Evaluating the genetic diversity and performance of peanut (*Arachis hypogaea* L.) lines

By

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Magister Scientiae

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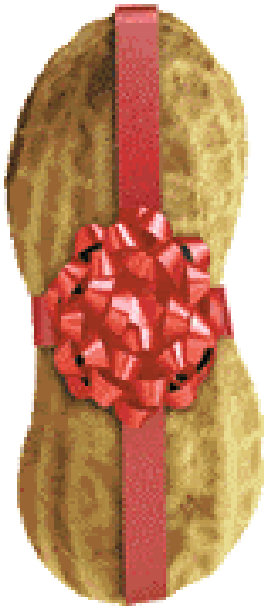
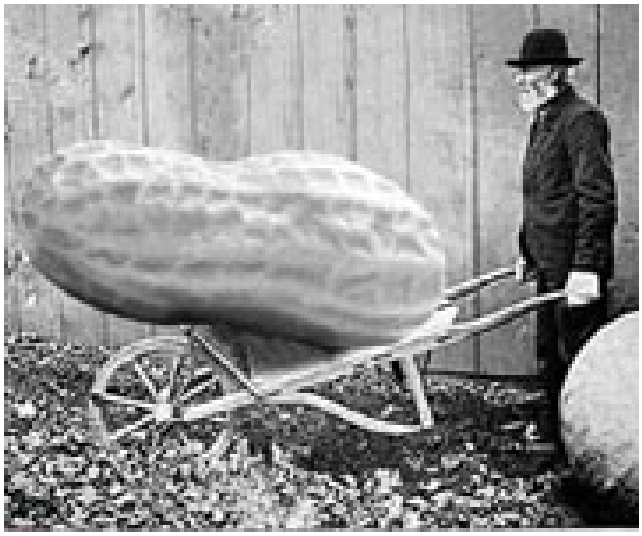
This in memory of Gedrud Kamburona (my dearest granny) and dedicated to Uejaa and Operi,

As the ALLMIGHTY gave me the serenity
to accept things I cannot alter and the
courage to alter things I can, regardless of
the circumstances!



Acknowledgments:

My Dear GOD, thank you for giving me the courage to wake up every day and believe that I could do something to make this world a better place for those around me and myself. **My family,** I am appreciative for putting your hopes in me, all these years, and not only encouraging me but also assuring me every single day that all will be well and fine, even during the most challenging times. **My supervisor, Prof. AM Botha-Oberholster,** you are one of a kind! Never have I met somebody as hardworking, self-sacrificing (noble) and accommodative as you! I am grateful for everything and have always felt privileged to have met you. In the same vain, I would like to acknowledge the efforts that my **co-supervisor, Dr. André Cilliers**, has put in along with the valuable advises and all the information he provided especially for my second chapter. I am also indebted to the great **Fabi Team** for all the support from technical assistance and the fabulous events that made me feel at home and part of one of the most remarkable centres of excellence in the entire world. All my **lab-mates** will also be thanked for all the good and difficult times we shared.



Declaration:

I, **Charline Kausana Kamburona**, hereby declare that this thesis/dissertation for the *Magister Scientiae* Degree at the University of Pretoria is my own work and has never been submitted by myself at any other University.

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium persulfate
ARC	Agricultural Research Council
ARC-GCI	Agricultural Research Council-Grain Crop Institute
ATP	Adenosine Triphosphate
BC	before Christ
BLAST	Basic Local Alignment Search Tool
bp	Base pair (s)
BSA	Bovine Serum Albumin
^{o}C	Degrees Celsius
$CaCl_2$	Calcium Chloride
$CaCO_3$	Calcium Carbonate
Chv	*chv*A and *chv*B regions on the chromosomes required for stable binding to plant cells
cm	Centimeter (s)
CPB	Colorado potato beetle
ddH_2O	double distilled water
DEB	DNA Extraction Buffer
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Oligonucleotides
DTT	dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
e.g.	*Exempli gratia* (for example)
et al.	*Et alii* (and others)
EtOH	Ethanol
g	Gram (s)
GUS	ß-glucuronidase
ha	hectare (s)
HAc	The dissociation of a weak acid (or base) where HAc is the acid form and Ac- is its conjugate base form.
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICRISAT	International Crop Research Institute for the Semi-Arid Tropics
IPM	Integrated Pest Management
IPTG	isopropylthio-ß-D-galactoside
ITS	Inter Transcribed Spacer
KCl	Potassium chloride
KOAc	Potassium acetate
KOH	Potassium hydroxide
L	Litre (s)
LB	Luria-Bertani medium
M	Molar
MAS	Marker assisted selection

MES	2-(N-Morpholino) ethanesulfonic Acid
mg	Milligram (s)
MgAc	Magnesium acetate
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MI	Marker index
min	Minute (s)
ml	Millilitre (s)
mm	Millimetre (s)
mM	Millimolar (s)
MUG	4-methyl umbelliferyl glucuronide
MU	4-methylumbellferone
m/s	metre per second
m/v	Mass per volume
NaCl	Sodium chloride
$NaCO_3$	Sodium carbonate
NaH_2PO_4	Sodium dihydrogen phosphate
$NaPO_4$	Sodium phosphate
ng	nanogram (s)
nm	nanometer (s)
PAC	Preharvest Aflatoxins Contamination
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PI	Proteinase Inhibitor
PIC	Polymorphic Information Content
pmol/µl	picomoles per microlitre
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Ristriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSA	Republic of South Africa
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s	Second (s)
SDS	Sodium dodecyl sulphate
SOC	Super Optimum Catabolite
SSLP	Simple Sequence Length Polymorphism
SSRs	Simple Sequence Repeats
STRs	Short tandem repeats
TB	Transformation buffer
TBE	Tris-borate-EDTA buffer
T-DNA	Transfer DNA
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Ti	tumour inducing
Tris-HCl	Tris-hydrochloride

U	Unit: One unit of *Taq* polymerase enzyme is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 minutes at 74 ^{o}C under standard analysis conditions.
UPGMA	Unweighted Pair Group Method based on arithmetic averages
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
V	Volt (s)
v/v	Volume per volume
Vir	virulence
µg	Microgram (s)
µl	Microlitre (s)
µM	Micromolar
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indol-*β*-D-galactoside

Research output:

1) Poster presentations:

18th Congress of the Genetic Society (4-7 April 2004, Stellenbosch, RSA):

Theme: Understanding life: Microbes to Man

Title: AFLP technique as tool in studying diversity of South African *Arachis hypogaea* lines.

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Preface:

The first chapter gives an overall introduction to *Arachis hypogaea* L., the crop that was studied in this thesis. The literature reviewed touched on various aspects of the crop in general such as the importance of peanut as a crop, its botanical description, origin and distribution world-wide. Different marker studies done on *A. hypogaea* L. and their extents have also been reviewed. The chapter also looked at the *oryzacystatin*-1 (*OC*-1) gene as a possible candidate gene for peanut improvement in South Africa (Chapter I).

Agronomic data was collected in an attempt to observe and evaluate variability amongst the 18 commercially cultivated peanut lines from South Africa. The growth habits, growth seasons, testa colours, total oil percentages and oleic and linoleic acid ratios were the agronomic characters used to study variation (Chapter II).

One South African commercially cultivated peanut line, JL 24, was transformed with the *oryzacystatin*-1 (*OC*-1) gene, which was isolated from rice. Three to four week old plantlets were vacuum infiltrated with *Agrobacterium* during the transformation process and various methods were used to analyze the putative transformants (Chapter III).

Two types of DNA markers along with phynotypic data were used to examine polymorphism among 18 South African peanut lines and a transformed line. The amplified fragment length polymorphisms (AFLPs) and simple sequences repeats (SSRs) were the two DNA markers used in this study to determine the level of variation amongst these lines (Chapter IV).

Chapter I

Literature review

1.1 Introduction

The lack of adequate and quality food to the ever-increasing world population is putting more and more pressure on the agricultural industry to deliver higher crop yields with better quality. This is more critical in Sub-Saharan Africa, which has a population growth of 2-3.5% and which is home to about 10% of the world population (Dar, 2002). With the imbalances in population growth versus food production, the region has emerged as a major locus of hunger (Dar, 2002).

Adverse effects of several biotic and abiotic stresses hinder sustainable peanut production in Sub-Saharan Africa and this call for the introduction of appropriate technologies. For a long time peanut has been considered a woman crop, because women are involved in the production process in this region (Future Harvest, 2002). Unfortunately, unfavourable prices, high labour requirements, shortage of land and the lack of equipment for harvesting and shelling have decreased the crop's popularity as a cash crop and not much cash can be obtained from the sale of the crop (Future Harvest, 2002).

1.2 Peanut as a crop

Peanut (*Arachis hypogaea* L.) has been grown widely in the pre-Colombian times mainly in Mexico, Central and South America (Stalker, 1997; Figure 1.1). Peanut is cultivated around the world in tropical, sub-tropical and temperate climatic conditions between 40^{o} South and 40^{o} North of the equator (Encyclopaedia of Agricultural Science, 1994; Figure 1.2).

Figure 1.1: The origin of the peanut plant is believed to be in Peru, South America (http://www.graphicmaps.com)

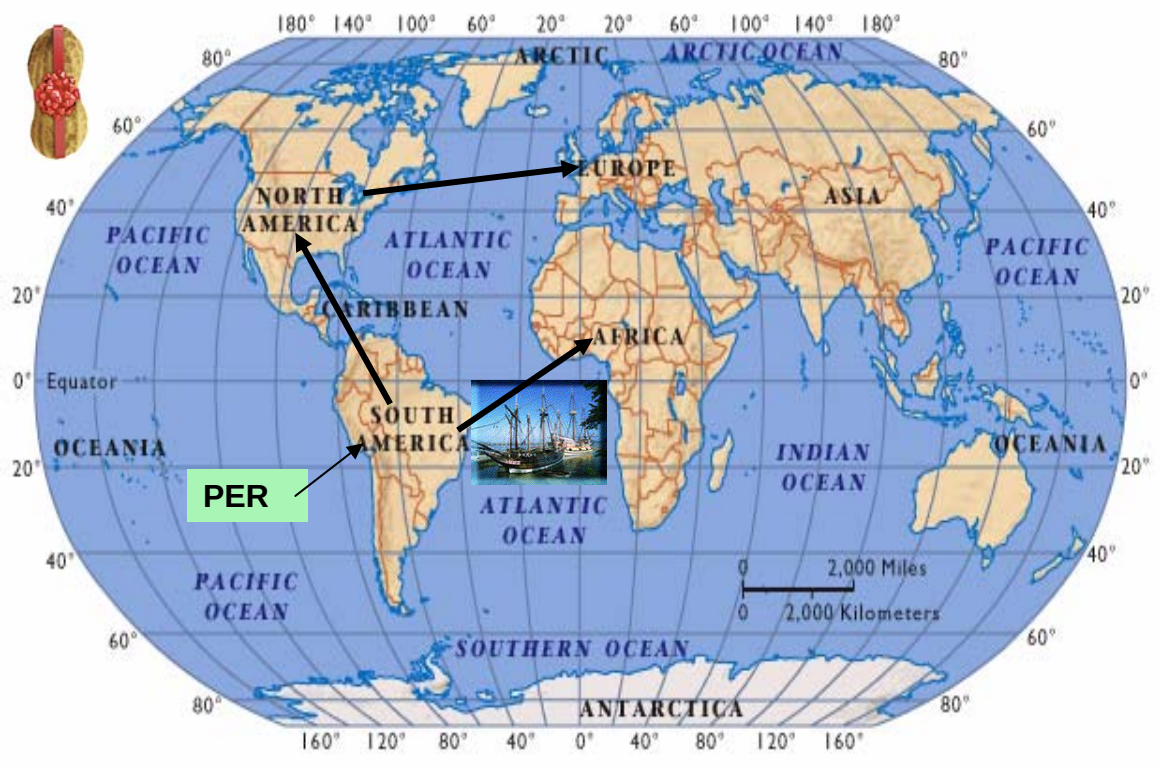

Figure 1.2: A world map illustrating the origin and distribution of the peanut plant (http://www.aboutpeanuts.com/infohis.html).

Most of the crop is produced where the average rainfall is 600 mm-1 200 mm and the mean daily temperatures are higher than 20^{o}C (Encyclopaedia of Agricultural Science, 1994). The largest producers are China and India followed by Sub-Saharan countries, as well as Central and South America [Encyclopaedia of Agricultural Science, 1994; Figure 1.3 (B)]. Seventy percent of global groundnut production is in the semi-arid tropics, and Sub-Saharan Africa accounts for nine million hectares (ha) of a total of 24 million ha cultivated over the whole world [Dar, 2002; Figures 1.3 (A and B)].

