used in ecological studies where species richness and composition of a particular community or ecosystem are evaluated. Recently, these indices have been applied to quantify genetic diversity of germplasm collections when phenotypic frequencies are collected. The greater the index value, the greater the genetic diversity. In Table 4-4 the values of the indices for each morphological descriptor are presented. The total genetic diversity was also calculated, and it is an indication of how different the accessions are in relation to the morphological features utilized in this research.

Diversity values were variable among traits, but in general all morphological features expressed high genetic diversity. According to Simpson's index, leaf shape (0.83), seed color (0.82), flower standard width (0.81), and seed length (0.75) were the descriptors with greatest diversity. In opposition, the lowest diversity values were related

to flower standard color (0.18), flower standard crescent (0.24), and flower hypanthium hairiness (0.24). The total Simpson's index to all morphological descriptors was 0.58.

Shannon-Weaver's diversity values were in general higher than Simpson's. Descriptors with higher values for the Shannon-Weaver index were leaf hairiness inferior surface (1.00), flower hypanthium color (1.00), leaf hairiness margins, and pod reticulation (0.97). Total genetic diversity was estimated as 0.71.

Shannon-Weaver diversity index values observed in this work are much higher than the ones observed by Upadhyaya et al. (2002), who applied 38 agromorphological descriptors to the whole *A. hypogaea* ICRISAT collection (13342 accessions). These authors found a total genetic diversity value of 0.50. Leaflet length (0.62) and shelling percentage (0.62) were the traits showing most variation.

These values are also higher than those reported by Upadhyaya (2003) who evaluated a core collection prepared using the results of the previous work. He obtained a total diversity index of 0.44 in 32 agromorphological traits. He concluded that the core collection had significant variation for the morphological and agronomic traits evaluated.

A principal components analysis (PCA) was performed with the goal of discriminating among accessions and grouping even further. Principal components analysis (PCA) can be used in sets of data with large number of variables. The goal of PCA is to provide a reduced dimension model that would indicate measured differences among groups. It also can contribute to a better understanding of the set of variables by describing how much of the total variance is explained by each one. With this objective the PCA was performed with the matrix of morphological data generated by applying the list of descriptors presented in Table 4-1.

Table 4-4. Simpson and Shannon-Weaver diversity indices for *Arachis. pintoi* morphological descriptors

Morphological Descriptor	Simpson	Shannon-Weaver
Flower/inflorescence	0.60	0.34
Flower standard width	0.81	0.89
Flower standard length	0.68	0.80
Flower standard color	0.18	0.47
Flower standard crescent	0.24	0.58
Flower wing width	0.56	0.33
Flower wing length	0.60	0.33
Flower keel length	0.54	0.74
Flower hypanthium length	0.73	0.89
Flower hypanthium width	0.73	0.46
Flower hypanthium color	0.50	1.00
Flower hypanthium hairiness	0.24	0.58
Stem internode length	0.50	0.58
Stem internode diameter	0.65	0.45
Stem color	0.47	0.96
Stem hairiness	0.63	0.96
Stem bristles	0.36	0.80
Leaf shape	0.83	0.83
Leaflet hairiness inf. surface	0.50	1.00
Leaflet hairiness margins	0.48	0.98
Leaf bristles margins	0.36	0.80
Leaf length	0.64	0.46
Leaf width	0.58	0.34
Leaf length/Leaf width	0.67	1.00
Leaf petiole length	0.74	0.84
Peg length	0.59	0.50
Peg width	0.46	0.71
Peg color	0.45	0.92
Peg hairiness	0.45	0.92
Pod weight	0.69	0.45
Pod length	0.47	0.74
Pod width	0.74	0.89
Pod beak	0.73	0.87
Pod reticulation	0.64	0.97
Seed width	0.69	0.48
Seed length	0.75	0.88
Seed weight	0.64	0.37
Seed color	0.82	0.89
Pod weight/Seed weight	0.57	0.87
Total	0.58	0.71

Variables pollen size and shape (FPSi and FPS) and leaf bristles superior and inferior surface (LGHU and LGHL) were not included because they did not show any variability. In addition, leaf length/leaf width ratio (LLLW) and pod weight/seed weight ratio (PodweSwe) were also left out because they are derived from two other variables. After the first analysis, the leaflet hairiness superior surface variable (LHU) was also excluded from the analysis because it contributed little to the exploration of total variance.

The first five principal components (PCs) were responsible for 67.7% of the total variation (Table 4-5). Values similar to these were reported by Stalker (1990), Upadhyaya et al. (2002), Upadhyaya (2003), and Upadhyaya et al. (2002), who worked with wild species of *Arachis*, groundnut, and chickpea germplasm collections, respectively, to explain their results. The first PC explained 30.0% of the variation, the second accounted for 15.2%, the third for 10.1%, the fourth for 6.6%, and the fifth explained 5.8% of the total variation.

Examining the variable loadings of the first five PCs (Table 4-5) we can clearly observe that the characteristics of pegs, pods, and seeds are the ones with highest contribution to PC1. Therefore, PC1 could be termed the “sexual reproduction axis”. Performing the same exam to PC2 we can conclude that the features related with flower and leaf dimensions were the ones with highest loadings. Therefore, PC2 could be called the “vegetative axis”. Finally, examining the loading of PC3 we note that features related to the shape, color, and hairiness of morphological structures were the ones with the most contribution. Those are all qualitative features and because of that we could call PC3 the “qualitative axis”.

Therefore, we could state that the principal components analysis was able to discriminate and separate the accessions in terms of these three dimensions, represented by “sexual reproduction”, “vegetative”, and “qualitative” axes. This is clearly observed when accessions were projected in two-dimensional graphs formed by PC1 and PC2, PC1 and PC3, and PC2 and PC3 (Figures 4-6, 4-7, and 4-8).

Table 4-5. Vector loadings and percentage of variation explained by the first five principal components for morphological characteristics of *Arachis pintoi*

	Principal components				
Characteristics	1	2	3	4	5
Variance explained (%)	30.01	15.15	10.09	6.63	5.76
Cumulative variance explained (%)	30.01	45.16	55.25	61.88	67.65
Flower/inflorescence	0.077	0.041	**0.215**	0.036	-0.019
Flower standard width	-0.060	**0.338**	-0.085	-0.092	0.027
Flower standard length	-0.068	**0.369**	-0.028	0.070	-0.014
Flower standard color	-0.060	-0.038	0.072	0.333	0.317
Flower standard crescent	0.145	-0.117	0.056	-0.132	0.248
Flower wing width	-0.065	**0.331**	0.006	-0.099	0.122
Flower wing length	-0.080	**0.350**	-0.017	0.025	0.085
Flower keel length	-0.084	**0.213**	0.146	0.200	0.141
Flower hypanthium length	-0.097	**0.299**	0.089	0.136	-0.142
Flower hypanthium width	0.017	**0.218**	0.273	0.235	-0.128
Flower hypanthium color	0.023	0.013	**-0.164**	-0.165	-0.382
Flower hypanthium hairiness	-0.009	0.087	0.133	0.124	-0.422
Stem internode length	-0.030	0.079	**-0.304**	-0.145	0.051
Stem internode diameter	-0.043	-0.036	**0.157**	-0.194	0.338
Stem color	-0.076	-0.042	0.090	0.203	-0.274
Stem hairiness	0.015	0.051	**0.438**	0.104	-0.115
Stem bristles	-0.033	0.198	0.013	0.085	0.112
Leaflet shape	-0.105	0.074	**-0.275**	0.231	0.115
Leaflet hairiness margin	-0.056	-0.063	**0.302**	-0.290	0.023
Leaflet hairiness inf. surface	0.006	-0.086	**0.366**	-0.180	0.139
Leaflet bristles margin	0.021	0.071	**-0.186**	0.285	0.290
Leaflet length	0.057	**0.285**	0.039	-0.310	0.011
Leaflet width	0.155	**0.233**	0.071	-0.245	-0.101
Leaf Petiole length	0.062	**0.251**	-0.138	-0.248	0.028
Peg length	**0.231**	0.070	-0.092	0.038	-0.165
Peg width	**0.256**	-0.001	0.015	0.185	-0.075
Peg color	0.138	-0.090	**-0.195**	-0.134	-0.140
Peg hairiness	0.109	-0.002	**-0.240**	0.113	-0.075
Pod weight	**0.273**	0.099	0.016	-0.029	0.095
Pod length	**0.293**	0.016	0.023	0.046	0.032
Pod width	**0.291**	0.033	0.047	0.079	0.035
Pod beak	**0.274**	0.017	0.003	-0.034	0.022
Pod reticulation	**0.288**	-0.016	0.024	0.111	-0.005
Seed width	**0.292**	0.044	0.044	0.066	0.041
Seed length	**0.293**	0.042	0.008	0.001	0.042
Seed weight	**0.268**	0.101	-0.005	-0.031	0.122
Seed color	**0.287**	-0.028	0.003	0.099	0.012

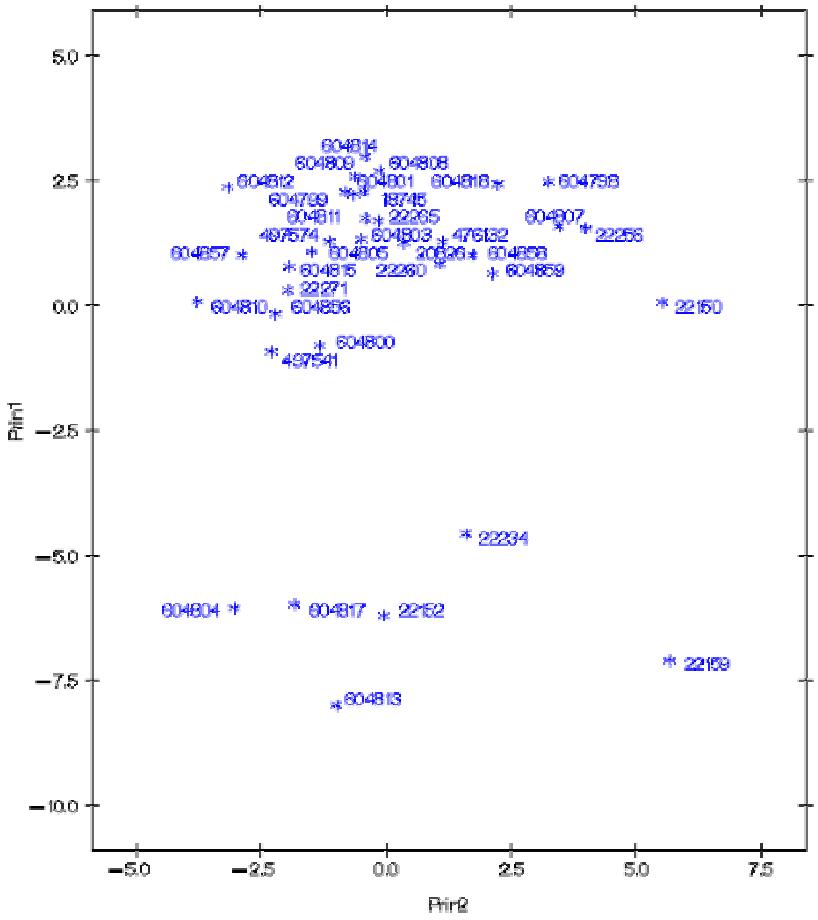

Figure 4-6. Projection of the 35 *Arachis pintoi* accessions in a two-dimensional graph defined by PC1 and PC2.

Figure 4-6 corresponds to the plane formed by PC1 and PC2 dimensions. PC1 values are on the Y axis and PC2 on the X axis. Moving from the bottom of the Y axis where coefficients were negative to the top where they were positive represents moving from lower values of peg, pods, seed dimensions, and weight. Doing the same to the X axis represented by PC2, and moving from the left (- coefficients) to the right (+ coefficients) means that we are moving from lower values of flower and leaf dimensions to higher values. Accessions were obviously discriminated, and two groups were formed. The group in the bottom was composed of six accessions and the other 29 in the top of the graph. These six accessions in the bottom are the ones with no pegs, seeds, and pods.

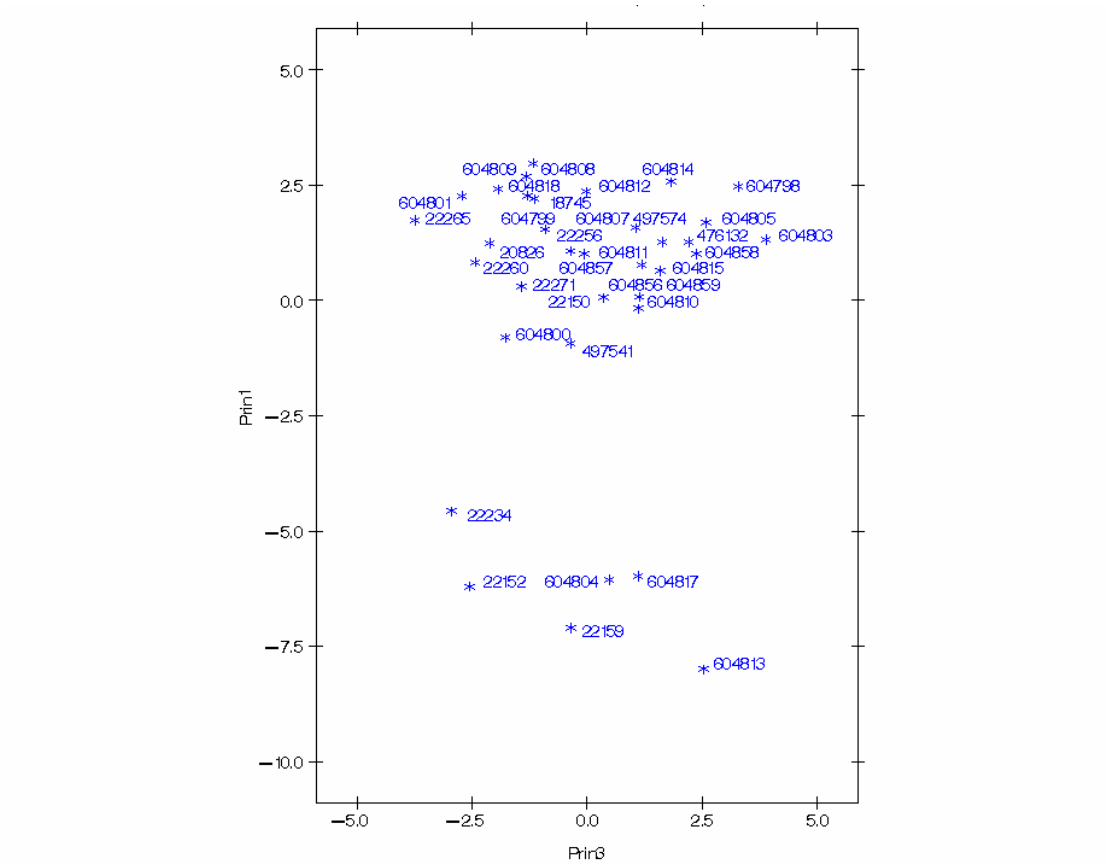

Figure 4-7. Projection of the 35 *Arachis pintoi* accessions in a two-dimensional graph defined by PC1 and PC3.

In the same way, figure 4-7 corresponds to the plane formed by PC1 and PC3 dimensions, where PC1 represents the Y axis and PC3 represents the X axis. Here, the Y axis indicates the same tendency as in the previous figure. PC3 is the represented by the qualitative characteristics (shape, color, and hairiness), and a more comprehensive reading of the tendency here is difficult to achieve. However, accessions were also discriminated with two groups formed. The group in the bottom was again composed of the six accessions with no pegs, seeds, and pods.

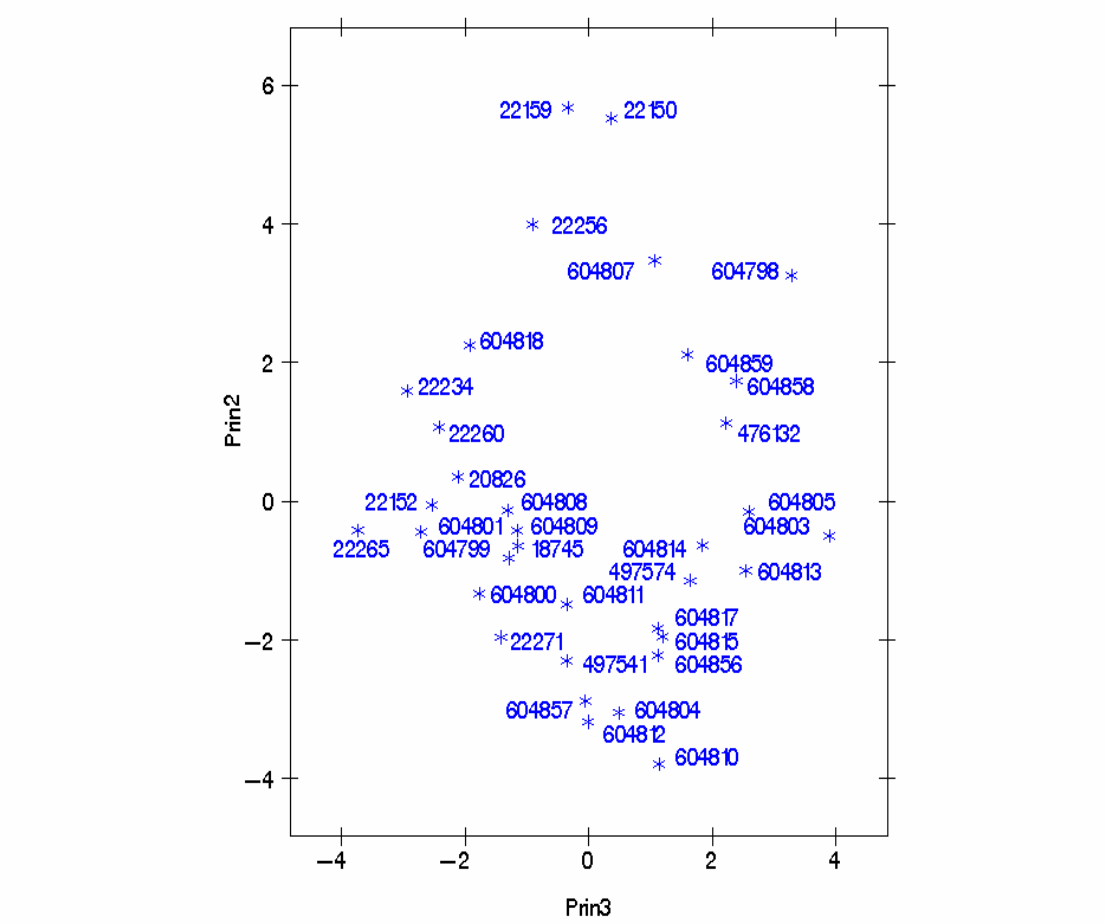

Figure 4-8. Projection of the 35 *Arachis pintoi* accessions in a two-dimensional graph defined by PC2 and PC3.

Finally, figure 4-8 represents the plane formed by PC2 and PC3 dimensions, where PC2 represents the Y axis and PC3 represents the X axis. Moving from the bottom (-coefficients) to the top (+ coefficients) means that we are moving from smaller to bigger flowers and leaves. Accession discrimination was also obtained. However, group formation was more difficult to obtain, once accessions were spread in the plane.

We could state that by using this set of descriptors we accomplished the discrimination of the *A. pintoi* germplasm. All the 35 morphological features used in the PCA presented high loading values at least once when the first five PC were analyzed, reinforcing the importance of each one as an *A. pintoi* descriptor.

Since discrimination was obtained by using of the genetic diversity indices and also by the principal component analysis, the next step was to perform a grouping or cluster analysis. The first nine principal components were used to execute a cluster analysis using the complete linkage clusters method. The dendogram resulting from this analysis is presented in Figure 4-9.

From the dendogram we can differentiate four distinct groups of accessions. Group 1 was composed of accessions PI 497541, 497574, 604800, 604810, 604811, 604812, 604815, 604856, 604857, and CIAT 22271. Group 2 was formed by PI 604799, 604801, 604808, 604809, 604814, 604818, CIAT 18745, 20826, 22260, and 22265. Group 3 was composed by PI 476132, 604798, 604803, 604805, 604807, 604858, 604859, CIAT 22150, and 22256. Finally, Group 4 was composed by PI 604804, 604813, 604817, CIAT 22152, 22159, and 22234.

Morphological characteristics of each of the four groups created by the cluster analysis are presented in Table 4-6. Based on these features we could characterize the four groups. Group 1 was composed by accessions with small leaves, flowers, pods, pegs, and seeds. Therefore, we could name this group as the “small type” group. In Figure 4-10, accession PI 497541, a member of this group is presented.

Group 2 was formed by accessions with intermediate size features and could be called as the “intermediate type” group. Accession PI 604814 is presented as a representative of this group in Figure 4-11.

Group 3 was formed by accessions with large leaves, flowers, pegs, pods, and seeds. It was named as the “large type” group due to their large features. In Figure 4-12 accession PI 604798 is displayed as a representative of this type.

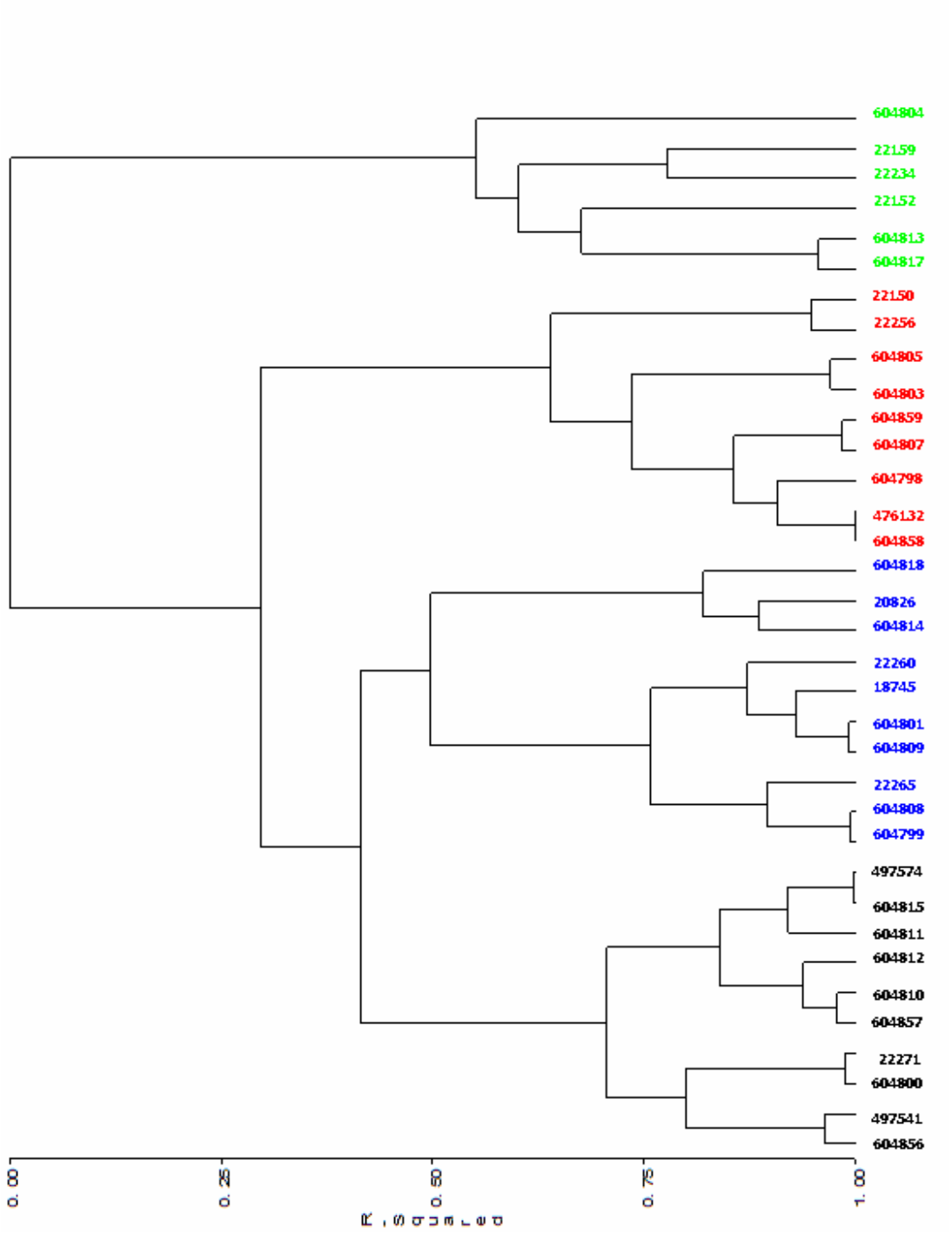

Figure 4-9. Dendogram of 35 *Arachis pintoi* accessions based on morphological descriptors and the first nine principal components.

Finally, Group 4 was composed of accessions which did not produce any pegs, pods, and seeds. Their leaves, flowers, and stems characteristics were relatively similar to

the ones displayed by the members of Group 3, which presented large sizes for these structures. So, based on the fact that this group did not produced seeds, this group could be called the "vegetative type" group. Accession 604817 is presented in Figure 4-13 as an exemplar of this group.

Table 4-6. Morphological characteristics of *Arachis pintoi* accession groups obtained by the cluster analysis

Characteristics	Group 1	Group 2	Group 3	Group 4
Quantitative descriptors†				
Flower/inflorescence	3.14 a[a]	3.03 a	3.34 a	3.00 a
Flower standard width	14.17 b	15.15 ab	16.23 a	16.07 a
Flower standard length	10.80 b	11.32 ab	12.40 a	12.06 a
Flower wing width	5.72 c	6.13 bc	6.81 a	6.62 ab
Flower wing length	7.53 b	7.85 b	8.64 a	8.49 a
Flower keel length	4.98 a	5.02 a	5.21 a	5.20 a
Flower hypanthium length	65.00 b	64.00 b	79.00 a	78.00 a
Flower hypanthium width	0.94 b	0.91 b	1.08 a	0.94 b
Stem internode length	26.40 a	32.27 a	28.13 a	31.94 a
Stem internode diameter	2.72 a	2.79 a	3.14 a	3.34 a
Leaflet length	20.68 b	26.61 a	27.80 a	24.69 a
Leaflet width	13.01 b	16.73 a	17.23 a	12.91 b
Leaf Petiole length	16.13 c	27.44 a	23.89 ab	21.42 b
Peg length	9.85 b	13.66 a	12.14 ab	0.00 c
Peg width	0.97 a	0.89 a	0.96 a	0.00 b
Pod weight	0.11 b	0.18 a	0.17 a	0.00 c
Pod length	10.23 b	12.07 a	11.53 a	0.00 c
Pod width	5.26 c	5.66 b	6.12 a	0.00 d
Seed width	4.17 c	4.80 b	5.15 a	0.00 d
Seed length	7.33 b	9.56 a	8.96 a	0.00 c
Seed weight	0.08 b	0.14 a	0.13 a	0.00 c
Qualitative descriptors††				
Flower standard color	Yellow	Yellow	Yellow	Yellow
Flower standard crescent	Present	Present	Present	Absent
Flower hypanthium color	Absent	Present	Absent	Absent
Flower hypanthium hairiness	Present	Present	Present	Present
Stem color	Absent	Absent	Absent	Present
Stem hairiness	Present	Absent	Abundant	Present
Stem bristles	Absent	Absent	Absent	Absent
Leaflet shape	Obovate	Obovate	Narrow ellipitc	Obovate
Leaflet hairiness sup. surface	Absent	Absent	Absent	Absent
Leaflet hairiness margin	Absent	Absent	Present	Present
Leaflet hairiness inf. surface	Present	Absent	Present	Absent
Leaflet bristles margin	Present	Present	Present	Present
Peg color	Present	Present	Absent	-
Peg hairiness	Present	Present	Absent	-
Pod beak	Moderate	Slight	Moderate	-
Pod reticulation	Moderate	Moderate	Slight	-
Seed color	Yellow brownish Orange yellowish		Orange yellowish	-

† Mean

†† Mode

[a] Differences between means of different groups were tested by Student Newman-Keuls test. Means followed by same letter are not different at p=0.05.

Figure 4-10. Group 1 representative accession (PI 497541).

Figure 4-11. Group 2 representative accession (PI 604814).

Figure 4-12. Group 3 representative accession (PI 604798).

Figure 4-13. Group 4 representative accession (PI 604817).

Summary and Conclusions

Thirty-five germplasm accessions of *Arachis pintoi* were morphologically characterized using a list of descriptors prepared by IBPGR/ICRISAT (1990 and 1992) Data from stems, leaves, flowers, pegs, pods, and seeds were collected and comparisons among accessions were made, based on the mean, standard deviations, and range of the quantitative features and the mode of the qualitative characteristics. Phenotypic correlations were conducted among descriptors, and Pearson's correlation coefficient and significance test were also calculated. Simpson and Shannon-Weaver's diversity index were computed for each descriptor to access the genetic diversity among the accessions for individual descriptors. Principal component analysis was then executed to discriminate the accessions, and finally a cluster analysis was performed to group the germplasm in accordance with its morphological similarities.