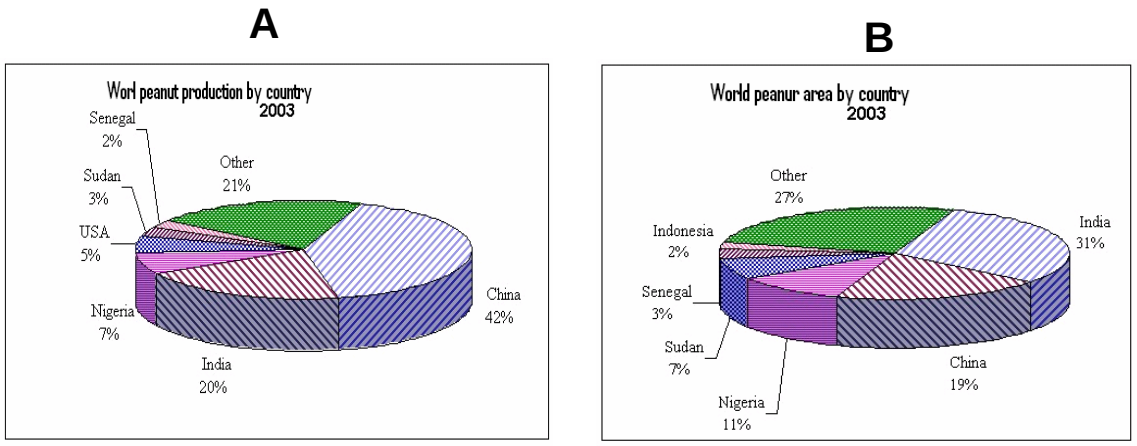

Figure 1.3: Percentage peanut production (A) and cultivation area (B) in the major peanut producing countries of the world. (http://lanra.anhtro.uga.edu/peanut/knowledgebase)

Peanut is a primary source of edible oil and has high oil (45% - 50%), fat (44%-54%) and a reasonable amount of digestible protein (25%-30%) (Encyclopaedia of Agricultural Science, 1994: Robertson, 2003). It is the richest plant source of thiamine and is also rich in niacin, which is low in cereals. Peanut is a valuable source of vitamins E, K, and B (Encyclopaedia of Agricultural Science, 1994; Robertson, 2003). Farmers in the USA and South America in general, make money from selling the crop for consumption since the international commodity market favours the sale of peanuts as edible seeds or roasted

nuts, peanut butter and confectionary products. The foliage serves as an important fodder for animals and the meal that remains after oil extraction is made into animal feed (Paik-Ro *et al.*, 1991). As consumers are becoming increasingly conscious about food safety and quality, peanut consumption is on the decline due to allergic effects. Peanuts are susceptible to *Aspergillus flavus* var. *columnaris* and this leads to the production of aflatoxins, which are carcinogenic (Holbrook and Stalker, 2003). It is because of this that testing of seeds has become a crucial exercise.

Breeders are focusing their attention towards breeding for disease and pests tolerance as these are increasingly causing heavy reductions in yields. Breeding for biotic stresses is an important objective especially in regions where pesticide and fungicide usage is not economical. Restrictions on pesticide applications have put even more pressure on breeders which forced them to shift their breeding objectives from the production of cultivars with high yields to the breeding of cultivars which contain resistance genes to plant and seed pathogens (Holbrook and Stalker, 2003).

A number of hybrids between *A. hypogaea* and other *Arachis* species have been produced through direct hybridization methods, as well as by first forming autotetraploids or amphidiploids prior to pollination. Studies have shown that there is a great deal of embryo abortion and a low level of introgression due to sterility after fertilization. The latter may be attributed to ploidy level differences, genomic incompatibilities, meiotic irregularities and restricted recombination among different species (Stalker and Moss, 1987). Most recombination is found in the F_1 (triploid) generation or in the early

hexaploid generation, and continuous selfing and backcrossing lead to the depletion of the genetic base (Garcia, 1995). Species manipulation for germplasm introgression has been reported to be impossible since no haploids have been obtained with the hybridization methods (Garcia, 1995). *A. hypogaea* has been reported to be cross compatible with *A. monticola* Krapov. and Rigoni with the F_1 produced being fertile. Hybridization with the wild diploid species on the other hand, is possible but more often than not, infertile triploids are produced (Paik-Ro *et al*., 1991).

Peanut production in the Republic of South Africa (RSA) is important for both commercial and communal farmers for the provision of plant protein and oil. Peanut production in RSA varies between 80 000 to 250 000 tons per annum, with most production coming from the commercial sector and mainly from farmers in the Northern Cape, North West and Free State Provinces. Apart from 'Spanish' peanut types, 'Virginia', 'Valencia' and 'Runner' types are also cultivated in RSA. Peanuts has a common ancestral background (i.e., 'Natal Common') and thus, peanut cultivars in RSA have presented difficulties in the establishment of their variation at the genetic level even though they differ extensively in terms of their morphology and phenotypic parameters.

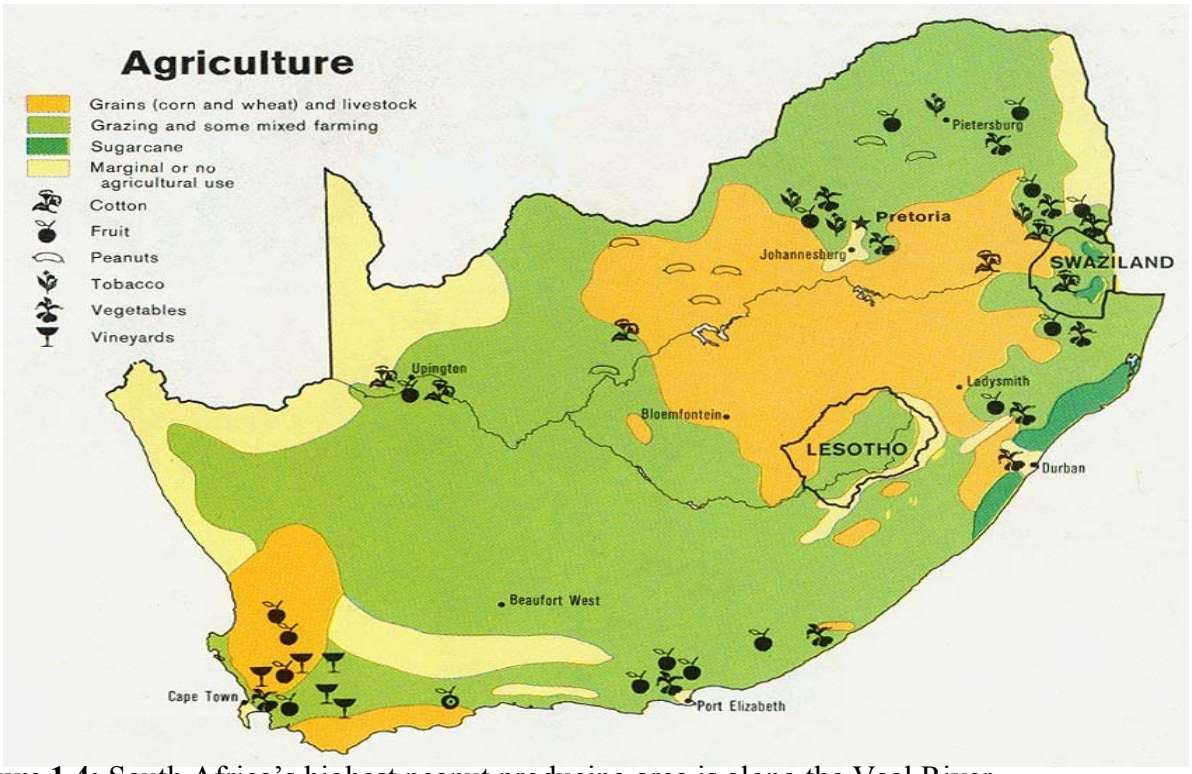

Figure 1.4: South Africa's highest peanut producing area is along the Vaal River. (http://www.lib.utexas.edu/maps/africa/south_africa_ag_1979.jpg)

Most of the varieties used by farmers in RSA are short growing season 'Spanish' types (Swanevelder, 1998), which were all developed at Agricultural Research Council (ARC) -Grain Crop Institute (GCI) at Potchefstroom, South Africa (Cilliers and Swanevelder, 2003). It is at the same institute where the peanut germplasm collection is maintained, preserved, documented and distributed. So far the collection consists of 849 accessions from 39 different peanut producing countries or research institutions all over the world, and it has served as the genetic pool for peanut breeding programs in the country since the 1940's (Cilliers and Swanevelder, 2003). This peanut germplasm collection is the only one in South Africa and not only serves as a base for peanut breeding programs, but also as an important source of genetic diversity. Other species of the genus *Arachis* are also kept there as well as varieties which resulted from interspecies crosses. Accessions

are renewed every five years (Cilliers and Swanevelder, 2003).

Peanut is known to grow reasonably well in semi-arid tropical regions where soils are poor in nitrogen and rainfall is erratic. Low nitrogen in the soil leads to reduced yields in many crops and increases the need for the application of nitrogenous fertilizers and that becomes a costly exercise for the small-scale farmer. Like many leguminous crops, peanuts are able to convert atmospheric nitrogen into ammonium and nitrate, which are absorbable by plants through their roots. Thus, peanut is not only valuable to humans as a food crop but also for soil improvement (Pimratch *et al*., 2004).

1.3 Botanical description of the plant

Peanut belongs to the tribe Aeschynomeneae, subtribe Stylosantinae, family Leguminosae (Stalker, 1997). Peanut is a member of the genus *Arachis*, which poses a unique characteristic that distinguishes it from all other plants namely that it is flowering above ground and producing fruits below ground (Figure 1.5), hence the name peanut (Holbrook and Stalker, 2003).

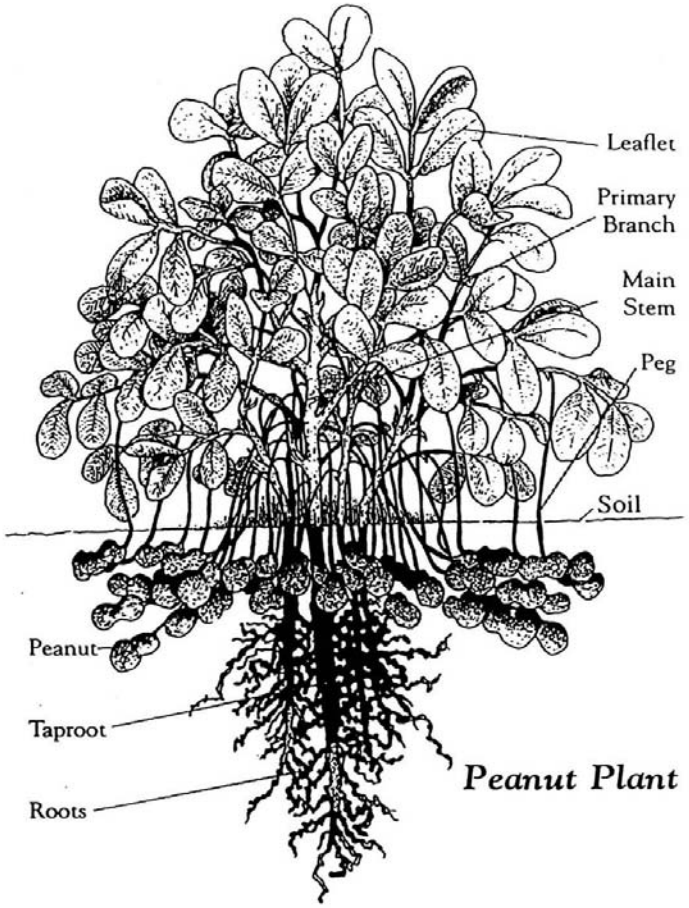

Figure 1.5: The peanut plant**:** The pegs, which give rise to the peanuts can be clearly seen above the ground and below (http://www.whitleyspeanuts.com/funfacts/).

The plant is an annual/perennial herb, with an undetermined mode of growth and a number of varieties belonging to either of the subspecies *A. hypogaea* ssp. *hypogaea* or *A. hypogaea* ssp. *fastigiata* (Stalker, 1997). The species and varieties are classified according to the location of the flowers on the plant, patterns of reproductive nodes on the branches, number of trichomes, as well as pod morphology (Krapovickas and Gregory, 1994).

A peanut seed consists of two cotyledons, a stem axis and leaf primordial, hypocotyls and primary root. Seed germination is of the epigeal mode and the cotyledons tend to change colour to green after emergence. The primary root system of peanut is a tap root system and numerous lateral roots are visible on the third day after germination (Gregory *et al*., 1973; Figure 1.5). Peanut roots do not have the normal root hair, but tuffs of hair can be seen on the lateral root. The former is only restricted to a root zone of 35 cm below the soil surface. Root hairs generally serve as invasive sites in most legumes, but this is not the case in peanut (Intorzato and Tella, 1960; Moss and Rao, 1995).

Cultivated peanut is generally self-pollinating although little out-crossing does occur with the assistance of bees, which pollinate the flowers (Wynne and Coffelt, 1982a). The wild peanut relatives are also assumed to be self-pollinating, although not much is known about their mating systems (Kochert *et al*., 1991). Kochert and colleagues (1991) reported that the geocarpic habit of peanut is a unique characteristic and could be responsible for dispersal and thus population structure. They further noted that much of the dispersal is by water and therefore the species distribution matches to a great extend the flow of major rivers.

Day length is crucial in the peanut's development as the plant produces more flowers under long day conditions, but reproductive efficiency is higher during short days. This phenomenon is an important factor to take into account during species cross hybridization programs (Wynne *et al*., 1973). The main difference between annual and perennial peanut plants, in terms of reproductive biology, is the morphology of the stigma. The

stigma of the perennial species would be covered with hair in order to hinder pollen attachment (Lu *et al*., 1990). Two days after pollination, fertilization will occur and the ovary will elongate geotropically to form a peg (Figure 1.5). In domesticated *A. hypogaea* the peg will only elongate for up to two days after soil penetration while in the wild species, it may continue to elongate for up to meters (Lu *et al*., 1990).

Despite the fact that some important traits in peanut are genetically controlled (Wynne and Coffelt, 1982a), only four cases have been reported. Thirty percent recombination was reported among genes responsible for growth habit and branching type, 40.4% recombination accounted for genes controlling growth habit and pod reticulation, and 31.5 % recombination among genes controlling stem hairiness and pod reticulation (Patel *et al*., 1936; Patil, 1965). Badami (1928) discovered the relationship between violet colour and stem hardiness, as well as small seed size and incidents of albino seedlings, respectively (Badami, 1928 and Coffelt and Hammons, 1973; as quoted by Halward *et al*., 1991a).