The germplasm presented great morphological variability with all the descriptors, except pollen size and shape, leaf bristles superior and inferior surface, showing polymorphism.

From 595 correlations computed, 96 were statistically significant. These 96 significant correlations could be divided in eight different groups. However, when only the biologically meaningful correlations ($r \geq 0.71$) were evaluated, the number of significant correlations dropped to 29, and only four groups were observed. These meaningful correlations were found between leaf length (LL) and pod weight (Podwe); leaf length (LL) and pod width (PodW); leaf length (LL) and seed weight (Swe); leaf length (LL) and seed width (SW); and finally leaf length (LL) and seed length (SL). Thus, leaf length could be used as a selection criterion in programs where increased seed size is one of the objectives.

Diversity values were variable among traits, but in general all morphological features expressed high genetic diversity. According to Simpson's index, leaf shape (0.83), seed color (0.82), flower standard width (0.81), and seed length (0.75) were the descriptors with most diversity. In contrast, the lowest values were related to flower standard color (0.18), flower standard crescent (0.24), and flower hypanthium hairiness (0.24). The total Simpson's index to all morphological descriptors was 0.58.

Alternatively, Shannon-Weaver's values were in general higher than Simpson's, with higher values displayed by leaf hairiness inferior surface (1.00), flower hypanthium color (1.00), leaf hairiness margins (0.80), and pod reticulation (0.97). Total genetic diversity was estimated as 0.71.

The first five principal components explained 67.7% of the total variation, with PC1 explaining 30.0% of the variation, PC2 15.2%, PC3 10.1%, PC4 6.6%, and PC5 5.8% of the total variation. The principal component analysis was able to discriminate and separate the accessions in terms of three dimensions, represented by the "sexual reproduction", "vegetative", and "qualitative" axes.

The cluster analysis based on the first nine principal components differentiated four distinct groups of accessions. Group 1 was composed by accessions with small leaves, flowers, pods, pegs, and seeds. Group 2 was formed by accessions with intermediate size features, Group 3 was formed by accessions with large leaves, flowers, pegs, pods, and seeds, and finally, Group 4 was composed of accessions which did not produce any pegs, pods, and seeds.

CHAPTER 5
AGRONOMIC EVALUATION OF *Arachis pintoi* GERMPLASM

Introduction

Agronomic evaluation is a very important step in germplasm characterization programs. Although molecular and morphological characteristics are relevant, plant breeders and ultimately producers have their attention focused on the potential of the plant to grow well in their environment and to produce forage, grain, or other economic products. Thus, agronomic evaluation will always be a key component in breeding programs.

When evaluating a species outside its original environment, it is important to assess its adaptation to the new ecosystem. Emphasis must be given to how soils, climate, and rainfall conditions will impact the growth of this "new species". Along with adaptation, several agronomic characteristics can be measured. The importance of each variable will be defined by the use of the plant and by the environment where it will be cultivated. In the case of a forage crop such as *Arachis pintoi* growing in a subtropical environment like Florida, forage yield, forage nutritive value, seed production, winter survival, and nematode resistance are just some of the characteristics that should be evaluated.

Arachis pintoi is native of and well adapted to certain tropical environments. According to Pizarro and Rincón (1994), *A. pintoi* was evaluated by the International Tropical Pastures Evaluation Network (RIEPT) in Brazil, Uruguay, Bolivia, Colombia, Peru, and Venezuela under savanna and humid tropical conditions. They concluded that it

presented a wide range of adaptation and grows best under humid tropical conditions with total annual rainfall ranging from 2000 to 4000 mm.

Fisher and Cruz (1994), however suggested that although *A. pintoi* grows well under high rainfall conditions it can tolerate periods of drought. They reported that *A. pintoi* was able to maintain a large proportion of its aerial parts at the expense of root tissue when exposed to 8 wk of water deficit. The same authors also revealed that *A. pintoi* did not tolerate long periods of flooding. They concluded that 3 wk of flooding severally restricted plant growth with severe leaf chlorosis and reduction of leaf area.

Pizarro and Rincón (1994) reported that plants growing in a subtropical environment in Pelotas-Brazil were exposed to severe frosts (Temperature < 0ºC) that reduced growth, but did not kill the plant stands. They recovered after the return of warm and rainy conditions.

The literature has abundant *A. pintoi* forage yield data collected in the tropics. In evaluations performed in Bolivia, Brazil, Ecuador, Colombia, and Peru, accession CIAT 17434 produced between 0 and 2.7 Mg ha^{-1} of DM during the rainy season and 0.04 to 2.8 Mg ha^{-1} of DM during the dry season with a growing period of 12 wk (Pizarro and Rincón, 1994). In Costa Rica, Argel and Valerio (1993) reported forage yields of 7, 12 and 7 Mg ha^{-1} of DM for accessions CIAT 17434, 18744, and 18748, with 20 mo of growth in Guapiles and San Isidro. In Puerto Rico, forage dry matter yields of 2.1 Mg ha^{-1} of DM were harvested 16 wk after planting from the accession CIAT 17434 (Argel, 1994).

Several publications also report forage nutritive value and seed production data of *Arachis pintoi* in the tropics. Crude protein values ranging from 120 to 220 g kg^{-1} and in

vitro digestibility (IVOMD) ranging from 560 to 700 g kg^{-1} were reported from Argel & Pizarro (1992); Rincón et al. (1992); Carulla et al. (1991); and Pizarro and Carvalho (1996).

Average seed yields of *A. pintoi* varied from 1 to 2 Mg ha^{-1} when harvested at 15 to 18 mo after planting (Ferguson et al., 1992). However, in Colombia, when planted in soils with high fertility, Ferguson (1994) reported yields of 7.3 Mg ha^{-1}. In Australia, Cook and Franklin (1988) reported seed yields of 1.4 Mg ha^{-1}, 12 mo after sowing cv. Amarillo. Cook and Lock (1993), also working with the cv. Amarillo in Australia, stated that seed yields of 2.8 Mg ha^{-1} were obtained in a commercial seed crop.

As stated before, the literature has several examples of research work done in the past where agronomic characterization of *Arachis pintoi* was the primary goal. However, most of this work wase done with a single germplasm accession, that latter was released as a commercial cultivars in several different countries. These studies also have in common the fact that most of them where executed in tropical regions. Therefore, there is a lack of information about other accessions of *A. pintoi* stored in germplasm banks, there are little or no existing data regarding the performance of the same germplasm in subtropical conditions.

The goal of this research was to evaluate the agronomic adaptation, forage yield, seed yield, forage nutritive value, and nematode resistance of several *A. pintoi* germplasm accessions stored at the USDA-NPGS germplasm bank.

Material and Methods

The Germplasm

Germplasm of *A. pintoi* stored at the USDA-NPGS germplasm bank located in Griffin, GA was transferred to the University of Florida on three different occasions. In

May 2001 the first set composed of 25 accessions was sent, the second set with 15 accessions, came in February 2002, and finally the third set with 13 accessions was sent in May 2003, for a total of 53 accessions. All accessions were received as vegetative material because seeds were not available at the time and also to ensure the genetic purity of each germplasm accession. A list with the identification number and geographical information about the site of collection is presented in Appendix A.

Field Evaluation

Adaptation and forage dry matter yield

In September 2001, 4 m² (2 m x 2 m) field plots of the original 25 accessions were established with four rooted cuttings in a randomized complete block design with two replications at the Agronomy Department Forage Research Unit of the University of Florida near Gainesville. The experiment was located on a Pomona sand (siliceous, Hyperthermic Typic Psammaquents – Entisol) soil type, a flatwood soil characteristic of north central Florida. The soil was prepared by disking and 190 kg ha^{-1} of 0-10-20 fertilizer was applied and incorporated into the soil. *Arachis glabrata* cultivars 'Florigraze' and 'Arbrook' were used as local standards and were also planted following the same scheme.

During the years 2001, 2002, and 2003 plots were maintained with periodical hand weeding and applications of the herbicide Cadre (Imazapic), in accordance with the recommended dosage for cultivated peanut (100 g ha^{-1}). Annual applications of 800 kg ha^{-1} of 0-10-20 were made in may 2002 and 2003.

Visual evaluations were made following the winter 2001/2002. In these evaluations number of plants per plot, plant survival and rate of spread were assessed. Plant survival was expressed as percentage of total plants alive and rate of spread in accordance with a

scale of 1 to 5 (1 < 10% plot coverage, 2 = 20%, 3 = 30%, 4 = 40%, and 5 > 50% plot coverage). These parameters were intended as indications of adaptation and winter survival. No dry matter harvest was made in 2002.

In 2003 visual evaluations of plot coverage and plant height were made. These evaluations were used as a criteria to determine which plots would be sampled to estimate forage dry matter yield (FDMY). FDMY was estimated on three dates during 2003, with an interval of 8 wk between harvests. These dates were 13 June (Harvest 1), 13 August (Harvest 2), and 21 October (Harvest 3). To estimate FDMY a sample of 0.25 m^2 was harvested at soil level using battery-powered hand clippers as a cutting tool. Pintoi peanut and weeds were manually separated and the samples were placed in a 65ºC forced-air drier for 72 h. Dry weight was measured using a digital scale. Plot borders were uniformly cut at soil level with a rotary lawn mowing tractor after each harvest.

FDMY was analyzed with Proc GLM of SAS (SAS institute, 1989) using the following model:

$Yij = \mu + Bi + Aj + eij$

Where:

μ = mean

Bi = block effect

Aj = accession effect

eij = experimental error

LSD at 5% significance level was used as the mean separation test.

This same model was used for forage nutritive value and seed production.

Forage nutritive value

Forage samples harvested on 13Aug 2003 were ground in a Wiley Mill, to pass through a 1 mm screen. This ground tissue was analyzed for crude protein and *in vitro* organic matter digestibility (IVOMD) in the Forage Evaluation Support Laboratory of the Agronomy Department, University of Florida.

The N analysis started with sample digestion using a modification of the aluminum block digestion procedure (Gallaher et al., 1975). In this procedure samples of 0.25 g and catalyst of 1.5 g of 9:1 K_2SO_4:$CuSO_4$ were used during a 4 h digestion at 375ºC using 6 ml of H_2SO_4 and 2 ml of H_2O_2. Digestate was then filtrated and N was determined by semiautomated colorimetry (Hambleton, 1977).

IVOMD was performed by a modified two-stage technique (Moore and Mott, 1974). Both crude protein and IVOMD were expressed on an organic matter basis.

Seed production

In February 2003 and 2004 plots were sampled to assess seed production. Samples were taken in each plot using a soil core sampler of 12.5 cm diameter and 24 cm depth (0.01227m^3). The soil was screened on a sieve with a 0.6 x 0.6 cm mesh to remove the pods from the soil. The pods were then dried at room temperature for 6 wk and weighed. Calculations were made to extrapolate the values in terms of production per hectare.

Nematode Response Evaluation

Stems of the *A. pintoi* germplasm accessions were cut and placed in vermiculate trays under an automated mist system (10 sec every 30 min) for rooting. After 45 d under the mist systems, the rooted cuttings were transferred to 150 cm^3 Conetainers® filled with methyl-bromide-fumigated fine sand topsoil. After transferring, plants were allowed to establish for 2 wk and then used in this experiment. On 12November 2003 plants were

inoculated with either *M. arenaria* race 1, *M. javanica*, or *M. incognita* race 1. During the experimental period, plants were watered daily and fertilized with 20-20-20 fertilizer every 2 wk. The green house temperature ranged from 15 to 25ºC during the 12 wk that the trial lasted.

Tomato plants were used to propagate the nematodes and then the Hussey and Barker (1973) method was used to extract eggs and juveniles. In this method, roots are cleaned, split in small pieces and washed in a 0.525 % sodium hypochlorite (NaOCl) solution for 2 min. The roots are then stirred strongly and passed through a 200-mesh sieve (openings 0.149-0.074 mm). The eggs and juveniles are collected on a 500-mesh (openings 0.028 mm) sieve placed under the 200-mesh one. Eggs are subsequently rinsed with H_2O, pored to a beaker and water is added to bring the volume to 1000 ml. A sample is taken, placed on a slide, and the number of eggs per ml is estimated by counting under the microscope. Prior to injecting the egg suspension into the soils, the solution was diluted to 300 eggs per ml. This procedure was followed for each one of the three nematodes used in this experiment.

To each container 5 ml of egg suspension was applied, which brings the total eggs per container to 1500 or 10 eggs cm^3 of soil. Application was delivered with a veterinarian surgical syringe, and during the whole process the eggs were kept in continual suspension by a magnetic stirrer.

The experimental design was a randomized complete block, with four replications for *M. arenaria*, and three replications for *M. javanica* and *M. incognita*. A single plant constituted each replication. *Arachis hypogaea* cv. ‘Florunner’ was used as a susceptible control to verify inoculum viability.

Twelve weeks after inoculation plants were removed from the containers and soil was carefully washed from the roots with tap water. Plants were then placed in a bucket with roots immersed in a 0.25% Phloxine B solution to stain the egg masses. Roots were rated for gall index (GI), gall size (GS), and percent galled area (GA) in a 1-9 scale and after that a damage index (DI) was calculated based on the same parameters (Sharma et al., 1999). DI was calculated by the following equation: DI = (GI+GS+GA)/3. GI, GS, GA and DI scales are presented in Table 5-1.

Number of egg masses (EI) was rated with a 1-9 scale similar to gall index, where 1 represented no egg masses and 9 more than 100 egg masses. Accessions with EI = 1 were considered highly resistant to nematode reproduction and with EI = 9 were highly susceptible. Intermediate values followed the DI scale.

Mean and standard error of the mean were computed for EI and DI and these variables were used to classify the accessions in relation to its nematode reaction.

Table 5-1. Gall Index, gall size, percent galled area and damage index values

Scale value	Gall index (GI)	Gall size (GS)	Percent galled area (GA)	Damage Index (DI)
1	No galls	No galls	No galls	Highly resistant
2	1-5 galls			Resistant
3	6-10 galls	10% increase	1-10% root galled	Resistant
4	11-20 galls			Moderate resistant
5	21-30 galls	30% increase	11-30% root galled	Moderate resistant
6	31-50 galls			Susceptible
7	51-70 galls	31-50% increase	31-50% root galled	Susceptible
8	71-100 galls			Highly susceptible
9	>100 galls	>50% increase	>50% root galled	Highly susceptible

Accessions with DI and/or EI =1, were considered highly resistant; DI and/or EI > 1 and ≤ 3, were classified as resistant; DI and/or EI > 3 and ≤ 5, were classified as moderately resistant; DI and/or EI > 5 and ≤ 7, were classified as susceptible; and finally accessions

with DI and/or EI > 7 and ≤ 9, were classified as highly susceptible. If there was discrepancy between DI and EI values, the higher value was applied.