1.4 *Arachis hypogaea* L.: Origin and distribution

The earliest records by archaeologists for *A. hypogaea* are reported to have been from coastal regions of Peru dated from 3 900-3 750 before Christ (BC) and this comes as no surprise as South America is the centre of genetic diversity (Hammons, 1994) (Figure 1.1; Figure 1.2). It has been reported that the domesticated species had already evolved into the different subspecies and varietal groups long before seeds were distributed to the old world by Spanish and Portuguese travellers (Stalker, 1997). Distribution is believed

to have occurred in two ways whereby two seeded types of Brazilian origin were taken to Africa, and three seeded types of Peruvian origin found their way to the west coast of South America, China and various islands in the western Pacific. It was later in the 1 700s, that the 'Spanish' types were taken to Europe where they were grown for oil and human consumption. Introductions to North America are presumed to have been from northern Brazil or The West Indies, where they are believed to have been loaded onto ships carrying slaves from Africa to the New World as food supplies (Hammons, 1982) (Figure 1.2).

Gregory and his co-workers (1973) used characteristics such as morphological comparisons, geographic distributions, cross compatibility relationships and hybrid fertility to divide the genus into seven sections. Today, at least 70 species belong to the genus *Arachis* (Stalker *et al.*, 1993). Fatty acid profiles and the conservation of oleic/linoleic ratios done on the sections *Exstranervosae* and *Trisiminalae* led to the conclusions that *Extranervosae* may be the oldest species in the genus, and that the oleic/linoleic ratios assist in conditioning endurance in arid environments (Stalker *et al.*, 1989). *Arachis hypogaea* belongs to one of the seven sections in the genus *Arachis*, namely *Arachis* (Gregory *et al*., 1973). The *Arachis* section consists of *A. monticola* as the only wild tetraploid species with 2n = 4x = 40 chromosomes, whereas the rest are diploid species with 2n = 2x = 20 chromosomes (Paik-Ro *et al*., 1991).

Studies have revealed that *A. hypogaea* can only be crossed with species from the section *Arachis* and not with other sections. This led to the conclusion that its progenitors should

have indeed been from the same section (Gregory and Gregory, 1976, 1979). Cytological evidence has shown that *A. hypogaea* ssp. *hypogaea* is an allotetraploid (2n = 4x = 40) species, which consists of two subspecies, namely *A. hypogaea* ssp. *hypogaea* and *A.hypogaea* ssp. *fastigiata* (Paik-Ro *et al*., 1991). The subspecies *hypogaea* is further divided into two botanical varieties, '*hypogaea'* and '*hirsuta*', with the former comprising the 'Runner' and 'Virginia' US market types and the latter the 'Peruvian' market type. Subspecies *fastigiata* on the other hand, is also divided into two botanical varieties namely: '*fastigiata'* and '*vulgaris*' or the 'Valencia' and 'Spanish' market types, respectively (Paik-Ro *et al*., 1991). A study on isozyme variation and species relationship in peanut and it's wild relatives showed evidence that *A. hypogaea* originated from just two diploids as the two subspecies showed continuous differences in two of thirteen putative loci tested (Lu and Pickersgill, 1993).

There are a number of species, which are thought to be the progenitors of *A. hypogaea* and amongst them *A. duranensis* Krapov. and W. C. Gregory and *A. ipaensis* Krapov and W. C. Gregory are the most likely progenitors (Kochert *et al*., 1996). The evidence concerning the two genomes of *A. hypogaea* was cytogenetically observed in 1936, through the observation of a significantly smaller single chromosome pair and afterwards through karyotyped accessions in both species (Stalker and Dalmacio, 1986; as quoted by Paik-Ro *et al*., 1991). *A. hypogaea* is thus a segmental allopolyploid, which most likely originated from two closely related species with similar genomes (Stalker, 1997). Garcia and colleagues (1995) further supported this by reporting that genes from a single diploid species could be introgressed into both genomes of *A. hypogaea* (Garcia *et al*., 1995).

In 1982, it was stated that qualitatively inherited traits were controlled by duplicated loci and this added evidence to the hypothesis that *A. hypogaea* is an allotetraploid species (Wynne and Coffelt, 1982b). However in 1995, the two genomes of *A. hypogaea* were separated by the restriction fragment length polymorphisms (RFLPs) technique (Garcia *et al*., 1995) and it was concluded that most agronomically important traits in *A. hypogaea* were quantitatively inherited (Wynne and Coffelt, 1982b).

Diploids in the section *Arachis* have been reported to produce hybrids with reduced pollen fertility though few hybrid seeds between two specific diploids, *A. batizocoi* Krapov. W. C. Gregory and *A. glandulifera* Stalker were found to be entirely sterile (Smartt *et al*., 1978; Singh and Moss, 1984; Stalker, 1991). This led to the assumption that there should be three genomes in the section *Arachis* namely, the A, B and D genomes (Stalker, 1991). The A genome occurs in most of the species and is comprised of both annuals and perennials. The B genome occurs in *A. batizocoi,* while the D genome only occurs in *A. glandulifera.* Both B and D genome species are said to be annuals (Lu and Pickersgill, 1993). The allotetraploids in section *Arachis* (*A. monticola* and *A. hyp*ogaea) are therefore comprised of AABB (Smartt *et al*., 1978; Singh and Moss, 1982, 1984; Singh, 1988; Stalker, 1991) and are entirely cross compatible. It is believed that they received their B genome from *A. batizocoi* or *A. ipaensis* Krapov. and W. C. Gregory, while the A genome originated from a number of others including *A. duranensis, A. cardenasii* Krapov. and W. C. Gregory, *A. villosa* Benth. and *A. spegazzinii* M. Gregory and W. Gregory (Varisai Muhammad, 1973; Krishna and Mitra, 1988; Singh, 1988; Kochert *et al*., 1991; Singh *et al*., 1991). It has been reported that the

subspecies originated autonomously from *A. batizocoi* and other two A genomes species, but little variation has been found between the two tetraploids with molecular tools such as RFLPs and random amplified polymorphic DNAs (RAPDs) (Halward *et al*., 1991b). Previous reports suggested that *A. monticola* was the allotetraploid progenitor of the cultivated peanut, but evaluation with molecular markers proved inconclusive as they produced similar banding patterns (Halward *et al.,* 1991b; Kochert *et al*., 1991).

Polyploids encompass 30-80% of all angiosperms and this includes most of the much-needed food, fibre and forage crops world-wide (Stebbins, 1971; Sanford, 1983; Masterson, 1994; Soltis and Soltis, 1995). Based on this, it is assumed that even though polyploidism leads to a genetic bottleneck, the usefulness of polyploids in nature suggest that there must be a significant benefit in having multiple genomes in a single nucleus (Burow *et al.*, 2001). Polyploid formation more often than not leads to speciation whereby the organisms involved will reproduce independently from their progenitors and allied species (Burow *et al.*, 2001).

Breeders have been using different techniques such as pedigree, modified pedigree, mass selection, mutation breeding and backcrossing to develop different cultivars of *A. hypogaea.* These methods limited genetic variation among genotypes in commercial peanut production over the years although diversity is believed to have increased in recent years (Stalker, 1997). Even though there is an abundant germplasm resource available to peanut breeders, they have for long been depended on the crossing of elite breeding lines for the development of improved cultivars. This practice led to the erosion

of the germplasm base of domesticated peanut (Halward *et al*., 1993). Consequently, a significant amount of morphological variation could be observed, but not much genetic polymorphism is detectable with molecular markers among *A. hypogaea* cultivars, as well as exotic lines (Wynne and Halward, 1989; Grieshammer and Wynne, 1990; Halward *et al*., 1991b, 1992). Epistatic relations have been reported to be involved in the modification of genetic ratios in segregating generations of some economically vital traits (Wynne and Rawlings, 1978; Layrisse *et al*., 1980; Isleib and Wynne, 1983).

The highest number of accessions of domesticated peanut is found at the International Crops Research Institute for Semi-Arid Tropics, ICRISAT as well as in the United States Development of Agriculture (USDA) at Griffin, and amount to 13 000 and 6 000, respectively (Bettencourt *et al*., 1989; Singh *et al*., 1991). Although these accessions are representative of most regions where peanut is grown, there is a great need for collections from Africa, Central and South America, Asia and China, because most of the landraces are unfortunately being replaced by modern cultivars (Stalker and Simpson, 1995).

The development of molecular markers and genetic transformation in *A. hypogaea* are two new developments in peanut molecular and genetic research, which might bring about great benefits in breeding programs for specific traits. Studies involving molecular markers suggested that *A. hypogaea* evolved from a single hybridization incident of two wild species, *A. duranensis* and *A. ipaensis*, thus cultivated peanut has only a single origin (Kochert *et al*., 1996). Hybridization of the two genomes paved the way for chromosome duplication and later polyploidization, an event that is believed to have led

to the separation of the cultivated tetraploid from the rest of the diploid species (Hopkins *et al*., 1999). Extremely low introgression occurred between related diploid species even though there is an enormous amount of morphological variation present in the different species. This explains the low level of genetic variation amongst peanut lines, and consequently the genetic bottleneck that is found today with the use of different molecular markers (Stalker, 1997; Hopkins *et al*., 1999)

The low molecular variation found in cultivated peanut species has limited the construction of a molecular map for the species. However, in 1993 a RFLP map for two diploid *Arachis* species was constructed (Halward *et al*., 1993). Gene collinearity between the diploid *Arachis* species and the tetraploid *A. hypogaea* limits the use of the map, and it would only be utilized in following genes from the diploid to the tetraploid *Arachis* species. The map serves another purpose in the improvement of selection efficiency in the development of nematode resistant germplasm (Garcia *et al*., 1996). A genetic map for cultivated peanut will not only aid in the improvement of the crop, but will clarify the genetic basis and evolution of the vast number of different agronomic traits, which are being used to isolate the subspecies within *A. hypogaea*. Sufficient amounts of polymorphisms detected with molecular markers are crucial for a linkage map to be constructed and to link important traits can easily be linked with the markers (Halward *et al*., 1991b).

1.5 Marker studies on *A. hypogaea*

1.5.1 Biochemical markers

A great number of chemical analyses have been carried out with the aim of characterizing cultivated peanut. Variation in seed storage proteins was found between the different cultivars but diversity was inadequate to be used for cultivar identification (Bianchi-Hall e*t al*., 1991). No more than three out of 25 isozymes tested on 68 *A. hypogaea* genotypes showed polymorphisms (Grieshammer and Wynne, 1990). Similarity values found in banding patterns of an analysis of crude protein extracts and six enzymes on 36 *Arachis* accessions confirmed the seven sections described by Gregory and colleagues, (1973) (Cherry, 1975). Another confirmation of Gregory and colleagues, (1973) came ten years later from Klozova and colleagues (1983), who analysed seed protein profiles by polyacrylamide-gel electrophoresis (PAGE).

Studies done on total protein electrophoresis could be used to differentiate between and evaluate three peanut growth habits or types namely, 'Virginia', 'Valencia' and 'Spanish' (Har-Tzook *et al*., 1969). A follow-up study done on more peanut cultivars revealed four major groups in which the peanut varieties could be grouped depending on the intensities of the protein components on electropherograms (Savoy, 1976). Savoy observed that the 'Spanish' cultivars fell in the same group, while the 'Valencia' and 'Virginia' genotypes were spread among all four groups, and his finding led him to conclude that peanut possesses a standard protein pattern with quantitative differences among individual proteins.

1.5.2 DNA Markers

The study and analysis of plant genomes is crucial for the development of the crop in question. It provides insight into the organization of the particular genome, practical usage as in the case of variety identification using DNA fingerprinting and genetic maps can be developed, which can be used to identify and select important economic traits as well as in evolutionary and phylogenetic studies.

Molecular markers broaden the understanding of plant systems and plant genetics and together with biochemical studies, promise much for the classical plant breeder. Although genetic markers support breeding programs, as well as evolution studies in many other crops (Mohan *et al*., 1997), application of molecular markers such as RFLPs, RAPDs, isozymes and AFLPs have been limited by the difficulty in finding variation in cultivated peanut (Kochert *et al*., 1991; Halward *et al*., 1991b, 1992; Paik-Ro *et al*., 1991; Stalker *et al*., 1994; He and Prakash, 1997).

There is, however a great deal of polymorphism detectable between wild peanut species in the section *Arachis* with isozymes, RFLPs and RAPDs (Halward *et al*., 1991b; Kochert *et al*., 1991; Stalker *et al*., 1994). Wild *Arachis* species have been reported to be important genetic resources for disease and insect resistance, tolerance to environmental stresses, and protein and oil quality, which could be utilized in breeding programs in order to increase genetic variability for the development of improved cultivars (Young *et al*., 1973; Amaya *et al*., 1977; Cherry, 1977; Moss, 1980; Subrahmanyam *et al*., 1982). Molecular markers have become extremely useful in the exploitation of these genetic

resources as they can be used to label and follow introgression of chromosome segments containing traits of interest from wild peanut relatives into cultivated ones (Halward *et al*., 1993). The development of a genetic linkage map would further speed up the transfer and location of these tagged chromosomal regions (Halward *et al*., 1993).

Earlier work done using RFLPs on American cultivars revealed abundant polymorphisms amongst wild varieties of peanut, while few were found amongst cultivated varieties (Kochert *et al*., 1991). As a result, genetic markers have merely been used to study variation among the species and introgression from wild crosses (Stalker *et al*., 1994; Garcia *et al*., 1995, 1996). RFLPs were used to understand the relatedness of different peanut species and the technique showed that *A. ipaenis, A. duranensis* and *A. spegazzinii* are the most closely related, and that wild *Arachis* species were rather closely related to the diploid progenitor species of the allotetraploid cultivated peanut (Kochert *et al*., 1991).

1.5.3 DNA marker tools

1.5.3.1 Amplified Fragment Length Polymorphisms (AFLPs)

RFLP, RAPD and microsatellites have long been used in the identification of polymorphic DNA markers in humans and animals, while AFLP only came into the picture later. Although SSR's have long been used on humans and animals, they have only recently been used on plants while the AFLP has extensively been used on plant species long before. The AFLP technique, which merges from both the RFLP and RAPDs techniques, involves restriction digestion and the polymerase chain reaction

(PCR) and thus can address some of the shortcomings that are discovered with the other techniques. AFLPs have been described as fragments of DNA that have been amplified with the use of directed primers from restriction digested genomic DNA (Karp *et al*., 1997; Matthes *et al*., 1998). According to Vos and co-workers (1995), the AFLP technique is basically the detection of restriction fragments by PCR. The technique has become very popular because of the fact that it detects a high number of polymorphic markers in a very short time frame, detects more point mutations than RFLP and is highly reproducible. AFLPs are said to be reliable and multilocus probes (Winfield *et al*., 1998).

The main advantage of AFLPs are the large amount of produced polymorphisms which can be used to differentiate individuals in a population, for Plant Variety registration, in gene flow research, in the investigation of genetic diversity, paternity analysis and the generation of a number of markers linked to targeted genes (Law *et al*., 1998; Barker *et al*., 1999; Krauss, 1999). The technique does not need any sequence information and the PCR technique is quick taking into account that the entire process of restriction, ligation, pre-amplification and amplification may be long (Rafalski *et al*., 1996).

Most of the shortcomings of the AFLP technique can be overcome, but the major disadvantage is that expert knowledge is obligatory and the technique is time consuming (Gift and Stevens, 1997). Computer detection of fragments is more efficient than scoring bands from auto-radiographs (Krauss and Peakall, 1998). AFLPs are dominant markers and are thus not as ideal for population genetics studies as SSRs (Robinson and Harris,

1999). Another aspect to be kept in mind is the fact that ploidy levels of the taxa in question have an effect on the amount of variation (Kardolus *et al.*, 1998).

1.5.3.2 Microsattelite markers/Simple sequence repeats (SSRs)

Microsattelite, otherwise called simple sequence repeats (SSRs), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs) are sequence repeats, generally less than five base pairs in their length, which are used to detect polymorphisms in genomes (Brudford and Wayne, 1993). These types of genetic markers have shown to be significantly more polymorphic than RAPDs or RFLP but less than AFLPs, and have been used in a number of genetic studies involving humans and other mammals, rice and soybean (Rongwen *et al.*, 1995; Dib *et al.*, 1996; Panaud *et al.*, 1996; Sun and Kirkpatrick, 1996). Simple sequence repeats come highly recommended because of their codominance and reasonable ease of detection using the PCR amplification. Little amounts of DNA are required for analysis, and SSRs are genetically defined and highly variable (Hopkins *et al.*, 1999). These markers are reproducible and this makes them perfect for population genetics studies as well as for genome mapping programs (Dayanandan *et al.*, 1998). SSRs provide high levels of polymorphism and require cloning and sequencing, if new once need to be developed, in order to obtain information on the flanking nucleotide sequences.

Microsattelite polymorphism is brought about by variation in the amount of repeat units. These variations are due to errors that occur during DNA replication, whereby the DNA polymerase slips while copying the repeat region thus resulting in a different repeat number (Jarne and Lagoda, 1996; Moxon and Wallis, 1999). Greater changes in repeat

number are sometimes observed and these are attributed to unequal crossing over and above those that occur during DNA replication (Strand *et al*., 1993). Microsattelites have been used to a great extent in diversity studies (Rossetto *et al*., 1999), which are important for taxonomy and conservation of genetic diversity, gene flow and mating systems as well as paternity analysis (Chase *et al*., 1996; Streiff *et al*., 1999). One distinct characteristic of microsattelite markers is the fact that they identify dissimilarities at individual loci and today SSRs designed for a particular species can be used across another species depending on the taxonomic distances between the species in question, a discovery that prompted more studies in phylogeny (Robinson and Harris, 1999).

1.6 Improvement of *A. hypogaea* with the *OC*-1 gene

1.6.1 Proteolysis and proteinacious inhibitors in plants

Proteolysis is an important process which governs the metabolism of all living cells. Proteases, on the other hand, are a group of enzymes responsible for various important proteolytic processes such as catabolism, hydrolysis of dietary proteins and selective degradation of proteins by cleaving peptide bonds (Michaud, 2000). These processes are important because they provide cells with the needed metabolites for plant growth and development. The implication is that both pest extra-cellular proteases and proteinase inhibitors (PIs) produced by certain plants are crucial for proteolitic reactions in the plant cells (Michaud, 2000).

Plants are challenged by numerous pathogens and the fact that they are not mobile place them even more at risk. In addition to that, a plant-insect interaction is a continuous and

ever-changing system. Peanut is no exception and the most serious peanut pests in general include ground weevils (*Protostrophus barbifrons* species), African Bollworm (*Helicoverpa armigera* Hübner), spotted maize beetle (*Astylus astromaculatus* Melyridae) and turnip moth (*Agrotis segetum* (Denis & Schiffermüller)] (van Wyk and Cilliers, 2000).

As one mechanism of plant defence, certain plants have been shown to produce a number of proteinaceous PIs in response to an attack by a predator or an infection. PI's are proteins which are produced in storage organs such as seeds and constitute 5-15% of total proteins. Wounding or exposure to exogenous phytohormones induces their synthesis (Ryan and Jagendorf, 1995; Rakwal e*t al*., 2001).

It has long been suggested that PI's are involved in the plant defence mechanism as concentrations by far surpass those needed in proteolysis (Jongsma and Bolter, 1997). Read and Haas measured PI's for the first time in plant tissues in 1938, as quoted by Jongsman and Bolter (1997). PI's can react with proteases produced in the midgut of herbivorous pests and pathogenic microbes (Jongsman and Bolter, 1997), as pseudo-substrate and obstruct their activities thus making them less destructive to the plant. It has been reported that as a result the pest will be affected by poor growth and fecundity and it will not degrade the host plant defence proteases (Michaud 1997; 2000). Therefore PI's basically function by causing an amino acid deficiency which will lead to compromised insect growth and development and ultimately leading to death either by inhibition of gut proteinases or owing to overproduction of the digestive enzyme, which causes less amino

acids to be available for synthesis of other proteins (Marcia *et al*., 2002). PI's can be synthesized locally but signals from wounding can induce the production of PI's throughout the whole plant body (Pearce e*t al.*, 1993).

Plant pests and plant seeds are believed to have coevolved, with the pest acquiring a method of obtaining nutrients from plant seeds and the seeds producing protenaceous inhibitors as a way of protecting themselves. The insect will then be adapted to the plant system and is from that point a pest (Michaud, 1997).

There are four types of proteases, classified based on the type of reaction that is catalysed, the chemical nature of the catalytic site and the structural evolutionary relationship between the different proteases. They are the serine, cysteine, aspartate and metallo-proteases. The latter proteases are inhibited by their respective PI's [i.e., serine PI's, cysteine PI's, aspartate PI's and metallo PI's (Michaud, 1997)]. These discoveries led to the use of PIs, either in transgenic systems or any other delivery system to protect plants from destruction caused by herbivorous insects, root-parasitic nematodes and fungal pathogens. This prompted the use of recombinant PI's in plants as a means of an effective method of controlling pathogenic organisms in plant systems and today a number of plants have been genetically modified with PI's by simply intergrading PI's into their genomes (Michaud, 1997). Plants resistant to insects through the expression of PI's were reported for the first time in 1987 (Hilder *et al*., 1987).

1.6.2 Cystatins in plants

Barret used the name cystatin for the very first time in 1981, as he was describing an inhibitor that was discovered and partly characterized from chicken egg whites (Barret, 1981). Cystatins forms a super family of tight and reversible binding inhibitors of the papain-like cysteine proteinases, which can be divided into three families, cystatin, stefins and kininogens (Michaud, 2000). The family poses a distinct characteristic, which is the presence of the two disulphide bonds next to the carboxyl terminal, and use their NH_2 terminal to bind to the enzyme (Michaud 2000; Garcia-Carréno *et al*., 2000).

Cysteine proteases are known to be the major type of digestive proteases in the Coleopteran gut and Michaud demonstrated that the OC1 protein is able to inhibit Colorado Potato Beetle (CPB) proteases *in vitro* (Michaud, 1997). Lecardonnel and colleagues in 1999 observed more than 50% death of Colorado potato beetle larvae with insects reared on a transgenic potato line expressing the *OC*-1 gene (Lecardonnel *et al*., 1999).

1.6.3 Effects of *OC*-1 in plant-pest relationships

The *OC*-1 is a naturally occurring cystatic gene isolated from rice that has been found to confer resistance against several insect pests in rice. The same gene has been used in the protection of a number of other plants such as potato, poplar and rapeseed (Michaud and Vrain, 1998).

Lecardonnel and colleagues (1999) established for the first time that *OC*-1 expression in transgenic plants confer resistance to coleopterans. It was later shown that *OC*-1 transgenic plants conferred resistance towards the nematode *Globodera pallida* ssp. (Vain e*t al*., 1998). They observed a weight and proteolytic activity increase in cabbage stem flea beetles (*Psylliodes chrysocephala* ssp.) feeding on a *OC*-1 expressing line of oilseed rape (Girard *et al*., 1998b); De Leo and his co-workers (1998) observed contradictory effects in *Spodoptera littorlis* ssp. on MTI-expressor transgenic lines. These findings led to a suggested threshold needed to induce deleterious effects below which the larvae could survive and even produce excessive amounts of proteases (De Leo *et al*., 1998). A study done on effects of *OC*-1 expressing potato on Colorado Potato Beetle (CPB) larvae showed reduction in body mass, which was inconsistent with previous results (Lecardonnel *et al*., 1999). These differences may be brought about by the amount of ingested PI, the form of PI used and most likely the type of bioassay used in each study (Lecardonnel *et al*., 1999). In a study done to determine the effects of *OC*-1 on different aphids it was found that, regardless of the weak toxic effects, the *OC*-1-transgenic oilseed rape plants continuously showed deleterious effects on *Myzus persicae* Sulzer especially on reproductive performance (Rahbe *et al*., 2003).

1.6.4 Insect adaptation to PI's

Herbivorous insects develop complementary adaptation mechanisms in response to host plant adaptations (Marcia and Marcio, 2002). One method by which insects adapt to PI's is by inducing the synthesis of proteases insensitive to the PI in question. Secondly they could over-express proteases in order to maintain adequate levels of activity (Lecardonnel *et al*., 1999). As another response, insects could detoxify the host defence

chemicals by oxidation, reduction, conjugation or hydrolysis of molecules (Scott and Wen, 2001). It has been reported that as a way of avoiding host plant poison, insects could sequester and deploy the poison for their own pheromone and defence systems (Nishida, 2002). The tobacco hornworm, for example, utilizes the nicotine produced by the tobacco plant (which is toxic to most insects) in its body to deter parasitoids (de Bruxelles and Roberts, 2001). It has recently been established that lepidopteran insects have constitutive trypsins and trypsins induced by ingestion of PI's that are insensitive to the inhibitors (as quoted by Marcia and Marcio, 2002).

1.6.5 The transformation process

The phenomenon of transformation was discovered in 1928, even though by that time there was no proof that DNA was involved (as quoted by Gardner *et al*., 1991). Purification of DNA became the concern, as proteins could also be the basis for transformation. In 1944, experimental results reported, showed evidence that DNA was indeed the basis for transformation and this was with the use of different enzymes (DNase, RNase) (as quoted by Gardner *et al*., 1991).

In eukaryotes the sexual process of meiosis and fertilization combines genetic material from two individuals in a single zygote. Meiosis and fertilization do not however occur in prokaryotes, and bacteria use another mechanism instead, which is divided into transformation, transduction and conjugation (Campbell, 1993). Transformation is a mode of recombination, thus an exchange or transfer of genetic information between organisms or even from one organism to the other that can be witnessed in some species

of bacteria. A simple definition of transformation would be that it is a process of the up-take of foreign DNA, usually plasmids, by bacteria. In this case, bacteria which have taken up the plasmid are then selected by growth on a plate containing an antibiotic to which the plasmid encodes resistance (Turner *et al*., 1998). The same technology is applicable in the transfer of plant genes from one plant to another.

Different methods such as particle bombardment and *Agrobacterium tumefaciens* mediation used for transformation of *A. hypogaea* resulted in different levels of transformation efficiency (Sharma and Anjaiah, 2000). The particle bombardment method of gene delivery has been seen as labour intensive and a high number of plants should be bombarded in order to achieve a few transformed cell lines. An improved *A. tumefaciens*-mediated transformation method used to transform *A. hypogaea* yielded 55% of the treated explants that resulted in independent transformants (Sharma and Anjaiah, 2000).