Results and Discussion

Adaptation and forage dry matter yield

The evaluations performed in 2002, eight, eleven, and twelve months after planting, revealed that although *A. pintoi* presented great reduction of green tissue during the winter, most plants did not die, and in fact, they fully recovered when the temperature warmed and soil moisture increased (Table 5-2). During the 2001/2002 winter, temperatures reached 0ºC or less on 24 different occasions (Appendix D).

The average plant survival (PS%) in Evaluation 1 was 79%, which shows that *A. pintoi* can tolerate winters where freezing and frosting are normal occurrences. However, when rate of spread (RS) was analyzed, most of the accessions were covering less than 50% of the plot area. Cultivars 'Florigraze'and 'Arbrook' presented 100% of plant survival, but they also covered less than 50% of the plot area.

This same trend was displayed in Evaluations 2 and 3, where average plant survival recorded was superior to 80% and plot coverage inferior to 50%. Actuality, this is one of the biggest problems that *A. pintoi* and also *A. glabrata* display, the fact that both species required a long period to establish themselves and cover the area where they were planted.

Even with general low plot cover, we can observe differences among accessions in relation to plant survival and plot coverage, which is an indication of variable in adaptation to the north Florida environment. Accessions PI 497574, 604798, 604800, 604803, 604807, 604814, 604817, and 604857 are the ones that appear better adapted, with values of plant survival and rate of spread superior to the others.

Table 5-2. Winter survival evaluations of *Arachis pintoi* at the forage research unit in Gainesville-FL

Accession	Evaluation 1 (04/25/2002)			Evaluation 2 (07/03/2002)			Evaluation 3 (08/23/2002)		
PI number	#PL †	PS (%)	RS	#PL	PS (%)	RS	#PL	PS (%)	RS
476132	3.0	75	1.0	3.0	75	2.4	4.0	100	2.0
497541	2.0	50	0.5	2.0	50	1.0	4.0	100	1.0
497574	4.0	100	2.5	3.0	75	1.5	4.0	100	2.5
604798	3.5	87	2.0	3.0	75	3.0	4.0	100	3.0
604799	3.0	75	3.0	3.0	75	2.5	4.0	100	2.0
604800	3.5	87	2.0	3.0	75	2.0	3.5	87	2.5
604801	1.0	25	0.5	1.0	25	1.0	3.5	87	2.0
604803	3.5	87	2.0	3.5	87	2.8	4.0	100	3.0
604804	4.0	100	1.5	3.0	75	1.5	3.0	75	1.5
604805	3.0	75	2.0	3.0	75	2.0	4.0	100	1.5
604807	3.5	87	2.5	3.5	87	2.0	4.0	100	3.0
604808	4.0	100	4.0	4.0	100	4.5	4.0	100	2.0
604809	3.0	75	2.0	3.0	75	3.0	3.5	87	2.5
604810	3.0	75	2.5	2.0	50	2.0	3.5	87	1.5
604811	4.0	100	2.5	4.0	100	2.0	4.0	100	1.5
604812	2.0	50	1.0	1.5	37	1.5	4.0	100	2.0
604813	4.0	100	1.5	4.0	100	1.5	4.0	100	1.5
604814	1.5	37	0.5	3.0	75	2.4	3.5	87	4.0
604815	2.0	50	2.0	0.5	12	1.0	4.0	100	2.0
604817	4.0	100	2.5	3.8	94	3.5	4.0	100	3.5
604856	2.5	62	1.0	3.0	75	1.5	4.0	100	2.0
604857	3.5	87	2.0	3.8	94	3.2	4.0	100	3.0
604858	3.5	87	3.0	3.3	81	3.5	4.0	100	2.5
604859	1.0	25	1.0	3.8	94	3.2	1.5	37	1.5
Arbrook	4.0	100	4.0	3.3	81	3.5	4.0	100	4.5
Florigraze	4.0	100	2.5	3.0	75	3.0	4.0	100	2.5

† #PL = number of plants per plot; PS (%) = plant survival; RS= Rate of spread

Pizarro and Rincón (1994) affirmed that *A. pintoi* is slow establishment is a limitation to adoption as producers want to see quick results. They suggested that when seeds are used as the propagation material the establishment time is shortened when compared to vegetative propagation. French et al. (1994) stated that although adequate establishment has been achieved in Florida with rhizoma peanut (*A. glabrata*) in a single year, a typical planting demands 2 to 3 yr for full development. Cook et al. (1994) agreed

with the authors previously cited. They believe that the major constraint to farmer acceptance of these species is the complexity and cost of establishment.

Slow establishment is particularly significant where weed pressure is high. This fact will require extra resources to assure an adequate initial coverage that will extend the period of utilization of the pasture or hay field. Research is necessary to determine the best planting method, using the different factors that could result in a fast and inexpensive pasture establishment.

In 2003, 21 mo after establishment, samples were harvested on three different occasions to determine FDMY. Before each harvest, evaluations were made to estimate percentage of plot covered (% cover) and plant height (Table 5-3).

At Harvest 1 (13 June 2003), average plot coverage was estimated as 62% among the *A. pinto*i germplasm. Accessions PI 497574, 604808, 604817, and 604858 showed the best plot coverage with values above 90%. In contrast, accessions 604801, 604804, 604812, 604814, and 604859 had plot coverage less than 25%. Plot coverage is reflected in the FDMY. In terms of height, the average was 6 cm with little variation. This number is very contrasts markedly with Florigraze and Arbrook that presented values of 11 and 20, respectively. It seems clear that the two species have a very different growth habit, and while *A. pintoi* exhibits a more prostrate habit, *A. glabrata* is more erect.

At Harvest 2 (13 Aug. 2003), average plot coverage was 66%, with a general increase in covered area by all accessions. The average height was similar to the previous evaluation. Harvest 3 (21 Oct. 2003) presented similar average plot coverage and plant height as Harvest 2. A general increase in covered area was also observed in this evaluation.

Table 5-3. Plot coverage and plant height before forage dry matter yield evaluations of *Arachis pintoi* at the forage research unit in Gainesville-FL in 2003

Accession	Harvest 1†		Harvest 2		Harvest 3	
PI number	% cover	Height (cm)	% cover	Height (cm)	% cover	Height (cm)
476132	47	5.5	30	7	40	8
497541	57	3	42	6.5	47	3
497574	95	7.5	95	8	100	6.5
604798	62	6	70	8.5	75	8.5
604799	87	5	75	5.5	65	5
604800	85	7	80	8	80	7
604801	27	4	42	5	55	5.5
604803	80	14	85	15.5	77	10.5
604804	17	2.5	15	3	10	1.5
604805	62	5	65	5.5	50	6
604807	57	6.5	40	5	40	4.5
604808	92	5.5	100	6	100	4.5
604809	50	6	60	8.5	65	6
604810	62	4	75	5	75	5
604811	67	5.5	85	7	85	7
604812	22	2.5	37	5.5	47	4.5
604813	67	5	80	6.5	90	3
604814	17	4	20	3.5	25	3
604815	82	8.5	100	9	95	8
604817	95	8	100	10.5	100	7
604856	60	4	72	5	80	5
604857	80	9	75	10	82	8.5
604858	97	7.5	95	7.5	100	6.5
604859	22	6.5	37	9	55	9
'Florigraze'	82	11.5	100	14	95	7.5
'Arbrook'	100	20.5	100	22.5	100	12

† Harvest 1: 13 Sep. 2003; Harvest 2: 13 Aug. 2003; Harvest 3: 21 Oct. 2003

A careful analysis of these evaluations demonstrates again that there are large differences among accessions in relation to environmental adaptation. Accessions PI 604801, 604804, 604812, 604814, and 604859 presented low values of plot coverage in each of the three harvest. This is very significant, since the Harvest 3 was completed at 25 months after plot establishment. Another significant fact that can be extracted from these evaluations is that in Harvest 3 the values of plot coverage and plant height

displayed a significant drop in relation to the values of the same variables for Harvest 1 and 2.

Forage dry matter yield (FDMY) was assessed three times during summer 2003 with an 8-wk interval between harvests. Analysis of variance table for Harvest 1, 2, and 3 is presented below (Table 5-4).

Table 5-4. Analysis of variance table of forage dry matter yield (FDMY) evaluations of *Arachis pintoi* at the forage research unit in Gainesville-FL in 2003

Source	df	Harvest 1†		Harvest 2		Harvest 3	
		MS	Pr>F	MS	Pr>F	MS	Pr>F
Accession	25	1.19	0.0061	2.28	0.0033	1.05	0.0060
Replication	1	0.82	0.1758	0.13	0.6824	0.15	0.5253
Error	25	0.42		0.74		0.37	
Total	51						

† Harvest 1: 13 Sep. 2003; Harvest 2: 13 Aug. 2003; Harvest 3: 21 Oct. 2003

Significant differences ($P < 0.01$) were present among accessions in all three FDMY harvests, indicating that accessions show variability in their environmental adaptation, an attribute that will consequently impact their forage production potential. Replication effects were not significant.

Forage dry matter yield at each harvest date in 2003 is presented in the Table 5-5. A large amount of variability was present among these *A. pintoi* accessions with respect to their FDMY. The average FDMY at Harvest 1 was 1.34 Mg ha^{-1}, ranging from 0.06 to 2.86 Mg ha^{-1}. More than half of the accessions presented superior performance producing above the average, but accessions PI 604801, 604804, 604812, and 604814 had very low yields. These low yields are a reflection of poor plot coverage for these accessions as they had less than 30% plot coverage at Harvest 1. In comparison, accessions PI 604817, 604815, 604803, 604808, 604810, 604857, and 497574 were the most productive, showing no differences in FDMY to the rhizoma peanut cultivars.

Table 5-5. Forage dry matter yield of *Arachis pintoi* germplasm at the Forage Research Unit near Gainesville, FL in 2003

Accession	Harvest 1†	Harvest 2	Harvest 3
	----------Mg ha^{-1}----------		
476132	0.79	0.75	0.98
497541	1.18	0.94	0.44
497574	1.85	3.00	2.50
604798	1.51	1.52	1.38
604799	1.35	1.74	1.60
604800	1.39	1.98	1.33
604801	0.34	1.10	0.93
604803	1.99	1.93	1.74
604804	0.06	0	0
604805	1.58	1.48	0.88
604807	1.38	0.78	0.56
604808	1.98	2.62	1.99
604809	1.47	0.38	1.14
604810	1.96	2.54	2.14
604811	1.06	1.45	1.63
604812	0.19	0.6	0.82
604813	0.98	1.72	1.01
604814	0.10	0	0
604815	2.32	2.12	2.30
604817	2.86	3.45	2.79
604856	1.12	1.90	1.56
604857	1.85	2.26	2.24
604858	1.59	2.25	1.87
604859	1.38	2.29	1.63
'Arbrook'	3.16	4.75	1.76
'Florigraze'	2.14	1.89	0.95
LSD	0.79	0.75	0.98

† Harvest 1: 13 Sep. 2003; Harvest 2: 13 Aug. 2003; Harvest 3: 21 Oct. 2003

At Harvest 2 the average FDMY of *A. pintoi* germplasm showed a 20% increase reaching 1.62 Mg ha^{-1}. Accessions PI 604804 and 604814 showed no production, which reflects low plot coverage and poor adaptation to the north Florida environment. Here once more, half of the accessions presented superior performance with FMY above the average. Accessions PI 604817 and 497574 were the most productive, showing no difference to Arbrook, which produced 4.75 Mg ha^{-1}. Florigraze however, was intermediate in FDMY and similar to a group of *A. pintoi* accessions. This is somewhat

surprising, since it is regarded by many as the most adapted and productive of the rhizoma peanut cultivars.

Harvest 3 presented mean FDMY of 1.39 Mg ha^{-1}, which corresponded to a drop of about 20% in relation to the preceding harvest. Arbrook and Florigraze exhibited a significant reduction in FDMY of ca. 50% compared to previous period. *A. pintoi* germplasm also exhibited this reduction, however with less decrease (Figure 5.1). This reduction in FDMY could represent a change in nutrient partitioning as a process to overcome restriction in growth due to the shortening days in autumn. Accessions PI 604817, 497574, 604815, and 604857 had the highest FDMY and were significantly higher than 'Florigraze' and 'Arbrook' rhizoma peanut.

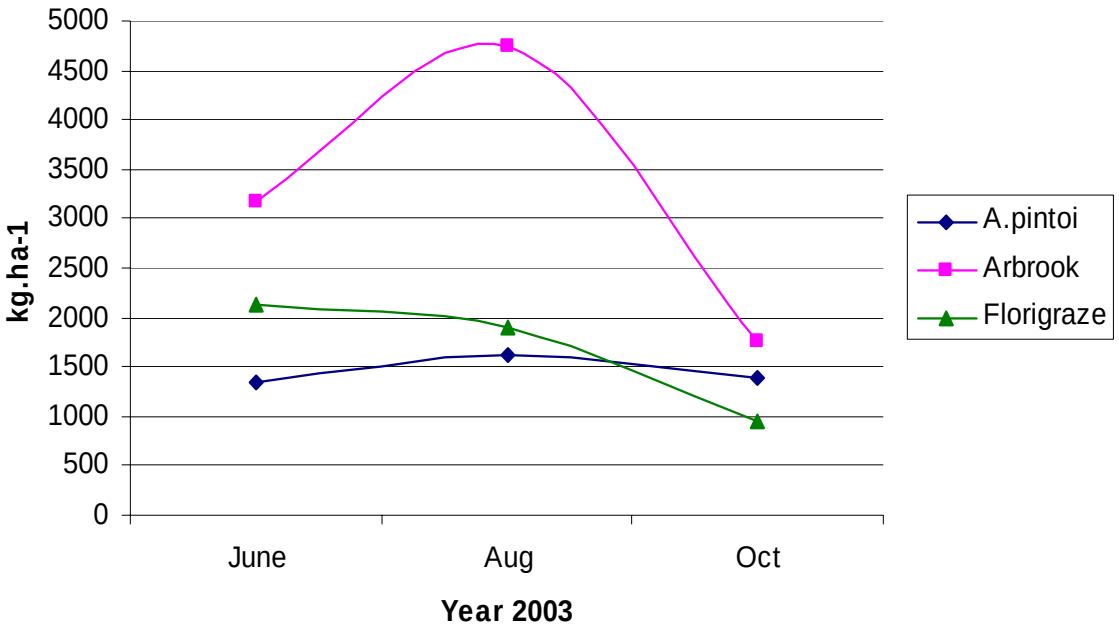

Figure 5-1. Forage dry matter yield (FDMY) of *Arachis pintoi* and *A. glabrata* cultivars at the forage research unit in Gainesville-FL in 2003.