Agrobacterium tumefaciens is a soil-borne bacterium, which causes tumours to infected plants through the integration of part of the plasmid, the tumour inducing (Ti) plasmid. The system is based on the natural ability of *A. tumefaciens* to infect susceptible plants and transfer genetic material form its Ti plasmid into the host genome. This causes the development of the crown gall tumour, or in the case of *A. rhizogenes*, the Ti plasmid causing the hairy root symptom. The T-DNA also carries a gene for the production of opines (special amino acids) which the bacteria utilizes for it's nourishment. Opine catabolism genes carried on the non-detached section of the Ti plasmid enable the

bacteria to metabolise these opines. The *chv* genes located on the bacteria chromosome are stimulated by the phenolic compounds released into the environment by the wounded plant cells. This enables the bacterium to recognize the injured plant and attach itself to the injured cell. After attachment, the Ti-plasmid detaches a section of its DNA, the T-DNA then enters the host plant cell and gets integrated into the plant genome. The process is facilitated by products of the *Vir* (virulence) genes, which are located on the non-detached section of the plasmid. Integration of T-DNA into the host plant genome results in the expression of the oncogenes leading to the production of plant growth hormones that promote uncontrolled proliferation of the affected cells. This is by far the most common crop transformation system used to date and it has been used extensively in the introduction of foreign genes into selected plants (Gardner *et al*., 1991).

1.7 Overall objectives of the study

The objective of this study was firstly to evaluate the agronomic performance of 18 South African peanut lines (Chapter II).

Secondly, to transform the wild-type JL24 peanut line with an exogenous cysteine proteinase inhibitor gene from rice in order to confer resistance against peanut storage pests and thus decrease or eliminate costly fumigation practices and then to evaluate the transformation efficiency of JL24 in the greenhouse (Chapter III).

Thirdly, to establish the genetic diversity amongst different South African commercial peanut varieties, including the genetically enhanced variety JL24 (OCI), using the AFLP technique and microsatellites markers (Chapter IV).

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Chapter II

Agronomic factors as a tool in determining variability in South African commercial lines of *Arachis hypogaea* L.

2.1 Introduction

Previous studies (Gregory *et al*., 1973; Norden *et al*., 1987; Stalker *et al*., 1993) have confirmed the abundance of morphological and physiological variation in *A. hypogaea* despite the fact that it has been difficult to determine variation at genetic level (Herselman, 2003). Taxonomic work done on the genus has divided it into seven sections using morphological characteristics, geographic distribution, cross-compatibility relationships and hybrid fertility (Gregory *et al*., 1973; Halward *et al*., 1991; Stalker *et al*., 1993).

Oil content determination is an important physiological characteristic to consider in the peanut industry. Two fatty acids, oleic acid (O) and linoleic acid (L) make up over 80% of peanut oil content in most commercial varieties. On average, a peanut seed has 55% oleic and 25% linoleic acid (Knauft *et al.*, 1993). Oleic acid is saturated and more stable compared to linoleic acid, which is a less saturated and less stable fatty acid. Oleic and linoleic ratios are crucial as they govern rancidity of peanuts and therefore determine their shelf-life. Norden and co-workers (1987) identified and reported a mutant peanut type with an extremely high O/L ratio. This highly desirable trait would be very valuable to the peanut industry.

Most South African commercially cultivated peanut lines share the same ancestral background and are of ‘Spanish’ type, a few ‘Virginia’ types, one ‘Valencia’ and two Florida ‘Runners’. These came about through extensive hybridization, intercrossing

events and selections by South African peanut breeders (Cilliers and Swanevelder, 2003a).

Traders who interacted with local South Africans introduced one of the first commercial varieties, 'Natal Common', which was used to develop four other cultivars through direct selection (Cilliers and Swanevelder, 2003). 'Sellie', a cross between 'Natal Common' and 'Namark', later replaced 'Natal Common'. 'Sellie' had even better characteristics than 'Natal Common' (van der Merwe and Vermeulen, 1977). Cultivars such as 'Harts' and 'Robbie' were selected for resistance to common diseases, namely black hull (*Chalara elegans* Nag Raj and Kendrick) and stem rot (*Sclerotium rolfsii* Saccardo), respectively (van der Merwe and van der Merwe, 1988). Also, a cross between 'Sellie' and 'Harts' paved the way for the release of three cultivars namely, 'Jasper', 'Kwarts' and 'Akwa', in 1991, 1991 and 1994, respectively (van der Merwe and Joubert, 1995). 'Anel' is the latest important cultivar selected from 'Natal Elite', and it was developed for dry land conditions, since it was drought tolerant (Swanevelder, 1998). 'Runner' types such as 'Norden' and 'Selmani' are not very popular among breeders and farmers because of their long growth seasons and furthermore, 'Norden' is susceptible to *Sclerotinia* stem rot (Cilliers and van Wyk, 1999).

It is because of this shared ancestral background that it has become increasingly important to establish morphological and physiological variation among these lines in order to determine and evaluate the gene pool. The objective of this study was to

determine variability between 18 South African commercially cultivated lines using agronomic characteristics.

2.2 Material

The eighteen South African peanut lines, bred and/or cultivated at the Agricultural Research Council (ARC)-Grain Crops Institute in Potchefstroom, RSA, used in the study are indicated in Table 2.1 below.

Table 2.1: Botanical types, varieties, subspecies and sources of the 18 lines used in this study (Herselman, 2003; Cilliers and Swanevelder, 2003).

Genotype	Botanical type	Variety	Subspecies	Source
'Harts'	'Valencia'	*fastigiata*	*fastigiata*	South Africa
'Kwarts'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Jasper'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Sellie'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Anel'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Natal Common'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
JL24	'Spanish'	*vulgaris*	*fastigiata*	South Africa*
'Norden'	'Runner'	*hypogaea*	*hypogaea*	South Africa
'Robbie'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Billy'	'Virginia'	*hypogaea*	*hypogaea*	South Africa
ICGV93415	'Spanish'	*vulgaris*	*fastigiata*	ICRISAT
PC222-9	'Spanish'	*vulgaris*	*fastigiata*	South Africa
'Akwa'	'Spanish'	*vulgaris*	*fastigiata*	South Africa
PC253K12	'Spanish'	*vulgaris*	*fastigiata*	South Africa
98Sel-37K1	'Virginia'	*hypogaea*	*hypogaea*	South Africa
98Sel-8K1	'Virginia'	*hypogaea*	*hypogaea*	South Africa
98Sel-3K1	'Virginia'	*hypogaea*	*hypogaea*	South Africa
'Selmani'	'Virginia'	*hypogaea*	*hypogaea*	South Africa

ICRISAT = International Crop Research Institute for the Semi-Arid Tropics
*Provided by Dr. A.J. Cilliers and Dr. C.J. Swanevelder, Agricultural Research Council-Grain Crops Institute, Private Bag X1251, Potchefstroom, 2520

2.3 Methods

2.3.1 Descriptive keys to symbols used in the study

The following parameters were used as descriptive keys to record the different agronomic characteristics during the study as obtained from the 'Catalogue of Groundnut Germplasm' (Cilliers *et al.*, 2001).

The angle between the vertical main stem and the side branches was used to describe the **growth habit** whereby 9.0 was used for the 'Runner' types and 4.5 for the bunch types. **Growth season**, referred to the number of days from planting to lifting of the plant and lifting was done once 70% of the pods were mature. Numbers were allocated to the different **testa colours** in order to measure their prevalence (1= Flesh, 2= Red, 3= Purple, 4= White, 5= Yellow, 6= Wine and 7= Variegated) (Cilliers *et al.*, 2001; Appendices, Table A.1.1-2)**.** Five of the cultivars studied were analysed in terms of their kernel sizes using the records given in the 'Catalogue of Groundnut Germplasm' (Cilliers *et al.*, 2001). Different grading screens were used to measure the **kernel sizes**. Grading screen 8.3 mm measured the percentage of kernels, by mass, not passing through the 8.3 mm grading screen; grading 7.6 mm measured the percentage of kernels, by mass, passing through the 8.3 mm screen, but not through the 7.6 mm grading screen; and grading 6.7 mm measured the percentage of kernels, by mass, passing through the 7.6 mm screen, but not through the 6.7 mm grading screen. Round kernels were measured by the percentage of round kernels, by mass, above the 6.7 mm grading screen (Appendices, Table A.2.1-2).

In terms of oil, **total oil percentage** was obtained from the oil content of kernels found on the 7.6 mm grading screen and C16:0 (the percentage palmitic acid obtained from a sample of kernels found on the 7.6 mm grading screen), C18:0 (the percentage stearic acid obtained from a sample of kernels found on the 7.6 mm grading screen), C18: 1 (the percentage oleic acid obtained from a sample of kernels found on the 7.6 mm grading screen) and C18:2 (the percentage linoleic acid obtained from a sample of kernels found on the 7.6 mm grading screen) was determined and analysed as obtained from the 'Catalogue of Groundnut Germplasm' (Cilliers *et al.*, 2001). **Oleic to linoleic acid ratios** (O/L) were calculated from the obtained data (Appendices, Tables A.3.1-2).

2.4 Results

2.4.1 Growth Habit

About 80% of all RSA cultivated peanut lines are of the bunch type as shown by the maroon colour and only about 20% are runners, namely 'Norden' and 'Selmani' indicated by the purple colour (Figure 2.1).

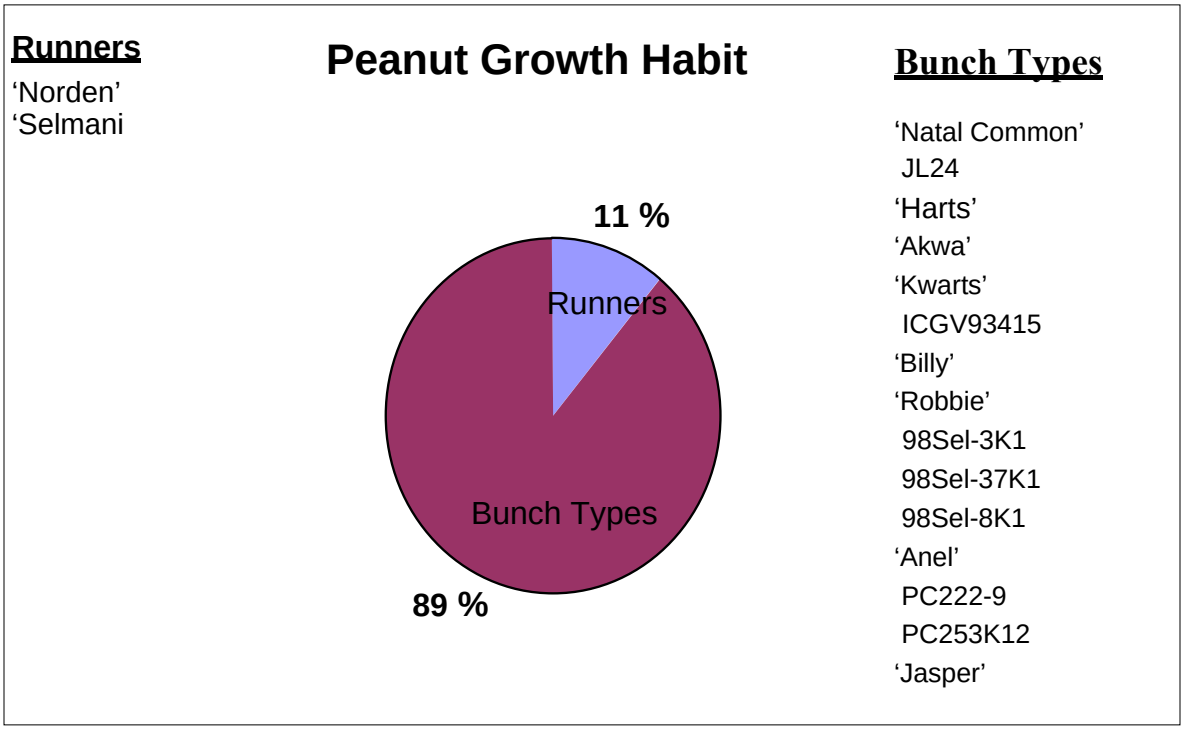

Figure 2.1: A pie-chart showing the growth habit of the 18 RSA peanut lines. The number of Runners and bunch types are given in percentages. The runners had a 9.0 angle between the vertical main stem and the side branches while the bunch types had a 4.5 angle

All the lines are from subspecies *hypogaea*, *fastigiata* and *vulgaris* varietal groups and 'Spanish', 'Valencia', 'Virginia' and 'Runner' botanical types (Table 2.1). Lines of 'Spanish', 'Valencia' and 'Virginia' types, showed a bunch type growth habit even though it is known that 'Virginia' types could be both runners and bunch types (Figure

2.1, blue colour). Only the cultivars of 'Runner' type origin ('Selmani' and 'Norden' in this case) are runners in terms of growth habit.

2.4.2 Growth season

The different growth seasons measured during this study are represented in Figure 2.2. The maximum number of days from planting to lifting recorded for the 18 cultivated peanut lines was 180 days (purple colour) representing the longest growth season. The shortest growth season was 120 days (Appendices, Table A.1.1-2).

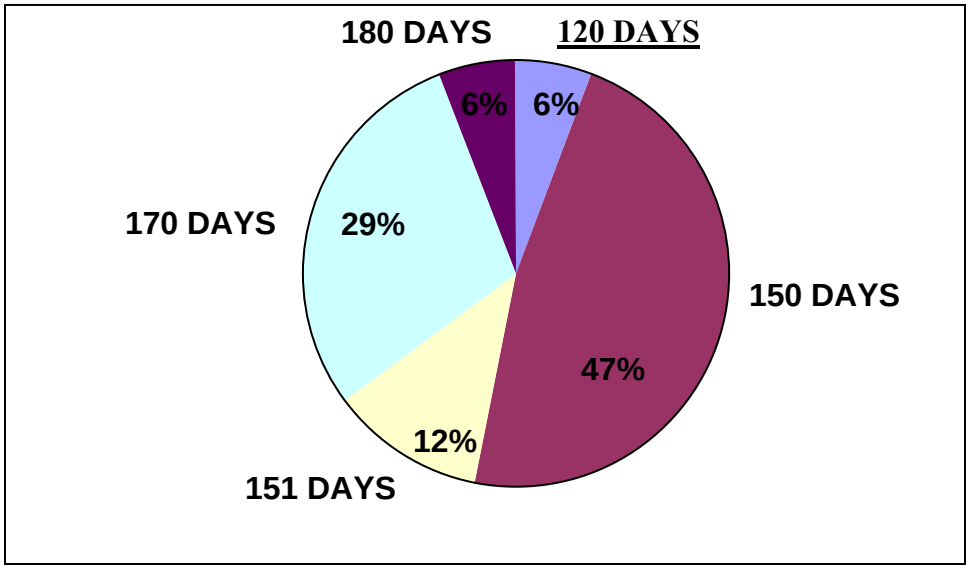

Figure 2.2: A pie-chart illustrating growth season of the 18 lines in number of days from planting to lifting. DAYS = Days from planting to lifting; **%** = Percentage lines

Most of the plants (47%) fell under the 150 days (maroon colour) growth season. Twenty nine percent of the plants fell under the 170 days (turquoise colour) growth season, 12% under the 151 days (yellow colour) and only 6% for both 120 and 180 days (blue and

purple colours respectively) growth seasons. The lines were considerably variable in terms of growth season.

The 'Runner' type, 'Selmani' had the longest growth season (180 days) followed by 'Norden', (170 days) and the 'Virginia' types, 'Billy', 98Sel-3K1, 98Sel-37K1 and 98Sel-8K1, which also recorded 170 days growth season. All the 'Spanish' types recorded 150 days growth season and the only 'Valencia' type, 'Harts', recorded the shortest growth season (120 days).

2.4.3 Testa colour

Only three different testa colours were observed among the peanut lines, flesh (F), red (R) and variegated (V) (Figures 2.3 and 2.4).

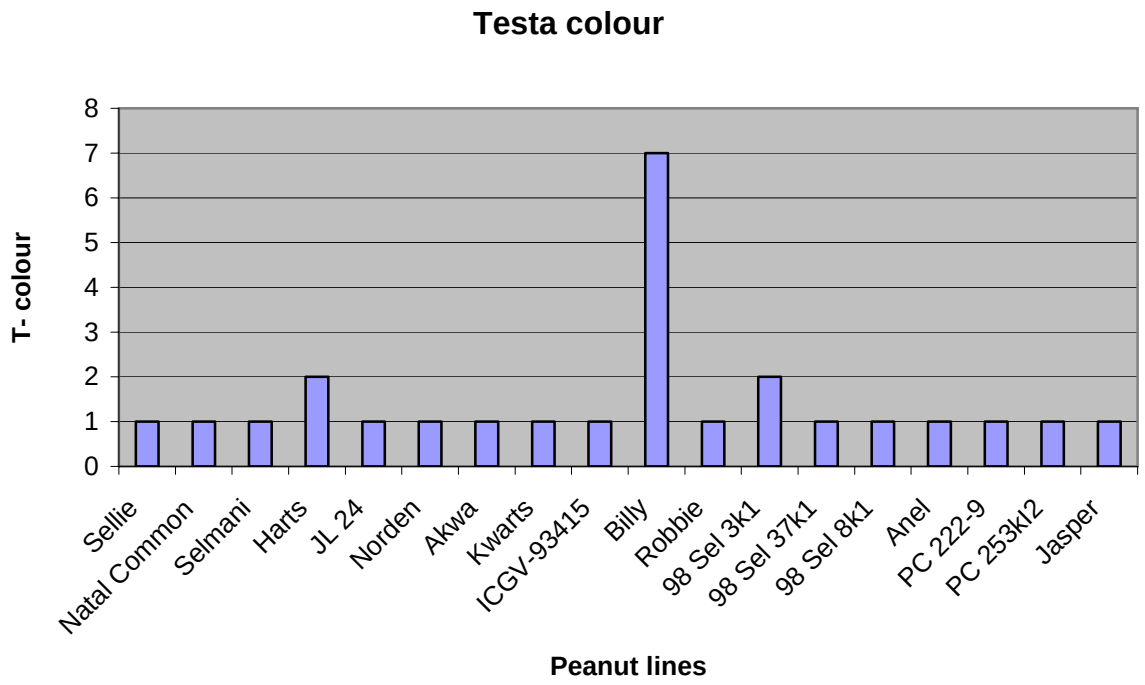

Figure 2.3: An illustration of testa (skin/ T colour) differences of all 18 RSA peanut lines. T-colour is shown by numbers, which have been allocated to the different colours as 1= Flesh, 2= Red, 3= Purple, 4= White, 5= Yellow, 6= Wine and 7= Variegated

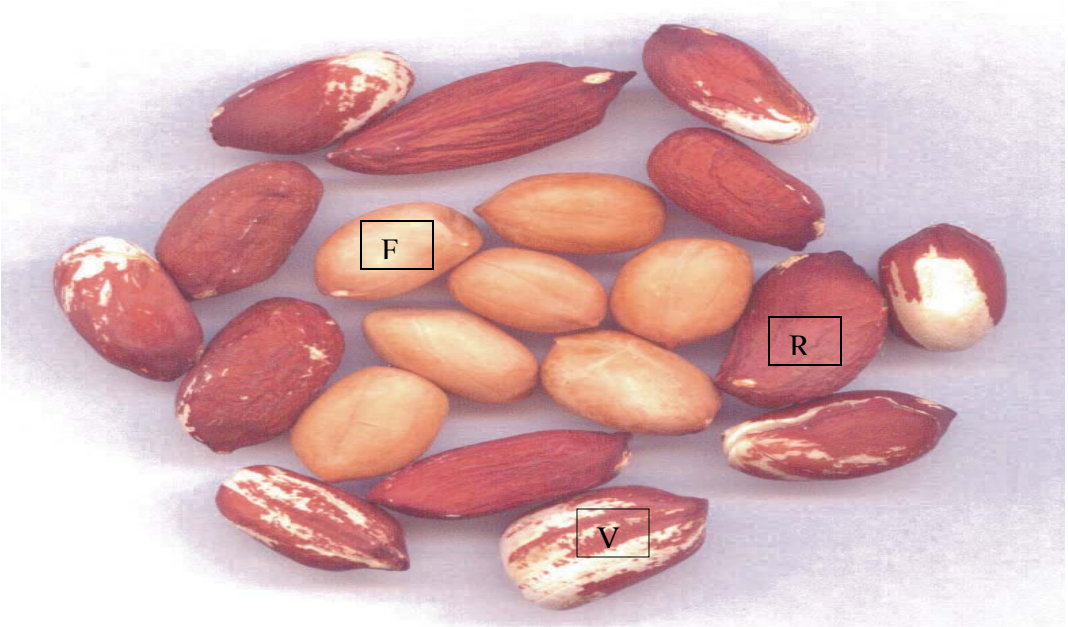

Figure 2.4: The three different testa colours observed on the lines. The difference can clearly be seen between flesh (F), red (R) and variegated (V)

Of the 18 lines, only three showed differing testa colours while the rest were flesh in colour. Two of the lines were red and only one was variegated. The 'Valencia' type 'Harts' and the 'Virginia' type 98Sel-3K1 had red testa colours, while 'Billy', a 'Virginia' type, had a variegated testa colour. These results were independent of the botanical types classifications in Table 2.1 as well as growth habits in Figure 2.1.

2.4.4 Total oil percentage

Three of the 18 lines were randomly selected and their oil percentages compared. The bunch 'Spanish' type, 'Sellie' had the highest percentage kernel oil followed by 'Norden' a 'Runner' and 'Selmani' a 'Runner' as well (Figure 2.5; Appendices, Table A.3.1-2).

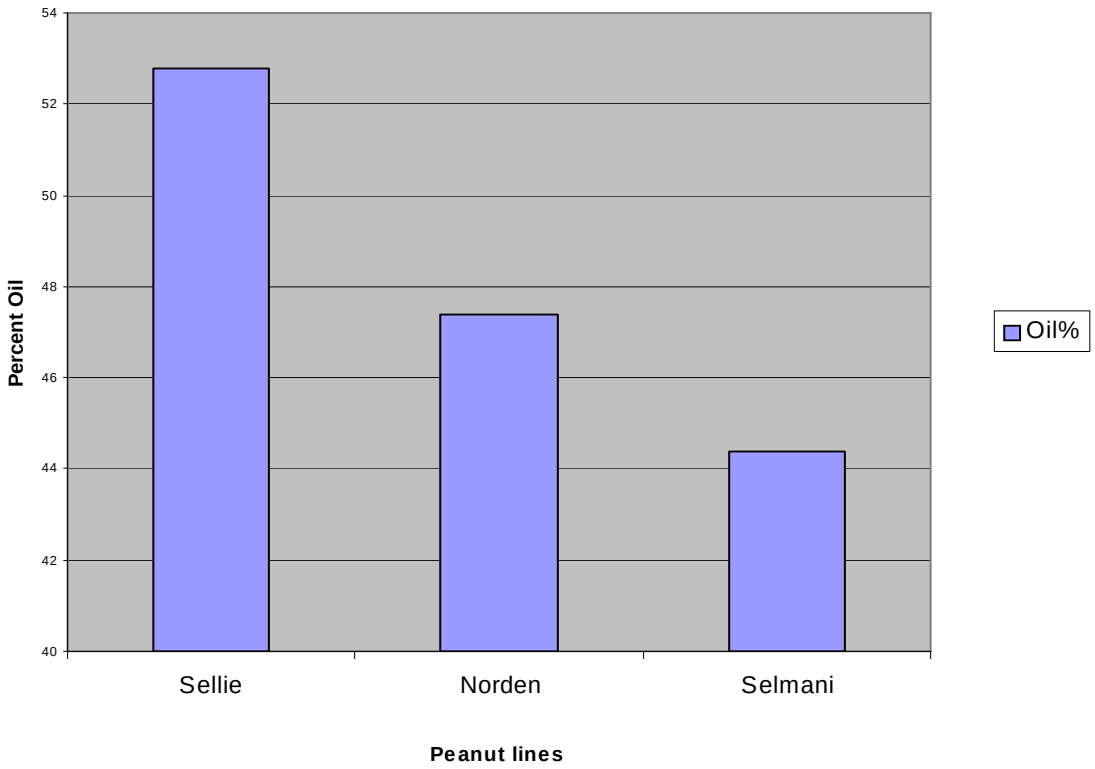

Figure 2.5: Oil percentage comparisons of kernels, which remained on the 7.6 mm grading screen, between three peanut lines, 'Sellie', 'Norden' and 'Selmani'

When the three lines were measured for their palmitic acid, stearic acid, oleic acid and linoleic acid, the lines showed variation for all fat types (Figure 2.6). Oleic and linoleic

acids were the highest in terms of percentage for the three lines, followed by palmatic acid while stearic acid was the lowsest of all. 'Sellie' and 'Selmani' had between 1.0 and 1.1 oleic/linoleic ratios respectively, while 'Norden' had a 1.2 oleic/linoleic ratio. Sellie had the highest total oil percentage contributed by high concentrations of palmitic and stearic acids. When statistical analysis was done, it was found that the three lines were significantly different from one another in terms of the contents of the four different types of oils namely, palmitic acid, strearic acid, oleic acid and linoleic acid (Jandel Scientific, Sigmastat, version 2.0; Appendices, Table A.3.2).

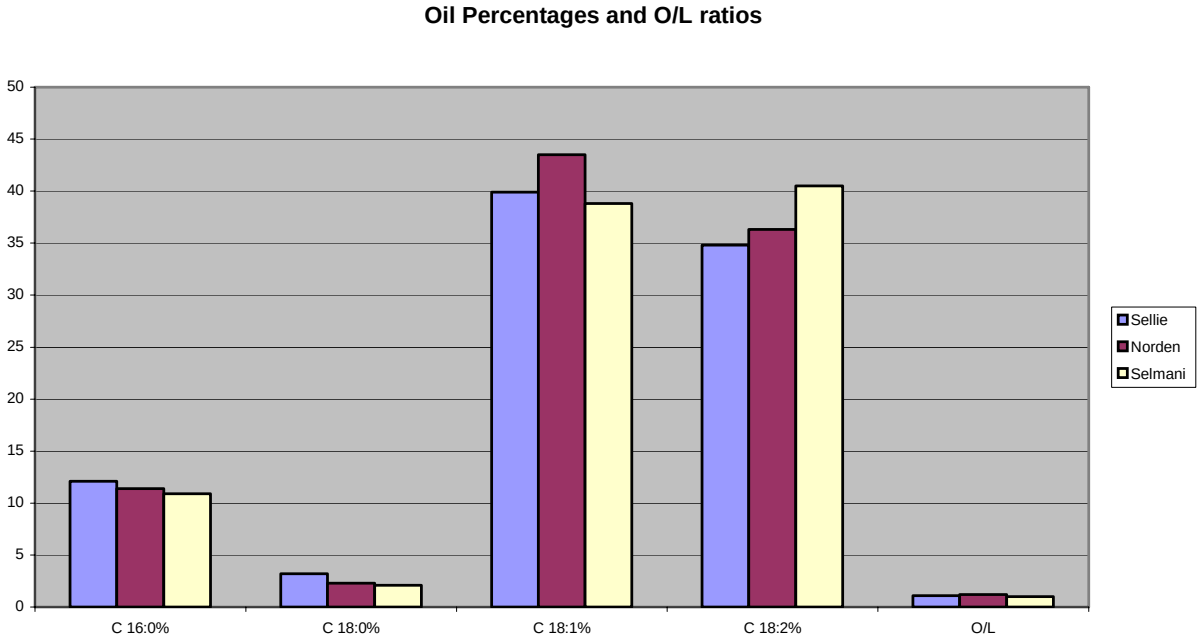

Figure 2.6: Palmitic acid (C 16:0 %), strearic acid (C 18:0 %), oleic acid (C 18:1 %), linoleic acid (C 18:2 %) and oleic to linoleic (O/L) ratios of the three lines ('Sellie', 'Norden' and 'Selmani')

2.5 Discussion and conclusion

Both peanut growth habits (i.e., runners and bunch types) were observed in the South African peanut lines. Only two (11%) of the lines were of the 'Runner' botanical types, while the rest (89%) were bunch types (Figure 2.1). The peanut growth habits results (11% 'Runner' and 89% bunch types) were exactly as expected, as the ancestral background of the lines was known and could be traced back. Therefore, not much variation was observed between the lines in terms of growth habits.