Adding the FDMY of the three individual harvests allows the analysis of the annual forage production of the germplasm. The analysis of variance table for total FDMY shows that there were significant ($P < 0.01$) differences among accessions.

Annual average FDMY for *A. pintoi* accessions was 4.36 Mg ha^{-1} (Table 5-7). Arbrook produced 9.67 Mg ha^{-1} and was the most productive. Accessions PI 604817, 497574, 604815, 604810, 604808, and 604857 were the most productive among the *A. pintoi* germplasm and were not different from Arbrook.

Table 5-6. Analysis of variance table of the annual forage dry matter yield (FDMY) of *Arachis pintoi* at the forage research unit in Gainesville-FL in 2003

Source	df	Total FDMY†	
		MS	Pr>F
Accession	25	11.48	0.0014
Replication	1	2.72	0.3726
Error	25	3.30	
Total	51		

†Total FDMY = Harvest 1 + Harvest 2 + Harvest 3

Several *A. pintoi* accessions yielded more than 5.00 Mg ha^{-1} which can be considered a good legume production in north central Florida. In fact this number is similar to yields displayed by other summer legumes used in Florida, and even could be compared to *A. pintoi* or others legume species yields obtained in tropical regions.

Kretschmer et al. (1988) reported 3-yr average FDMY for *Desmodium heterocarpon* cv. 'Florida', *Macroptilium atropurpureum* cv. 'Siratro', and *Arachis Kretschmeri* 'Pantanal' of the order of 2.97, 2.39, and 1.16 Mg ha^{-1} respectively. Annual yields of *Stylosanthes guianensis* cv. 'Savanna', *Alysicarpus vaginalis* and *Indigofera hirsuta* cv. 'Flamingo' ranged from 5 to7 Mg ha^{-1}, in plantings at Brooksville, FL during years 1989 and 1990 (Williams et al., 1993). Mislevy and Martin (2001) reported similar annual FDMY (5.70 Mg ha^{-1}) for *Aeschynomene evenia* at Ona, FL.

Table 5-7. Total forage dry matter yield of *Arachis pintoi* at the Forage Research Unit near Gainesville, FL in 2003

Accession	Total FDMY†
	----------Mg ha^{-1}----------
476132	2.52
497541	2.56
497574	7.36
604798	4.42
604799	4.69
604800	4.71
604801	2.37
604803	5.67
604804	0.06
604805	3.93
604807	2.73
604808	6.59
604809	2.99
604810	6.63
604811	4.14
604812	1.61
604813	3.71
604814	0.10
604815	6.74
604817	9.10
604856	4.58
604857	6.35
604858	5.71
604859	5.30
Arbrook	9.67
Florigraze	4.98
LSD	3.74

†Total FDMY = Harvest 1 + Harvest 2 + Harvest 3

In tropical areas, comparable yields were presented by Souza et al. (1992) who evaluated *Stylosanthes* and *Centrosema* germplasm in central Brazil. Average FDMY of 1.50 Mg ha^{-1} were harvested in a 12-wk growing period during the summers of 1989 and 1990. Pizarro et al. (1996c) reported annual FDMY of *Calopogonium mucunoides* of the order of 1.72 Mg ha^{-1} in research conducted at several different locations in South America.

Data from diverse agronomic characterization of *A. pintoi* are also similar to those presented in this work. Argel and Pizarro (1992) stated that cv. 'Amarillo' produced 2.30 Mg ha^{-1} in Planaltina, Brazil, during the rainy season. In Australia, Cook et al. (1990) stated that Amarillo yielded 6.50 and 7.30 Mg ha^{-1} yr^{-1} in unirrigated and irrigated conditions respectively. Also in Australia, Cook et al. (1994) presented average annual yields of 5.80 Mg ha^{-1} for Amarillo in two years of evaluation.

Although the yields obtained in this research were similar to those of other summer legumes in Florida, these data are relative to just 1 yr of evaluation. Supplementary information must be obtained if broader application of the data are to be made, because persistence is one of the major issues with forage legumes.

Forage nutritive value

The analysis of variance table for CP and IVOMD demonstrated that significant variation ($P < 0.01$) was observed among accessions for both variables (Table 5-8).

Table 5-8. Analysis of variance table of crude protein (CP) and in vitro organic matter digestion (IVOMD) of 8-wk regrowth of *Arachis pintoi* at the Forage Research Unit near Gainesville, FL in 2003

Source	df	CP†		IVOMD	
		MS	Pr>F	MS	Pr>F
Accession	25	2381.7	0.0002	301.9	0.0045
Replication	1	470.2	0.0001	392.4	0.0568
Error	25	495.2		97.5	
Total	51				

†CP= Crude Protein and IVOMD= In vitro organic matter digestibility

Crude protein and IVOMD values of *A. pintoi* accessions from this research confirm those reported in the literature, and support the information that this species produces high quality forage. The results endorse the claim of some that *A. pintoi* could be considered a "tropical alfalfa" (Table 5-9).

Average crude protein concentration across *A. pintoi* accessions was 180 g kg^{-1} ranging from 139 (PI 604800) to 225 g kg^{-1} (PI 604858). Florigraze CP was 172 g kg^{-1} while that of Arbrook was 153 g kg^{-1}. When CP of the *A. pintoi* germplasm was compared to the rhizoma peanut cultivars, the majority of the accessions demonstrated higher values than either rhizoma peanut cultivars.

Average IVOMD of the *A. pintoi* germplasm was 670 g kg^{-1}, and ranged from 600 to 730 g kg^{-1}. Accession PI 604812 had the lowest value, while PI 604801 was the highest. Florigraze presented the highest IVOMD value (740 g kg^{-1}), however several *A. pintoi* accessions were not different ($P \geq 0.05$) from it.

CP and IVOMD displayed in this research could be considered high and comparable to those reported for *A. pintoi* in other investigations. Average values of CP in the leaves of accession CIAT 17434 varied from 122 to 218 g kg^{-1} in Colombia, during dry and rainy seasons, respectively. In stems the same accession presented values of 93 and 135 g kg^{-1} during the same seasons (Argel and Pizarro, 1992). In Brazil, Purcino and Viana (1994) reported total-herbage CP values of 183, 157 and 161 g kg^{-1}, for accessions BRA-013251 (PI 338447), BRA-015253 (PI 604859) and BRA-015598 (PI 604815), respectively. Rincon et al. (1992) reported CP values for the whole plant of 130 and 180 g kg^{-1}, during the dry and rainy season, respectively. Average values of IVOMD during the same periods were 67 and 62 g kg^{-1}.

Average IVOMD of 168 d growth forage from three *A. pintoi* accessions in Brazil was 610 g kg^{-1} (Pizarro and Carvalho, 1992). In Australia, Amarillo IVDMD was 730 g kg^{-1} in 2 yr of evaluation (Cook et al., 1994).

Table 5-9. Crude protein (CP) and in vitro organic matter digestibility (IVOMD) of 8-wk regrowth of *Arachis pintoi* at the Forage Research Unit near Gainesville, FL in 2003

Accession	CP (g kg^{-1})	IVOMD (g kg^{-1})
476132	178	690
497541	147	630
497574	184	640
604798	170	680
604799	189	700
604800	139	700
604801	188	730
604803	195	670
604805	151	690
604807	156	680
604808	189	620
604809	148	620
604810	187	610
604811	189	690
604812	183	600
604813	166	660
604815	194	690
604817	212	670
604856	180	710
604857	222	720
604858	225	720
604859	173	710
'Arbrook'	153	690
'Florigraze'	172	740

+ LSD CP (0.05) = 30.3 g kg^{-1}, LSD IVOMD (0.05) =65 g kg^{-1}

Seed production

Another important factor for which genetic variability should be investigated is seed production. In February 2003 and 2004, respectively, 18 and 30 mo after planting, samples were collected in each plot to assess this trait.

The analysis of variance table for seed production (Table 5-10) showed significant variation ($P < 0.01$) among accessions for both the 2003 and 2004 years.

In 2003, the average seed production was 0.32 Mg ha^{-1}, ranging from zero to 2 Mg ha^{-1} (Table 5-11). Only 60% of the *A. pintoi* germplasm yielded seeds, however, if we

consider only those accessions with production above 0.1 Mg ha^{-1} this number falls below 50%. Accession PI 604857 was the most productive yielding 2 Mg ha^{-1}.

Table 5-10. Analysis of variance table of seed production of *Arachis pintoi* at the Forage Research Unit near Gainesville, FL in 2003 and 2004

Source	df	Seed Production 2003†		Seed Production 2004†	
		MS	Pr>F	MS	Pr>F
Accession	25	707.75	0.0001	929.12	0.0062
Replication	1	706.52	0.0023	850.83	0.1504
Error	25	47.46		40.120	
Total	51				

† pod weight

In 2004, with the exception of three accessions, all accession produced seeds. The average seed production was higher than the previous year reaching 0.43 Mg ha^{-1}, ranging from zero to 1.58 Mg ha^{-1} (Table 5-11). In general, yields of most accessions increased when compared to the 2003 evaluation. Exceptions were PI 604803, 604809, 604815, and 604857. Curiously, these accessions were among the most productive in 2003. Although 87% of the accessions produced some seeds, six of then yielded less than 0.10 Mg ha^{-1}, amount that would not be useful for commercial harvest. PI 604799 had the highest yield (1.58 Mg.ha^{-1}) in 2004 although it was intermediate in seed yield in 2003.

Seed production obtained in this research for some of the accessions was comparable to that obtained by other authors working with *A. pintoi* in tropical conditions. For legumes this trait has particular importance because it can be related to persistence. A species with high seed production capacity may have an advantage over one lacking this characteristic, because in theory, pasture establishment by seeds is simpler and cheaper than when vegetative material is used. Also, plants established by seeds will grow much faster, and consequently will have higher ground covered area. *A.*

pintoi has also a notable characteristic that differentiates it from other legumes in relation to seed production, in that the seeds are located below ground.

Table 5-11. Seed production of *Arachis pintoi* at the Forage Research Unit near Gainesville, FL in 2003 and 2004

Accession	Seed Production 2003†	Seed Production 2004†
	----------Mg ha^{-1}----------	
476132	0.01	0.07
497541	0.00	0.03
497574	0.00	0.36
604798	0.00	0.69
604799	0.75	1.58
604800	0.03	0.16
604801	0.00	1.10
604803	0.76	0.20
604804	0.00	0.00
604805	0.59	1.21
604807	0.29	0.36
604808	0.30	1.01
604809	0.91	0.43
604810	0.07	0.06
604811	0.00	0.02
604812	1.30	1.32
604813	0.00	0.00
604814	0.00	0.10
604815	0.47	0.17
604817	0.00	0.00
604856	0.08	0.05
604857	2.00	0.62
604858	0.06	0.27
604859	0.00	0.63
'Arbrook'	0.00	0.00
'Florigraze'	0.00	0.00
LSD	0.48	0.90

† pod weight

As previously stated, yields in this research were comparable to others found in the literature. Cook and Franklin (1988) reported mechanized harvest yield for Amarillo in Australia of 1.40 Mg ha^{-1} 12 mo after planting. In other work, seed yield of Amarillo in Australia reached an average production of 2.80 Mg ha^{-1} (Cook and Loch, 1993).

In a Costa Rica location with 4260 mm annual rainfall, Diulgheroff et al. (1990) obtained seed yields of 1.95 Mg ha^{-1}, 12 mo after sowing the cv. 'Maní Forrajero perenne'. Also in Costa Rica, Argel and Valerio (1993) reported average yields of 0.59, 0.54, 0.50, and 0.52 Mg ha^{-1} over the accessions CIAT 17434 , 18744, and 18748, at 8, 12, 16, and 20 mo after sowing, respectively. Rincon et al. (1992) stated that seed yields of 2 Mg ha^{-1} were obtained for cv. 'Maní Forrajero perenne' in mixed pasture with *Brachiaria* under grazing. This supports the idea of excellent persistence potential for *A. pintoi* due to seedling recruitment.

Nematode response evaluation

Reaction to *Meloidogyne arenaria*, *M. javanica*, and *M. incognita* was established in accordance with the methodology proposed by Sharma et al. (1999). The analysis of variance of *A. pintoi* reaction to *M. arenaria* showed significant ($P < 0.01$) differences among the accessions (Table 5-12).

M. arenaria reaction of *A. pintoi* germplasm is presented in Table 5-13. Large genetic variability was observed among the accessions with respect to this characteristic. Among the 44 accessions evaluated, 12 were classified as highly resistant, 14 were classified as resistant, 15 were considered moderately resistant, 2 were considered susceptible, and one was considered highly susceptible. Overall 93 % of the accessions presented some level of resistance and only 7% were classified as susceptible.

The *A. pintoi* accessions also demonstrated significant variation ($P < 0.01$) in response to infestation with *M. javanica* (Table 5-14). Although, significant variation was presented in *M. javanica* reaction, all 39 accessions evaluated were classified as highly resistant or resistant (Table 5-15).

Table 5-12. Analysis of variance table of *Arachis pintoi* germplasm reaction to *M. arenaria*

Source	df	DI†		EM††	
		MS	Pr>F	MS	Pr>F
Accession	44	8.61	0.0001	8.44	0.0001
Replication	3	0.99	0.5515	0.44	0.7428
Error	78	1.09		1.05	
Total	125				

† Damage index; †† Egg mass

In the case of *M. incognita* reaction significant differences were observed among accessions only for DI (Table 5-16). All except two accessions showed no galling or egg mass production (Table 5-17). Other reports have shown that in general *Arachis pintoi* have near immunity to *M. incognita*. In fact, *A. hypogaea* is used as a non-host differential for *M. incognita* in the standard test to characterize populations of root-knot nematodes in to major species and races. It is however generally susceptible to *M. arenaria*.

Nematode resistance is a valuable attribute for any species that will be incorporated into agriculture systems. It is more important with perennial plants that will have long-term exposure to soil borne problems. For a forage crop, nematode susceptibility can impact the ability to persist over a long period in the pasture. In the case of *A. pintoi,* which is known as multiple use legume, this characteristic could improve its utilization as ground cover and in crop rotations with cultures that are susceptible to root-knot nematodes. This is the case of the common peanut planted in the southeastern USA, which requires a crop rotation with bahiagrass (Paspalum notatum). The introduction of *A. pintoi* in bahiagrass pasture could improve nematode control and additionally improve the nutritive value of the pasture.

Table 5-13. Reaction of *Arachis pintoi* germplasm to *M. arenaria* race 1

Accession	Egg Mass (EM)		Damage Index (DI)	
CIAT / PI	mean	SE	mean	SE
Highly resistant				
20826	1	0	1	0
22175	1	0	1	0
22232	1	0	1	0
22233	1	0	1	0
22238	1	0	1	0
22241	1	0	1	0
22259	1	0	1	0
22268	1	0	1	0
604799	1	0	1	0
604813	1	0	1	0
604815	1	0	1	0.3
604858	1	0	1	0
Resistant				
22151	1	0	1.6	0.5
22152	1	0	1.6	0.5
22159	1	0	2.7	0
22234	1	0	2.7	0
22289	1	0	2.3	0.4
22324	1	0	2.7	0
22339	1	0	2.1	0.5
476132	1	0	1.6	0.5
497574	1	0	1.8	0.6
604798	1	0	2.1	0.5
604810	1	0	2.7	0
604814	1	0	2.7	0
604817	1	0	2.1	0.5
604856	1	0	2.7	0
Moderately resistant				
22150	2.3	0.7	4.6	0.9
22159	2	0.5	4.2	0.7
22174	2	0.6	2.5	0.1
22236	2	0	2.3	0.4
22256	2.3	0.2	2.7	0
22260	2	0	3.5	0.6
22271	3.3	1.4	4.8	1.8
22325	1.8	0.4	1.8	0.5
604800	2	0.6	3.7	0.5
604803	2	0	3.7	0.6
604805	1.3	0.3	2.1	0.5
604809	1.5	0.3	1	0
604811	1	0	4.3	0.9
604812	2.3	0.7	4.3	1
604859	1	0	3.8	0.5
Susceptible				
22265	4	0	7.2	1.3
22154	6	1.9	5.2	1.1
Highly Susceptible				
604808	9	0	7	0.5
Florruner	8.7	0.3	7.3	0.8

Table 5-14. Analysis of variance table of *Arachis pintoi* germplasm reaction to *M. javanica*

Source	df	DI†		EM††	
		MS	Pr>F	MS	Pr>F
Accession	39	0.203	0.0054	0.011	0.0002
Replication	2	0.241	0.0905	0.033	0.3986
Error	55	0.096		0.012	
Total	96				

† Damage index; †† Egg mass

In the case of *A. pintoi*, nematode resistance is remarkably important to permit a wide use of the species as forage crop or even as a cover crop. Also it is important due to the fact that the species could be considered a useful source of genes for its relative *A. hypogaea*, which is worldwide cultivated. Since direct crossing among the two species is not possible, some authors include *A. pintoi* in the tertiary gene pool of *A. hypogaea*. However, with the recent progress of molecular biology tools, direct transfer could be achieved even for non-related species of the genus, which makes this source of resistance potentially important.

Even though, knowledge about sources of nematode resistance is extremely important to the general use of the species and for its use in breeding programs of *A. hypogaea*, little was know about *A. pintoi* germplasm accessions response to root-knot nematodes. Information available is usually restricted to one or a few accessions. Sharma et al. (1999) studied *M. javanica* race 3 reaction of 161 accessions of wild *Arachis* species.

They reported that of the nine accessions of *A. pintoi* evaluated, eight were considered susceptible or highly susceptible, but a single accession was classified as moderately resistant. By contrast, all *A. pintoi* accessions were highly resistant to the M. javanica population used in this research (Not classified as a race).

Table 5-15. Reaction of *Arachis pintoi* germplasm to *M. javanica*

Accession	Egg Mass (EM)		Damage Index (DI)	
PI / CIAT	mean	SE	mean	SE
Highly resistant				
20826	1.0	0.0	1.0	0.0
22150	1.0	0.0	1.0	0.0
22151	1.0	0.0	1.0	0.0
22152	1.0	0.0	1.0	0.0
22154	1.0	0.0	1.0	0.0
22159	1.0	0.0	1.0	0.0
22174	1.0	0.0	1.0	0.0
22175	1.0	0.0	1.0	0.0
22232	1.0	0.0	1.0	0.0
22233	1.0	0.0	1.0	0.0
22234	1.0	0.0	1.0	0.0
22236	1.0	0.0	1.0	0.0
22238	1.0	0.0	1.0	0.0
22241	1.0	0.0	1.0	0.0
22256	1.0	0.0	1.0	0.0
22259	1.0	0.0	1.0	0.0
22265	1.0	0.0	1.0	0.0
22268	1.0	0.0	1.0	0.0
22271	1.0	0.0	1.0	0.0
22289	1.0	0.0	1.0	0.0
22324	1.0	0.0	1.0	0.0
22325	1.0	0.0	1.0	0.0
476132	1.0	0.0	1.0	0.0
497574	1.0	0.0	1.0	0.0
604798	1.0	0.0	1.0	0.0
604799	1.0	0.0	1.0	0.0
604800	1.0	0.0	1.0	0.0
604803	1.0	0.0	1.0	0.0
604805	1.0	0.0	0.7	0.3
604809	1.0	0.0	1.0	0.0
604810	1.0	0.0	1.0	0.0
604812	1.0	0.0	1.0	0.0
604813	1.0	0.0	1.0	0.0
604815	1.0	0.0	1.0	0.0
604818	1.0	0.0	1.0	0.0
604858	1.0	0.0	1.0	0.0
604859	1.0	0.0	1.0	0.0
Resistant				
22339	1.7	0.3	2.1	0.6
604808	1.0	0.0	1.6	0.6
'Florunner'	1.0	0.0	2.1	0.6

Queneherve et al. (2002) examined *A. pintoi* reaction to *Radopholus similis*, *Pratylenchus coffeae*, *Hoplolaimus seinhorsti*, *Meloidogyne incognita* and *M. mayaguensis*. Forty-five days after inoculation *R. similis*, *H. seinhorsti* and *P. coffeae* multiplied in the roots. *A. pintoi* did not allow the multiplication of *M. incognita* and *M.*

mayaguensis, indicating the inability of *A. pintoi* to act as a host to these two root-knot nematodes.

Table 5-16. Analysis of variance table of *Arachis pintoi* germplasm reaction to *M. incognita*

Source	df	DI*		EM**	
		MS	Pr>F	MS	Pr>F
Accession	39	0.0955	0.0015	0.0000	-
Replication	2	0.0364	0.3969	0.0000	-
Error	48	0.0386		0.0000	
Total	89				

* Damage index; ** Egg mass

Santiago et al. (2002) investigated the *A. pintoi* reaction to *M. paranaensis* and *M. incognita* races 1, 2, 3, and 4. They reported that no root penetration by *M. incognita* and *M. paranaensis* juveniles had occurred, and hence there was no gall or egg mass formation. They concluded that in general *A. pintoi* accessions had an antagonistic effect on the nematodes, suggesting that they could be used as an interccrop or cover crop to reduce *M. paranaensis* and *M. incognita* populations. This research supports this conclusion and includes populations of *M. arenaria* since many accessions presented resistance to this species.

Information published and available seems to support the results obtained in this research with respect to nematode reaction of *A. pintoi*. The great majority of the accessions evaluated presented some level of resistance to *M. arenaria* race 1, *M. javanica* and *M. incognita* race 1. Once more, the source of resistance of these accessions could be used in breeding programs of *A. hypogaea* and more important qualify *A. pintoi* as potential forage, at least by this criteria, in environments where nematode infestation is a factor. Another positive outcome of this result is the ability of the species to suppress

the multiplication of nematodes, and then be an important cover crop to species with nematode susceptibility problems.

Table 5-17. Reaction of *Arachis pintoi* germplasm to *M .incognita*

Accession	Egg Mass (EM)		Damage Index (DI)	
PI / CIAT	mean	SE	mean	SE
Highly resistant				
20826	1.0	0.0	1.0	0.0
22150	1.0	0.0	1.0	0.0
22151	1.0	0.0	1.0	0.0
22152	1.0	0.0	1.0	0.0
22154	1.0	0.0	1.0	0.0
22159	1.0	0.0	1.0	0.0
22174	1.0	0.0	1.0	0.0
22175	1.0	0.0	1.0	0.0
22232	1.0	0.0	1.6	0.6
22233	1.0	0.0	1.0	0.0
22234	1.0	0.0	1.0	0.0
22236	1.0	0.0	1.0	0.0
22238	1.0	0.0	1.0	0.0
22241	1.0	0.0	1.0	0.0
22256	1.0	0.0	1.0	0.0
22259	1.0	0.0	1.0	0.0
22265	1.0	0.0	1.0	0.0
22268	1.0	0.0	1.0	0.0
22289	1.0	0.0	1.0	0.0
22290	1.0	0.0	1.0	0.0
22324	1.0	0.0	1.0	0.0
22325	1.0	0.0	1.0	0.0
22339	1.0	0.0	1.0	0.0
476132	1.0	0.0	1.0	0.0
497574	1.0	0.0	1.0	0.0
604798	1.0	0.0	1.0	0.0
604800	1.0	0.0	1.0	0.0
604803	1.0	0.0	1.0	0.0
604804	1.0	0.0	1.0	0.0
604805	1.0	0.0	1.0	0.0
604808	1.0	0.0	1.0	0.0
604811	1.0	0.0	1.0	0.0
604812	1.0	0.0	1.0	0.0
604813	1.0	0.0	1.0	0.0
604815	1.0	0.0	1.0	0.0
604817	1.0	0.0	1.0	0.0
604818	1.0	0.0	1.0	0.0
604859	1.0	0.0	1.0	0.0
Resistant				
22271	1.0	0.0	2.7	0.0
'Florunner'	1.0	0.0	1.0	0.0

Summary and Conclusions

Arachis pintoi germplasm displayed great variability with respect to adaptation, dry matter yield, nutritive value, seed production, and nematode reaction. Average FDMY during the year 2003 was 4.35 Mg ha^{-1}, and ranged from zero to 9.10 Mg ha^{-1}. Crude protein and IVOMD were high and confirmed the fact that *A. pintoi* has excellent nutritive value. Average CP was 180 g kg^{-1} of DM and ranged from 130 to 220 g kg^{-1} of DM. IVOMD averaged 670 g kg^{-1} of DM and ranged from 600 to 730 g kg^{-1} of DM. Some accessions produced high seed yields reaching values above 1.00 Mg ha^{-1}, 18 and 30 mo after sowing. The average seed production was 0.32 Mg ha^{-1} in 2003, and 0.43 Mg ha^{-1} in 2004. Overall, accessions PI 604817, 497574, 604815, 604810, 604808, and 604857 were the best adapted to the north central Florida environment and with the best agronomic characteristics. *Arachis pintoi* germplasm presented high levels of resistance to *M. arenaria*, *M. javanica*, and *M. incognita*. Overall, 93% of the accessions were classified as resistant to *M. arenaria*, and all of them presented resistance to *M. javanica*, and *M. incognita.* Although it sounds obvious, this research demonstrates again, that general conclusions about the adaptation and agronomic value of a species to a particular environment should not be based on a single or a few germplasm lines.

CHAPTER 6
CONCLUSIONS

Germplasm accessions of *Arachis pintoi* Krap. and Greg. stored at the Southern Regional Plant Introduction Station of the National Plant Germplasm System (NPGS) located at Griffin, GA were transferred to the University of Florida, where they was characterized and evaluated at the molecular, morphological and agronomic levels during the period 2001 to 2004. The three different levels of germplasm accession characterization employed in this research were effective in demonstrating differences among the accessions, and furthered the utilization of the germplasm based on the information gathered at these three levels.

Molecular characterization was achieved with RAPD molecular markers, which proved to be very informative and efficient to characterize the genetic diversity and relationship among germplasm accessions of the species. DNA amplifications were obtained with eight primers (A4, B4, B5, C2, D4, D13, E4, and G5), which amplified 100 different bands with ninety eight showing polymorphism. Individual germplasm accessions were discriminated based on their RAPD band profile. Ten bands were accession specific and with further work (sequencing) they can be used as markers to identify these individual germplasm accessions. The molecular parameters used to assess the genetic diversity of the germplasm indicated that a large amount of genetic diversity exists among the germplasm evaluated in this research. These same parameters allowed a construction of a dendogram that separated the accessions in four distinct groups.

Accessions from different groups could be selected as parents in breeding program based on their genetic divergence.

Two tissue culture protocols were used to assess their organogenesis ability and variations on RAPD profile of regenerated plants were examined. Callus induction was achieved by both protocols 28 d after incubation, and Protocol 1 (Rey et al., 2000) was superior to Protocol 2 (Ngo and Quesenberry, 2000) for callus rating and weight. The germplasm presented large variability for these two variables, and the accession by protocol interaction was significant. In Protocol 1, shoot regeneration was obtained for accessions PI 604856, 604857, 604805, 604811, 604809, 604814, 604818, and CIAT 22234, 20826, 22152, and 22265. In Protocol 2, shoot regeneration was attained by accessions PI 604856, 604805, 604799, 604804, 604818, 604809, 604810, 604800, 604813, 604857, and CIAT 22256, 22234, 20826, 22152, and 22265. Although shoot regeneration was achieved for several accessions, root induction was very difficult to obtain, and many shoots died during this process. At the end, just 16 regenerated plants were recovered from both protocols.

Although differences in callus rating and weight were observed we can conclude that both protocols were equivalent in *A. pintoi* plant regeneration. Even though differences between protocols were not observed, large differences in individual accessions plant regeneration were present. *In vitro* regeneration is one of the requirements for genetic transformation, so one or more of the 10 accessions that showed effective regeneration could be used to develop a system to *A. pintoi* transformation.

RAPD band profiles of regenerated tissue culture plants were identical to their parent plant. Although our sample was small, the results suggest that the germplasm

could be stored *in vitro*, thus reducing the space to store large numbers of accessions if further tests confirmed that no somaclonal variation was generated in the tissue culture process. This should lower the costs when compared to growing and organizing plants annually in the field

This *A. pintoi* germplasm presented great morphological variability with all the descriptors, except pollen size and shape, leaf bristles superior and inferior surface, showing polymorphism. Meaningful correlations were found between leaf length (LL) and pod weight; leaf length and pod width; leaf length and seed weight; leaf length and seed width; and finally leaf length and seed length. Thus, leaf length could be used as a selection criterion in programs where increased seed size is one of the objectives.

The large amount of genetic diversity that exits among the germplasm accessions evaluated in this research, suggests for most traits, breeding and selection for specific attributes should be possible.