It was found that the growth season of the lines, from planting to lifting, was between 120 and 180 days (Figure 2.2). There were four observed and recorded growth seasons, 120, 150, 170 and 180 days. Forty seven percent of the lines required 150 days from planting to lifting, while 29% required 170 days, and 12% would be ready for lifting after 151 days. Only 6% of the lines needed 180 or 120 days before lifting. The lines were thus notably different in terms of growth season.

According to Robinson (1984), 'Runner' and 'Virginia' types required 130 to 150 days to maturity however our study showed that 'Runner' and 'Virginia' types required between 170 to 180 days to maturity. Robinson (1984) also recorded 90 to 120 days to maturity for his 'Spanish' types, while we recorded 150 days before lifting for our 'Spanish' types and 120 days for the only 'Valencia' type, 'Harts'. We can therefore conclude that South African peanut lines need a longer growth season compared to varieties cultivated in Minnesota (Robinson, 1984) and this could be attributed to various factors such as soil types, rainfall patterns and temperature.

More than 80% of the lines showed similarities in testa colour, i.e. flesh colour. Two of the lines were red and only one was variegated in testa colour (Figures 2.3 and 2.4). The lines were therefore not extremely different in testa colour. It has been observed that consumers prefer the flesh colour and show less preference towards red skinned peanuts (van der Merwe and van der Merwe, 1988).

Of the three lines selected for oil percentage determination, 'Sellie' had the highest total oil percentage, palmatic and stearic acids and 'Norden' had the highest oleic acid and oleic/linoleic ratio, while 'Selmani' had the highest linoleic acid percentage (Figures 2.5 and 2.6). The three lines showed significant variation in oil content and since they were randomly selected to represent the whole sample, it can be concluded that oil content in RSA peanut lines does vary considerably. O/L ratio, which is one of the most important factors in the peanut production industry, did not vary. All three lines tested for oleic acid content showed low percentages of oleic acid (35% to 45%) compared to the normal (55% to 60%) and this character is believed to occur naturally as a result of reduced oleoyl-pc desaturase (the main enzyme responsible for the production of linoleate) activity because of a mutation of aspartate 150 to asparagines (Andersen *et al*., 1998; Bruner *et al*., 2001). The lines had low O/L ratios (between 1.0 and 1.2) compared to typical O/L ratios, which normally range from 1.7 to 1.8, a factor that is important in measuring the shelf live of peanut seeds (http://lubbock.tamu.edu/peanut/breedingandgenetics/seedquality_6.htm). Peanut sales rely greatly on the rate at which oxidation is taking place and the latter is seen as the discouraging factor for peanut development, quality, flavour and shelf life. It has been

shown that high temperatures lead to oxidative rancidity, which lowers the product shelf life as well as its market value (Bednar and King, 2003).

In conclusion, enough variation for the growth season, oil % and O/L ratioS was observed between lines. More experiments on, for an example protein content and preharvest aflatoxins contamination (PAC), would have given even more conclusive results to the RSA peanut breeders, but this could not be done due to financial constraints. PAC is increasingly becoming important to the peanut industry because of the carcinogenic effects the fungal infection has on humans. Mehan and co-workers (1991) and Cole and his co-workers (1995) already reported resistance to infection by *A. flavus* by screening genotypes using *in vitro* colonization by the fungus of dehydrated mature kernels. Correlation between resistance in the field and *in vitro* was earlier observed in Africa (Zambettakis *et al.*, 1981; Waliyar *et al*., 1994). The author suggests and encourages more commercial lines to be included in studies such as this for more comprehensive understanding and conclusions to be made in order to improve peanut breeding programs in RSA.

2.6 Acknowledgements

I would like to show my gratitude to my co-supervisor Dr. Cilliers and the ARC-Grain Crops Institute team at Potchefstroom for supplying me with the seeds as well all information regarding the plant material. I would also like to encourage them to continue the work on improving the peanut germplasm with new, better and high quality varieties.

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Chapter III

Improvement of an *Arachis hypogaea* L. line (JL 24) with the *oryzacystatin*-1 gene

3.1 Introduction

Many scientists have tried in vain to introduce feasible variability from diploid wild type peanut into cultivated peanut in an attempt to reduce the effects of pests and diseases, but this led to unstable genotypes, which presented difficulties in management (Burow *et al*., 2001). Genetic transformation is a biotechnological approach through which novel genes, which otherwise will be hampered by sexual incompatibility, can be introduced into plant genomes for a specific trait of interest. A number of reports have been released on efforts to transform *Arachis* species, but these show few positive results (Sharma and Anjaiah, 2000; Cheng *et al*., 1996, 1997).

Plant genetic resistance today is being seen as very crucial for Integrated Pest Management (IPM) programs (Michaud, 2000). Several studies have revealed that plant inhibitors had harmful effects on the growth and development of Coleopteran insects and today cysteine protein inhibitors (PI's) are produced commercially to protect plants against these pests (Liang *et al*., 1991; Girard *et al*., 1998a). The oryzacystatin-1 proteinase inhibitor, isolated from rice has been used extensively in several studies, either in artificial diets fed to insects or when expressed in transgenic plants (Abe *et al*., 1987; Chen *et al*., 1992; Leplé *et al.,* 1995; Edmonds *et al*., 1996; Kuroda *et al*., 1996). Liang and his colleagues (1991) confirmed that *OC*-1 does inhibit the midgut cysteine proteinases of coleopteran insects when they tested its effect on two stored grain coleopteran insects, the rice weevil (*Sitophilus oryzae* L.) and the red flour beetle (*Tribolium castaneum* Herbst). A number of economically important crops have thus been transformed with the *OC*-1 gene in order to confer resistance to several pests such

as oilseed rape, potato and sweet potato (De Leo *et al*., 1998; Michaud, 1997; Michaud *et al*., 1998; Bonadé-Bottino *et al*., 1999; Lecardonnel *et al*., 1999; Bouchard *et al*., 2003a, 2003b; Ferry e*t al*., 2003; Rahbe *et al*., 2003). More than 50% mortality of Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) larvae was reported, after being fed on transgenic potato expressing the *OC*-1 gene (Lecardonnel *et al*., 1999). Girard and his co-workers (1998b) reported two strains of cabbage seed weevil, which showed differing susceptibilities to *OC*-1 transgenic oilseed rape plants. Transgenic oilseed rape plants expressing *OC*-1 were used to determine the effect of various aphids and results suggested that *OC*-1 affected the potato aphid (*Myzus persicae* Suzler) through digestive tract targets as well as by reaching the haemolymph (Rahbe *et al*., 2003).

Molecular interactions between insect predators and their herbivore preys were investigated and results proved that *OC*-1 does not interfere with the predation capacity of the two-spotted stink bug (*Perillus bioculatus* F.) when transgenic potato plants were fed to its prey (Bouchard *et al*., 2003a). The multicoloured Asian ladybird *(Harmonia axyridis* Pallas) was used as the beneficial predator and the diamondback moth *(Plutella xylostella* L.) as the pest in a study on oil seed rape plants expressing *OC*-1. Results showed that the ladybird digestive enzymes where inhibited *in vitro* but not *in vivo* (Ferry *et al*., 2003). In another study, the *OC*-1 gene was used in tobacco plants and resulted in a conditional growth phenotypic expression as well as improved chilling tolerance (Van der Vywer *et al*., 2003).

These findings regarding the *OC*-1 gene and its potential usefulness in enhancing resistance in plants made the *OC*-1 gene a very good candidate gene to use in conferring resistance against storage pests of peanut. With the *OC*-1 gene, we aimed at targeting the following insects and insect groups: Saw-toothed Grain Beetle *(Oryzaephilus surinamensis* L.), rice weevil *(Sitophilus oryzae*), grain weevil *(Sitophilus gramarius* L.), confused flour beetle *(Tribolium confusum* Jacquelin du Val), maize weevil *(Sitophilus zeamais* Motschulsky), and the leave miner *(Aproaerema modicelia* Deventer) (www.africancrops.net and www.new-agri.co.uk/00-5/newsbr.html). The maize weevil causes enormous damage to stored grains while the leaf miner is a mayor pest of groundnut which causes huge injury to leaves (van Wyk and Cilliers, 2000).

The objective in this chapter was to transform the peanut line JL24 with the *OC*-1 gene, select and analyse the recombinants in attempt to determine the gene's (*OC*-1), effectiveness in enhancing resistance towards peanut storage pests and therefore reducing the damage caused by the later as well as the need for insect fumigation during storage.

3.2 Materials and Methods

3.2.1 Plant material, plasmid and the *Agrobacterium* strain

Seeds of cultivar JL24, *Aproaerema moducelia* (leave miner) and *Sitophiluz zeamais* (maize weevil) were obtained from ARC-Potchefstroom and ARC-Roodeplaat, respectively. The cultivar JL 24, was selected for transformation by ARC-Potchefstroom in accordance with their interest. The two insects were selected to be used in the insect studies. Plants were grown in 60 litters capacity pots containing sandy-loamy soil under greenhouse conditions of 25-28^{o}C and 54-50% relative humidity. Seeds were planted and three generations of putative transformants were grown (T_0 = Transformed plants, T_1 = F_1 generation and T_2 = F_2 generation). The T_0 generation plants were phenotypically evaluated and the following morphological characteristics assessed; plant height, number of pods and number of seeds. Watering was done twice a week and plants were grown to maturity, lifted and seeds harvested. Transformed tobacco plants were provided by Professor Karl Kunert (Department of Botany, University of Pretoria, RSA) and served as controls.

The *Agrobacterium tumefaciens* strain (C58pMP90) and the pKYΩ'OC1 binary plasmid vector were used for the transformation process. The *A. tumefaciens* strain (C58pMP90) was also generously provided by Professor Karl Kunert while the pKYΩ'OC1 plasmid vector was kindly provided by Prof. L. Jouanin (INRA Versailles, France).

3.2.2 Transformation

3.2.2.1 Preparation of plasmid containing the gene construct

The pKYΩ'OC1 plasmid vector encodes the *OC*-1 coding sequence (Figure 3.1) under the control of a double CaMV 35S promoter (P70) from cauliflower mosaic virus between the flanking regions. The transfer DNA (T-DNA) contained a Ω leader sequence for gene expression enhancement. The construct had a kanamycin resistance gene (*npt*II) controlled by the CaMV 35S promoter (P35S NPTII) used as a selectable marker and an intron-containing the *gus* gene (GUS) encoding β–glucuronidase (P35S GUSint) under the control of a 35S promoter as well. Also found on the construct were a number of restriction sites: *EcoR*1, *Hind* III, *Xba* I, *Bam* HI and *Pst* I.