Principal component analysis of the morphological data proved to be a useful technique to work with a large number of accessions and variables. The principal components analysis was able to discriminate and separate the accessions in terms of three dimensions, represented by “sexual reproduction”, “vegetative”, and “qualitative” axis. The cluster analysis based on the first nine principal components differentiated four distinct groups of accessions. It is important to comment that the molecular and the morphological dendograms were different.

Arachis pintoi germplasm displayed large variability with respect to its adaptation, dry matter yield, nutritive value, seed production, and nematode reaction. Annual average dry matter yield for some *A. pintoi* accessions was close to the forage production

presented by both rhizoma peanut cultivars. Crude protein and IVOMD were high and confirmed the fact that *A. pintoi* has superior nutritive value. Some accessions produce elevated seed yields reaching values superior to 1 Mg ha^{-1} 18 and 30 mo after sowing, which would give a pintoi peanut cultivar a great advantage over a rhizoma peanut cultivar, in terms of pasture establishment and seedling recruitment, if similar forage yields and persistence were present. *Arachis pintoi* germplasm showed somewhat variable but generally high levels of resistance in response to *M. arenaria*, *M. javanica*, and *M. incognita*. Overall 93% of the accessions were classified as resistant to *M. arenaria*, and all were resistant to *M. javanica*, and *M. incognita.* Susceptibility to root-knot nematodes, especially *M. arenaria,* is one of the major problems that groundnut cultivars face in the southeastern USA. *A. pintoi* is considered by some authors as representative of the tertiary or quaternary gene pool of *A. hypogea*, and so the germplasm could be a source of resistance to *M. arenaria* if genetic barriers could be overcome.

The great genetic diversity presented at molecular, morphological, and agronomic levels by the germplasm evaluated shows that effort to collect germplasm in the wild are in the right direction. The information generated by this research reaffirms once more for plant selectors and plant breeders that conclusions about a species' genetic diversity, adaptation to environments, and agronomic characteristics must not be based on a single or small number of germplasm accessions. This information will also be useful for genetic resources management, germplasm selection and plant breeding programs, and genetic studies in general. Finally we believe that a great contribution for the utilization

of the genetic resources of *A. pintoi* stored in various germplasm banks was made by this work.

APPENDIX A
LIST OF *A. pintoi* GERMPLASM EVALUATED AT UF

Table A-1. List of *A. pintoi* germplasm evaluated at UF

UF number	PI number	Lat. (South)	Long. (West)	Altitude (meter)
01	604856	16º 53’	42º 07’	360
02	604857	13º 23’	44º 05’	450
03	604858	15º 26’	47º 21’	700
04	604798	16º 18’	46º 58’	630
05	604803	14º 25’	44º 22’	510
06	604805	16º 59’	45º 57’	570
07	604812	14º 28’	46º 29’	500
08	604810	13º 06’	46º 45’	600
09	604811	13º 51’	46º 52’	490
10	604799	16º 19’	46º 51’	580
11	604800	16º 41’	46º 29’	540
12	604809	13º 02’	46º 45’	610
13	604817	18º 38’	44º 04’	630
16	604815	15º 49’	47º 58’	1080
17	604814	15º 52’	39º 08’	50
18	497541	18º 38’	44º 04’	640
20	604813	14º 27’	47º 00’	480
21	604801	16º 42’	46º 25’	560
22	604804	14º 20’	44º 25’	560
23	604808	13º 18’	46º 42’	500
24	604807	13º 18’	46º 48’	510
26	604818	-	-	-
29	338447	15º 52’	39º 08’	50
31	476132	16º 08’	47º 12’	690
32	497574	13º 23’	44º 05’	450
33	604859	17º 03’	42º 21’	360

Table A-1. Continued.

UF number	CIAT number	Lat. (South)	Long. (West)	Altitude (meter)
34	22256	16º 10’	46º 01’	580
35	22234	13º 14’	46º 44’	463
36	20826	-	-	-
37	22159	15º 17’	47º 23’	650
38	22152	16º 52’	46º 35’	550
39	22150	15º 07’	44º 08’	510
40	22265	-	-	-
41	22260	14º 04’	47º 18’	720
42	22271	15º 47’	47º 56’	1040
43	18745	16º 05’	42º 05’	280
44	22339	-	-	700
45	22259	14º 29’	47º 09’	510
47	22233	-	-	-
49	22238	15º 11’	39º 29’	256
50	22174	16º 12’	46º 54’	580
53	22241	12º 40’	39º 05’	53
54	22231	13º 18’	46º 24’	500
55	22175	16º 12’	46º 54’	600
56	22290	15º 06’	39º 16’	120
58	22325	15º 06’	39º 16’	120
59	22289	16º 09’	46º 10’	560
60	22232	-	-	-
61	22236	12º 49’	38º 24’	37
62	22151	16º 18’	46º 50’	590
63	22268	-	-	-
66	22324	16º 09’	46º 12’	550
67	22154	16º 44’	46º 12’	580

APPENDIX B
CTAB DNA EXTRACTION PROTOCOL

Solutions and Reagents

2x CTAB Extraction Buffer

- Prepare 100ml
- 2% CTAB 2g
- 1.4 M NaCl 28ml 5M NaCl Stock
- 100 mM Tris-Cl (pH 8.0) 10ml 1M Tris-Cl Stock
- 20 mM EDTA (pH 8.0) 4ml 0.5M EDTA Stock
- 1% PVP 10ml 10% PVP Stock
- Complete with ddH_2O to 100ml
- 2% (v/v) β-Mercaptoethonol 20µl/ml (Add just before use)

CTAB Precipitation Buffer (Add just before use)

- Prepare 100ml
- 1% CTAB 1g
- 50 mM Tris-Cl (pH 8.0) 5ml 1M Tris-Cl Stock
- 10 mM EDTA (pH 8.0) 2ml 0.5M EDTA Stock
- Complete with ddH_2O to 100ml

High Salt TE Buffer (Add just before use)

- Prepare 100ml
- 10 mM Tris-Cl (pH 8.0) 1ml 1M Tris-Cl Stock
- 1 mM EDTA (pH 8.0) 0.2ml 0.5M EDTA Stock
- 1 M NaCl 20ml 5M NaCl Stock
- Complete with ddH_2O to 100ml

10% CTAB

- Prepare 100ml
- 10% CTAB 10g
- 0.7M M NaCl 14ml 5M NaCl Stock
- Complete with ddH_2O to 100ml

(Modification of Rogers, S.O. and Bendich, J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissue. Plant Molecular Biology, 5:69-76)

Procedure

- 0.1 g grinded peanut leaf tissue
- Add 400 µl of pre-warmed (65ºC) 2x CTAB precipitation buffer
- Incubate in a 65 ºC water bath for 60 minutes and occasionally mix
- Add an equal volume of chloroform:isoamyl alcohol (24:1), mix gently but thoroughly
- Centrifuge @ 10,000 rpm for 5 minutes at 4 ºC
- Transfer supernatant to new tube and add 1/10 volume of 65 ºC 10% CTAB
- Repeat steps 4 and 5
- Transfer supernatant to new tube and add an equal volume of CTAB precipitation buffer and mix well by inversion
- Centrifuge @ 10,000 rpm for 5 minutes at 4 ºC
- Remove the supernatant and rehydrate pellet in 50-100 µl of high salt TE buffer by heating to 65 ºC for 10 minutes
- Re-precipitate the DNA with 0.6 volumes of isopropanol
- Centrifuge @ 10,000 rpm for 15 minutes at 4 ºC
- Wash pellet with 80% ethanol and dry
- Resuspend DNA in a small volume (10 – 50 µl) of DDW

APPENDIX C
MORPHOLOGICAL DESCRIPTORS CORRELATION TABLE

Table C-1. Morphological descriptors correlation table

	FPI	FSW	FSL	FSC	FSCr	FWW	FWL	FKL	FHL	FHW	FHC	FHH	SIL	SID	SC	SH	SGH	LS
FPI		0.0143* 0.9421¶	0.1313 0.5052	0.0370 0.8517	-0.037 0.8486	-0.034 0.8635	0.0300 0.8793	0.0576 0.7709	0.1088 0.3104	0.2248 0.2501	-0.185 0.3456	-0.204 0.2960	-0.068 0.7279	0.3517 0.0664	0.2129 0.2766	0.4547 0.0150	0.1109 0.5739	-0.135 0.4913
FSW			0.7815 0.0001	-0.119 0.5457	-0.092 0.6406	0.8180 0.0001	0.7402 0.0001	0.1992 0.3095	0.5253 0.0041	0.2200 0.2605	0.2164 0.2687	0.0457 0.8172	0.3348 0.0815	0.1129 0.5673	-0.294 0.1287	-0.111 0.5732	0.2431 0.2125	-0.076 0.7003
FSL				-0.115 0.5586	-0.318 0.0989	0.6398 0.0002	0.7495 0.0001	0.3867 0.0421	0.7251 0.0001	0.5685 0.0016	0.0902 0.6480	0.1311 0.5060	0.3092 0.1093	0.1762 0.3696	-0.237 0.2246	0.1649 0.4016	0.3667 0.0549	-0.072 0.7149
FSC					0.1885 0.3366	0.1117 0.5714	0.1477 0.4530	0.3146 0.1029	-0.084 0.6675	0.1367 0.4878	-0.346 0.0709	-0.120 0.5430	-0.147 0.4525	-0.040 0.8377	0.2786 0.1510	0.0351 0.8590	-0.066 0.7361	-0.235 0.2275
FSCr						0.1457 0.4592	0.0545 0.7829	0.0164 0.9337	-0.102 0.6051	-0.215 0.2719	0.2041 0.2975	-0.141 0.4729	-0.193 0.3230	0.0792 0.6886	0.1194 0.5450	-0.103 0.5997	-0.235 0.2273	-0.265 0.1714
FWW							0.8531 0.0001	0.2884 0.1366	0.4839 0.0091	0.2156 0.2705	0.1016 0.6067	0.0600 0.7614	0.2756 0.1557	0.2632 0.1775	-0.235 0.2268	0.0336 0.8650	0.1799 0.3594	-0.200 0.3065
FWL								0.4417 0.0186	0.6665 0.0001	0.5087 0.0057	0.1016 0.6067	0.0608 0.7614	0.2756 0.1557	0.2623 0.1775	-0.235 0.2268	0.0336 0.8650	0.1799 0.3594	-0.200 0.3065
FKL									0.2849 0.1417	0.4173 0.0271	-0.175 0.3703	0.0497 0.8017	-0.161 0.4123	0.2028 0.3005	-0.040 0.8383	0.2290 0.2409	0.1392 0.4798	-0.170 0.386
FHL										0.5460 0.0026	-0.077 0.6940	0.1320 0.5030	0.2150 0.2718	0.2699 0.1648	-0.144 0.4642	0.3555 0.0633	0.4061 0.0320	-0.231 0.2364
FHW											-0.110 0.5763	0.3542 0.0644	0.0114 0.9540	0.3544 0.0642	-0.082 0.6767	0.5758 0.0013	0.1454 0.4604	-0.126 0.5211
FHC												-0.115 0.5585	0.3486 0.0690	-0.158 0.4201	-0.073 0.7115	-0406 0.0320	-0.247 0.2043	0.1474 0.4539
FHH													0.0470 0.8121	-0.055 0.7804	0.0422 0.8311	0.3634 0.0573	-0.066 0.7361	0.0028 0.9886
SIL														0.0352 0.8587	-0.126 0.5219	-0.333 0.0827	-0.017 0.9285	0.0449 0.8203
SID															-0.124 0.5270	0.3883 0.0412	0.1753 0.3723	-0.089 0.6495
SC																0.1262 0.5222	0.0422 0.8311	0.0772 0.6958
SH	*** Pearson Correlation Coefficients (N = 28)**																0.2931 0.1301	-0.152 0.4393
SGH	**¶ Prob > |r| under H0: Rho=0**																	-0.241 0.2116

Table C-1. Continued

	LHL	LHM	LGHM	LL	LW	LPL	PegL	Pegw	PegC	PegH	Podwe	PodL	PodW	PodB	PodR	SW	SL	Swe
FPI	0.2564 0.1877	0.1952 0.3194	-0102 0.6028	0.1894 0.3343	0.1369 0.4873	0.0030 0.9878	-0.170 0.3852	0.0653 0.7404	0.0350 0.8595	-0.002 0.9914	0.3400 0.0767	0.3115 0.1066	0.4076 0.0313	-0.125 0.5244	0.0034 0.9862	0.3653 0.0559	0.2432 0.2122	0.3225 0.0942
FSW	0.0075 0.9695	0.0362 0.8548	-0.004 0.9800	0.3841 0.0432	0.3035 0.1163	0.3140 0.1037	0.0795 0.6874	-0.144 0.4637	-0.034 0.8605	0.0703 0.7219	0.3768 0.0481	0.1551 0.4304	0.4408 0.0189	0.2867 0.1391	-0.342 0.0743	0.4135 0.0287	0.2892 0.1355	0.3333 0.0831
FSL	-0.029 0.8806	-0.041 0.8331	0.1160 0.5563	0.3525 0.0657	0.3811 0.0990	0.2725 0.1606	0.0686 0.7286	-0.261 0.1796	-0.235 0.2268	0.1616 0.4112	0.2514 0.1968	0.0937 0.6351	0.3961 0.0369	0.1224 0.5349	-0.223 0.2527	0.3917 0.0392	0.2357 0.2271	0.2360 0.2266
FSC	0.1405 0.4755	-0.166 0.3966	0.3333 0.0830	-0.263 0.1775	-0.369 0.0527	-0.429 0.0224	-0.219 0.2628	0.3192 0.0977	-0.258 0.1846	-0.258 0.1846	0.0105 0.9575	-0.050 0.7970	0.1213 0.5385	-0.170 0.3850	-0.088 0.6529	0.1082 0.5835	-0.198 0.3104	0.0134 0.9460
FSCr	0.4385 0.0196	0.3535 0.0649	-0.235 0.2273	-0.101 0.6071	0.0125 0.9495	-0.177 0.3656	-0.082 0.6754	0.1659 0.3987	0.1217 0.5372	-0.091 0.6441	-0.109 0.5802	-0.050 0.7970	0.1213 0.5385	-0.170 0.3850	-0.088 0.6529	0.1082 0.5835	-0.198 0.3104	0.0134 0.9460
FWW	0.1723 0.3805	0.1883 0.3371	-0.086 0.6629	0.5312 0.0036	0.3290 0.0873	0.2401 0.2183	0.1427 0.4687	-0.153 0.4348	-0.143 0.4653	-0.041 0.8333	0.4399 0.0191	0.1940 0.3223	0.5754 0.0014	0.2270 0.2454	-0.204 0.2962	0.5303 0.0037	0.3372 0.0793	0.4224 0.0251
FWL	0.0547 0.7821	0.0565 0.7751	0.0764 0.6989	0.4038 0.0331	0.3128 0.1050	0.1827 0.3520	0.1462 0.4578	-0.049 0.8015	-0.256 0.1876	-0.061 0.7546	0.3385 0.0780	0.1447 0.4625	0.4675 0.0121	0.2780 0.1519	-0.320 0.0960	0.4674 0.0121	0.2715 0.1622	0.3561 0.0628
FKL	0.0396 0.8411	0.0870 0.6597	0.3039 0.1159	0.2430 0.2127	0.1902 0.3321	0.2732 0.1595	-0.030 0.8777	-0.044 0.8233	-0.518 0.0047	0.0219 0.9117	0.1655 0.4000	-0.036 0.8534	0.1217 0.5372	-0.117 0.5506	0.0950 0.6305	0.2372 0.2242	0.0823 0.6770	0.1635 0.4057
FHL	0.0106 0.9572	-0.118 0.5467	-0.048 0.8067	0.1806 0.3575	0.2292 0.2407	0.0987 0.6172	0.1541 0.4335	-0.070 0.7232	-0.378 0.0468	-0.074 0.7073	0.0178 0.9280	-0.234 0.2307	0.2345 0.2296	0.1626 0.4082	-0.130 0.5072	0.1619 0.4103	-0.051 0.7965	0.0154 0.9377
FHW	-0.028 0.8843	0.1356 0.4913	0.0683 0.7297	0.1472 0.4545	0.2272 0.2449	0.0188 0.9242	-0.083 0.6718	0.1908 0.3307	-0.418 0.0265	-0.245 0.2085	0.1026 0.6032	0.1141 0.5629	0.3248 0.0917	-0.057 0.7728	-0.049 0.8019	0.3388 0.0778	0.1030 0.6019	0.1003 0.6112
FHC	-0.358 0.0614	-0.144 0.4637	-0.082 0.6765	0.0458 0.8167	0.3060 0.1132	0.2828 0.1448	0.2688 0.1666	-0.071 0.7172	0.4472 0.0170	0.2981 0.1233	0.1037 0.5993	0.1133 0.5657	-0.133 0.4986	0.2762 0.1547	-0.384 0.0431	0.0084 0.9659	0.1828 0.3517	0.0622 0.7531
FHH	-0.090 0.6453	0.3000 0.1209	0.0666 0.7361	0.0590 0.7655	-0.036 0.8552	0.0292 0.8827	0.1762 0.3698	0.0090 0.9621	-0.017 0.9307	-0.258 0.1846	-0.231 0.2368	-0.259 0.1816	-0.078 0.6925	0.0394 0.8422	-0.088 0.6529	-0.106 0.5908	-0.227 0.2451	-0.268 0.1679
SIL	-0.452 0.0157	-0.336 0.0797	0.0876 0.6573	0.2531 0.1936	0.0366 0.8532	0.1508 0.4436	0.3853 0.0428	-0.160 0.4141	0.2207 0.2590	-0.082 0.6754	0.2671 0.1694	0.1160 0.5566	0.2562 0.1881	-0.047 0.8084	-0.179 0.3596	0.2503 0.1988	0.2443 0.2101	0.2624 0.1772
SID	0.4422 0.0184	0.3568 0.0623	-0.122 0.5363	0.5379 0.0031	0.5611 0.0019	0.1336 0.4977	0.0087 0.9647	0.0357 0.8567	-0.216 0.2692	-0.385 0.0430	0.4273 0.0233	0.3511 0.0669	0.5263 0.0040	0.2264 0.2465	0.1685 0.3914	0.4716 0.0113	0.4563 0.0147	0.4199 0.0261
SC	0.0157 0.9368	-0.105 0.5930	-0.042 0.8311	-0.158 0.4200	-0.134 0.4963	-0.224 0.2513	0.2703 0.1642	0.1361 0.4898	-0.163 0.4057	-0.010 0.9561	-0.236 0.2259	-0.325 0.0909	-0.188 0.3366	-0.257 0.1852	-0.253 0.1934	-0.028 0.8867	-0.315 0.1018	-0.147 0.455
SH	0.2981 0.1234	0.4250 0.0242	-0.058 0.7670	0.2096 0.2842	0.2472 0.2047	0.0649 0.7428	-0.072 0.7156	0.1936 0.3236	-0.348 0.0695	-0.348 0.0695	-0.020 0.9174	-0.062 0.7530	0.2225 0.2549	-0.265 0.1719	0.1856 0.3443	0.2056 0.2938	-0.075 0.7029	-0.033 0.8671
SGH	0.0413 0.8345	0.0000 1.0000	0.3333 0.0830	0.2816 0.1465	0.1272 0.5188	0.2230 0.2540	0.0707 0.7207	0.1600 0.4158	-0.086 0.6632	0.0860 0.6632	0.0239 0.9038	-0.106 0.5912	0.2303 0.2384	0.0093 0.9622	0.0000 1.0000	0.1956 0.3184	0.0062 0.9748	0.0552 0.7801
LS	-0.082 0.6755	0.1170 0.5529	-0.354 0.0629	0.0272 0.8905	0.1057 0.5922	0.0917 0.6423	-0.059 0.7622	-0.306 0.1128	-0.087 0.6563	0.1429 0.4682	-0.083 0.6737	0.0663 0.7372	-0.106 0.5885	-0.131 0.5050	-0.073 0.7108	-0.075 0.7013	-0.014 0.9430	-0.098 0.6195

Table C-1. Continued

	LHL	LHM	LGHM	LL	LW	LPL	PegL	Pegw	PegC	PegH	Podwe	PodL	PodW	PodB	PodR	SW	SL	Swe
LHL		0.5168 0.0049	-0206 0.2912	0.1238 0.5300	0.1638 0.4048	-0.152 0.4392	-0.304 0.1155	0.0691 0.7268	0.0533 0.7873	-0.245 0.2079	-0.014 0.9430	-0.002 0.9908	0.1114 0.5723	0.2036 0.2986	-0.110 0.5765	0.1343 0.4956	0.0184 0.9259	-0.002 0.9915
LHM			-0.333 0.0830	0.2748 0.1570	0.1428 0.4684	-0.017 0.9278	-0.287 0.1374	-0.156 0.4273	0.0430 0.8279	-0.107 0.5858	-0.048 0.8083	0.0794 0.6878	0.1050 0.5949	0.0328 0.8683	-0.069 0.7255	0.0674 0.7332	0.0149 0.9398	-0.099 0.6139
LGHM				-0.114 0.5614	-0.167 0.3936	0.0470 0.8123	0.0154 0.9379	0.3608 0.0592	0.0860 0.6632	0.0860 0.6632	0.0208 0.9160	0.0171 0.9309	0.0317 0.8726	0.0656 0.7399	0.0000 1.0000	-0.006 0.9739	-0.071 0.7192	0.0385 0.8457
LL					0.7944 0.0001	0.7660 0.0001	0.4667 0.0123	-0.111 0.5715	-0.071 0.7183	-0.205 0.2947	0.7168 0.0001	0.5248 0.0041	0.7082 0.0001	0.1951 0.3196	-0.011 0.9548	0.7382 0.0001	0.7255 0.0001	0.7098 0.0001
LW						0.7746 0.0001	0.3786 0.0469	0.0180 0.9274	-0.155 0.4285	-0.111 0.5721	0.5963 0.0008	0.4659 0.0125	0.5482 0.0025	0.3316 0.0847	-0.003 0.9843	0.6248 0.0004	0.6532 0.0002	0.6062 0.0006
LPL							0.5087 0.0057	0.0044 0.9822	-0.031 0.8723	0.0057 0.9767	0.5699 0.0015	0.3719 0.0513	0.4228 0.0250	0.1943 0.3216	-0.034 0.8597	0.4906 0.0080	0.5636 0.0018	0.5485 0.0025
PegL								0.0280 0.8875	-0.004 0.9832	-0.148 0.4523	0.2357 0.2271	-0.011 0.9524	0.1156 0.5577	0.2483 0.2025	-0.255 0.1887	0.1716 0.3824	0.1789 0.3622	0.2446 0.2096
PegW									0.0446 0.8214	-0.273 0.1592	0.1271 0.5191	0.1792 0.3614	0.1496 0.4473	0.1614 0.4119	-0.134 0.4949	0.2339 0.2309	0.0408 0.8364	0.1874 0.3395
PegC										0.0666 0.7361	-0.023 0.9067	0.0961 0.6263	-0.179 0.619	0.1865 0.3419	-0.258 0.1846	-0.152 0.4381	0.0777 0.6941	-0.084 0.6694
PegH											-0.141 0.4739	0.0090 0.9634	-0.244 0.2108	0.1526 0.4382	0.1434 0.4665	-0.207 0.2892	-0.088 0.6550	-0.097 0.6203
PodWe												0.8475 0.0001	0.8549 0.0001	0.1641 0.4039	0.0380 0.8475	0.8744 0.0001	0.9218 0.0001	0.9701 0.0001
PodL													0.7082 0.0001	0.2147 0.2725	0.0849 0.6675	0.7120 0.0001	0.8892 0.0001	0.8367 0.0001
PodW														0.0939 0.6344	0.0070 0.9718	0.8915 0.0001	0.7340 0.0001	0.8375 0.0001
PodB															-0.306 0.1127	0.0204 0.9177	0.1888 0.3359	0.1419 0.4713
PodR																-0.060 0.7604	0.0786 0.6906	0.0760 0.7006
SW																	0.8162 0.0001	0.9042 0.0001
SL																		0.9204 0.0001

APPENDIX D
CLIMATOLOGICAL DATA AT THE FORAGE RESEARCH UNIT IN GAINESVILLE-FL, DURING THE PERIOD OF THE AGRONOMIC EVALUATION FIELD TRIAL.

Table D-1. Climatological data at the forage research unit in Gainesville, FL, during the period of the agronomic evaluation field trial

		Temperature ºC			Rainfall (mm)	Nº days drought	Nº of days below temperature		
Year	Month	Min.	Max.	Mean			0 ºC	4 ºC	12 ºC
2001	Sep	23.22	10.18	34.34	292.2	13	0	0	1
	Oct	19.46	-0.11	31.48	2.9	29	1	4	11
	Nov	17.51	-0.22	28.88	27.1	26	1	3	14
	Dec	15.21	-5.44	28.24	38.4	27	6	9	18
2002	Jan	12.77	-7.31	29.11	133.0	19	6	14	19
	Feb	12.41	-8.09	28.8	27.7	25	6	16	24
	Mar	17.45	-6.37	31.65	83.1	22	4	8	19
	Apr	21.92	3.64	33.15	9.5	23	0	1	4
	May	23.11	6.42	35.57	39.5	26	0	0	7
	Jun	24.89	15.08	37.47	104.7	10	0	0	0
	Jul	25.71	18.05	36.84	138.3	13	0	0	0
	Aug	25.05	11.94	35.61	265.3	13	0	0	1
	Sep	25.66	17.13	33.99	128.2	19	0	0	0
	Oct	22.14	6.82	34.88	41.6	21	0	0	4
	Nov	14.07	-4.31	30.08	133.1	22	7	13	23
	Dec	10.96	-3.01	26.44	183.1	21	11	18	27
2003	Jan	8.22	-8.33	24.17	4.5	27	17	23	31
	Feb	13.42	-0.82	28.42	172.5	17	4	12	23
	Mar	18.69	2.03	30.64	194.4	19	0	1	14
	Apr	19.22	-0.26	30.99	41.4	26	1	2	15
	May	24.05	11.64	34.81	50.9	24	0	0	1
	Jun	25.27	16.44	34.49	297.8	12	0	0	0
	Jul	25.92	19.91	36.04	121.4	11	0	0	0
	Aug	25.53	19.55	34.04	206.2	9	0	0	0
	Sep	23.87	13.62	33.17	211.0	17	0	0	0
	Oct	20.66	7.61	31.34	103.7	25	0	0	7
	Nov	17.39	-2.63	31.62	34.1	25	2	4	15
	Dec	10.28	-6.46	25.16	18.9	24	12	21	31
2004	Jan	10.98	-6.16	27.29	33.9	24	13	17	28
	Feb	12.35	-1.76	27.82	143.6	15	2	9	24
	Mar	16.54	-1.35	29.00	25.5	27	1	6	25
	Apr	17.95	1.69	31.09	40.3	25	0	6	23

Table D-1. Continued

Year	Month	Temperature ºC			Rainfall (mm)	Nº days drought	Nº of days below temperature		
		Min.	Max.	Mean			0 ºC	4 ºC	12 ºC
2004	May	23.92	5.41	36.13	16.4	28	0	0	4
	Jun	25.77	18.03	36.15	174.3	16	0	0	0
	Jul	25.99	18.22	35.28	254.8	13	0	0	0
	Aug	25.69	20	34.94	150.7	12	0	0	0

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BIOGRAPHICAL SKETCH

Marcelo Ayres Carvalho, the older son of Milton de Sousa Carvalho and Magaly Ayres Carvalho, was born in Brasilia, DF, Brazil, on May 4th 1965. When Marcelo was 12, his father died in a car accident, and his mother moved to her parents' house, where he and his sister Patricia grew up under their mother and grandparents' guardianship. He inherited his father's interest in agronomy on several visits during school holidays to his grandfather Policarpo's farm in the north of Minas Gerais state. In 1983, Marcelo began his college studies in Agronomy at the University of Brasilia, where he received his Agronomist degree in 1988. During his time in college he spent 1 year in EMBRAPA doing an internship in the forage department.

In 1990, Marcelo was hired as a Research Assistant at the collaborative Project EMBRAPA\CIAT\IICA, where he worked under the supervision of Dr. Esteban Pizarro conducting research with tropical grasses and legumes. The time spent with Dr. Pizarro was extremely important in establishing Marcelo's passion for agronomic research. Encouraged by Dr. Pizarro, Marcelo received his M.S. degree in Agronomy from the University of Brasilia in 1996, working under the guidance of Dr. Jose F.M. Valls.

In 1995, he was hired by EMBRAPA to work at the Cerrados National Research Center located in Brasilia, where he is responsible for research projects with genetic resources and evaluation of tropical forage legumes and grasses.

In 2001, after being awarded with scholarships from EMBRAPA and CNPq, he started his Ph.D. program at the Agronomy department of the University of Florida. He is

studying evaluation of genetic resources of *Arachis pintoi* under the guidance of Dr. Kenneth H. Quesenberry. After the conclusion of his program he will return to Brazil, where he will reassume his position in EMBRAPA.

Marcelo married his lovely wife Aline in September of 1998. Aline gave him her entire support and dedication during the time spent at the University of Florida.