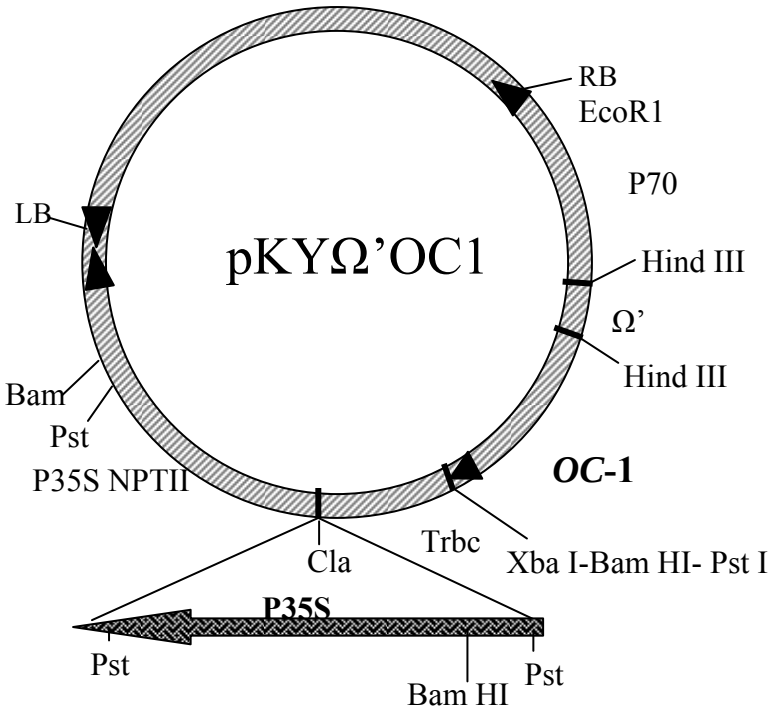

Figure 3.1: A detailed map of the pKYΩ'OC1 plasmid vector which contains the *OC*-1/ GUS intron and which was used in the transformation of the peanut line JL24 (Van der Vywer *et al.*, 2003)

A BLAST (Basic Local Alignment Search Tool, BLAST; Altshul *et al*., 1997) search was done with the *OC*-1 access number M29259 and the predicted protein (id = ‘AAA33912.1) to retrieve the complete cds sequence of the *OC*-1 gene (Womack *et al*., 1999; Figure 3.2). The gene contains two introns and two exons with exon-1 (539..652), exon-2 (989..1183) and TATA box at 400..405 (http://www.ncbi.nlm.nih.gov.BLAST).

```
   1 tcttggagct caaaggctca aagctcagcc acatacagcc aggcgaaaaa aaaaatcgaa
  61 aacttgtcaa atatctgtag caataaccaa tgatatctct actagtcatc tcagcggccg
 121 gtggcacgcg aaccggagtt atatcaaaca aacaccaccg acgttgccat cgtcacggta
 181 ccagactcct gcgttccacc actccaccag tgaccgtgac gtgatacatg gagataacgc
 241 cgcctggcaa ctggcgagat aattaaagaa accgggaaac actggttcca accgctcgct
 301 cggctcggtt ccaatttcca aacgtgtcgc ccattctaca ccgtctccat ccatctcatc
 361 tcgtcgtctt ccccccccgcg agtccgcgac cccccgcgct ataaaatcca aggccgcgat
 421 cgagatgcgg aaatatcgag tcgccggatt ggtagccgcc ctgctcgtgc tgcattcgct
 481 agccacgccg tccgctcagg ccgaggcgca tcgcgcaggg ggagaagggg aggagaagat
 541 gtcgagcgac ggagggccgg tgcttggcgg cgtcgagccg gtggggaacg agaacgacct
 601 ccacctcgtc gacctcgccc gcttcgccgt caccgagcac aacaagaagg ccgtaagcgc
 661 cccgctctac cctcctccct acttctcctc ttcgcaaggc cggtcgtcca actagatctg
 721 tgttctatgg actaattatg caacgcatga cacacggctc tatttaattt ttaaattgat
 781 tctcgccaag gaagtaattt tgattcctag tcatgtaaac ctgctcgagt cggtgagaaa
 841 aaaaaagatg cggtgttgcg tatgtttgtt aaaagtgcaa ttaggcaatt ttttgtgttc
 901 aaagattgtg ctgtgttctc ttttatgaca atatggaaca ggaggttctg gactgattag
 961 catttaatct tgatactacc atgcgcagaa ttctctgctg gagttcgaga agcttgtgag
1021 tgtgaagcag caagttgtcg ctggcacttt gtactatttc acaattgagg tgaaggaagg
1081 ggatgccaag aagctctatg aagctaaggt ctgggagaaa ccatggatgg acttcaagga
1141 gctccaggag ttcaagcctg tcgatgccag tgcaaatgcc taaggtctag acatgtttgg
1201 tttagatacc cgccaaacaa aattttcacc tgttccatgc attgaccatg tataatatat
1261 atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat
1321 atatatatat gtccttttct cattagaaaa ccagagcttg tgccccatcc tacttggtta
1381 aggccattga tgaatatgtt tgttattctc ttagtaagaa tacactttta agtttttcga
1441 tcaaataatt aagatattca actttctaaa taaacctttt ttcttttctc tagatattat
1501 atcactgaat catttattgc atattgaatt gacatgtcaa atctactaat ttcaggccca
1561 tctcgtatcc tatgtgtatc aagttatcaa gaagatgggg aataatatgg tgtggatata
1621 gctattggac atgttaatta tccacatgat aatatggctt ggatataagg atctcacacg
1681 aataatatgg cttggatata tagctattaa agattttacc tatggcatat ttcaatgtgt
1741 attagtacta agtaagaatg attgcaaggt gtattaacta caaatattgc aataaaagtc
1801 cctgttacta cacttacagt attttaagta gtccttgact ggatagactc tgtaatcatg
1861 tcacgtgcct gttgactgta gttgattgta cagacgtatc atgtcagcta cgttaatgct
1921 atatattact acggcga
```

Figure 3.2: Complete *OC*-1 gene cds sequence (http://www.ncbi.nlm.nih.gov)

3.2.2.2 Preparation of competent cells

Firstly, the most suitable *E. coli* cell culture, cloned in pBluescript, was selected before preparing competent cells as described by Tang and his colleagues (1994). The *E. coli* strain was incubated at 37^{o}C overnight. Hundred millilitres Luria-Bertani (LB) medium

(1 g/100 ml Bacto Tryptone, 0.5 g/100 ml Bacto Yeast extrct, 1 g/100 ml NaCl and 1.5 g/100 ml of Bacto-Agar) was inoculated with the overnight cultures and shaken for 2 hours at 37°C. Measurements of OD_{600} values were taken every 30 minutes until it was 0.94 and the medium was kept on ice. Cells were centrifuged at 4°C for 5 minutes at 5000 rpm in two 32 ml tubes and washed with sterile water. This step was repeated twice. The supernatant was removed and the pellets resuspended in ice-cold 8 ml Transformation Buffer (TB; 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 6.7; 55 mM $MnCl_2$; 15 mM $CaCl_2$; 250 mM KCl), made up to 35 ml total volume and set to a pH 6.7. This was stored on ice for 10 minutes before centrifuged at 0°C for 5 min at 5000 rpm. The pellet was resuspended in 4 ml ice-cold TB and 280 µl Dimethylsulfoxide (DMSO) was added to reach a final concentration of 7%. After a gentle swirl, cells were incubated on ice for 10 minutes and 200 µl dispensed with liquid nitrogen in 15 ml polypropylene tubes.

3.2.2.3 Transformation of plasmid in cell culture

Five microlitres plasmid solution was added to the propolene tubes and incubated on ice for 30 minutes (Inoue *et al*., 1990). Cells were heat-pulsed at 42°C for 30 seconds without agitation and transferred to ice. Fifty microliters $MgCl_2$ and 100 µl glucose were added before adding 0.8 ml SOC [2% (m/v) Tryptone, 0.5% (m/v) Yeast Extract, 0.4% (m/v) glucose, 10 mM NaCl, 2.5 mM KCl and 5 mM $MgSO_4$] and this was followed by an incubation step at 37°C for an hour while shaking vigorously. The solution was centrifuged and resuspended in 100 µl Tris/EDTA (10 mM Tris pH 8 and 0.1 mM EDTA) buffer.

3.2.2.4 Preparation of plates

A mixture of 0.1 mM of isopropylthio-ß-D-galactoside (IPTG) and 60 µl/ml 5-bromo-4-chloro-3indol-ß-D-galactoside (X-gal) was spread on each plate (LB and antibiotic) containing the transformed competent cells. These were left to dry and blue and white colonies, plasmid with or without DNA respectively, were visible. Plasmid purification was done following the standard miniprep using the Magic Minipreps^{TM} system from PROMEGA.

3.2.2.5 Vacuum infiltration

Three different three to four week-old JL24 plantlets were surface sterilized for 5 min in 70% (v/v) EtOH and thoroughly rinsed in ddH_2O. Leaves of the plants were punched about 50x, vacuum infiltrated for 10 min with infiltration medium (1liter) [2.2 g Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), 1X B5 vitamins (Inositol, Nicotinic Acid, Pyridoxine·HCl, Thiamine), 5 % (m/v) sucrose, 0.5 g 2-N-Morpholino ethanesulfonic Acid (MES) pH to 5.7 with potassium hydroxide (KOH), 0.044 µM benzylaminopurine, 200 µl Silwet] and the *Agrobacterium* mix (Figure 3.3). The transformed plantlets were placed inside a plastic bag for five days to retain humidity and *Agrobacterium* was allowed to infiltrate the plantlets (http://www.bch.msu.edu/pamgreen /vac.html). Plants were removed from the plastic bag, replanted in pots with soil and allowed to grow to maturity followed by testing for the presence of the foreign gene. Experiment included a JL24 plantlet transformed without the *OC*-1 insert, which was used as a control in consequent analysis.

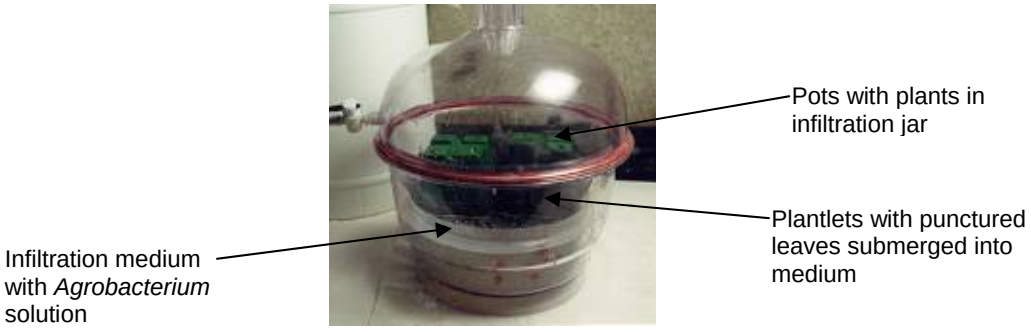

Figure 3.3: Vacuum infiltration system used for the transformation of JL24 plantlets

3.2.2.6 Cultivation of putative transformants

After replanting in pots containing autoclaved sand and soil, the three plants and the control, were allowed to grow to maturity in the greenhouse under controlled environmental conditions at 25-28^{o}C temperatures and 54-50 % relative humidity. Watering was done twice a week and plants were grown to maturity, phenotypically evaluated, lifted and seeds harvested.

3.2.3 *OC*-1 gene detection and selection of stable peanut transformants

3.2.3.1 Histochemical analysis of putative transformants

Transgenic whole peanut plants were cut and placed into an eppendorf tube and 0.5 ml colourless X-Gluc substrate buffer [50 mM NaH_2PO_4, pH 7.0; 0.01 % (v/v) Tween-20; 0.5 M EDTA; 0.1 % (m/v) X-Gluc] was added, vacuum infiltrated for 10 minutes, after which eppendorf tubes were tightly covered and incubated at 37^{o}C for six hours, since the GUS reaction is light sensitive. Tissues were washed with 1 ml 70 % (v/v) EtOH to improve contrast and observed under a stereomicroscope (Harborne, 1998). 0.1

3.2.3.2 Fluorometric assay on putative transformants

Fifty micrograms of the whole transformed peanut plants was homogenized in an Eppendorf tube in 0.5 ml of extraction buffer [50 mM $NaPO_4$ buffer, pH 7.0; 0.1 % (w/v) Triton X-100; 10 mM Na_2EDTA; 0.1 % (m/v) sodium lauryl sarkosine; 10 mM β-Mercaptoethanol and 1 % (v/v) Polyclar (insoluble polyvinyl pyrollidone)]. A pinch of sea-sand was used for better grinding. All cell debris was spinned down by a 10 min centrifugation at full speed (13 000 rpm) in an Eppendorf centrifuge at 4°C. The supernatant was transferred into a new Eppendorf tube and stored on ice. The supernatant (50µl) was mixed with 200 µl reaction buffer [1% (v/v) Polyclar, 1 mM 4-methyl umbelliferyl glucuronide (MUG)] and pipetted into every consecutive well of an alternative open microtiter plate (Harborne, 1998).

After mixing well, 50 µl of the mixture was transferred to the second wells of the plate and immediately 0.95 ml of the stop buffer [0.2 M sodium carbonate (Na_2CO_3)] was added to stop the reaction and obtain fluorescence at time zero. This was repeated at 30 minutes, 60 minutes, 90 minutes and 120 minutes, and visual observations were done under UV whilst fluorescence was measured with a fluorometer using an excitation wavelength of 370 nm and emission wavelength of 460 nm. Amount of 4-methylumbellferone (MU) produced/mg tissue/hour was determined by using a standard curve drawn from standard values (0.25; 0.5; 1.0 and 2.0 µg/ml MU). Expression of each sample was calculated using the following formula:

Expression = Average sample value – Average standard value

3.2.3.3 PCR-based gene detection of putative transformants

3.2.3.3.1 DNA Extraction:

Leaves of the three putative transformed plants were cleaned with 70 % (v/v) ethanol, air dried and cut into strips with a sterile pair of scissors. This was placed into a 1.5 ml eppendorf tube with sterile river sand and the DEB buffer (0.5 M Tris-HCl (pH 8.0); 0.3 M NaCl (pH 8.0); 0.1 M EDTA (pH 8.0); 0.5 % (m/v) SDS) (Surridge *et al*., 2002). The peanut leave material was grounded in an eppendorf tube using a sterile, plastic pestle that was kept in 0.25 M HCl; while the tobacco leaves were grounded using the Bio101 Fastprep Fp120 instrument (Bio 101, USA) using cylinders and balls at 4.0 m/s for 20 seconds. Equal volumes of phenol-chloroform (1:1) was added to the grounded material, vortexed and centrifuged at 10 000 rpm for 20 minutes. This step was repeated until there was no interface visible and was followed by an overnight ethanol precipitation at –20^{o}C. DNA was pelleted by a centrifugation step at 11 000 rpm for 30 minutes after which it was washed with 70 % (v/v) ethanol. Another centrifugation at 11 000 rpm for 5 minutes was done and the pellet was dried at 30^{o}C in an eppendorf concentrator (Savent) under reduced pressure. The pellet was resuspended in 200 µl sterile water.

DNA concentration of the samples were analyzed using a 1 % agarose gel, stained with Ethidium bromide and visualised under UV light. Samples were quantified with the GENE QUANT Pro RNA/DNA Calculator before being diluted to 50 ng/µl for AFLP analysis and 30 ng/µl for the microsatellite analysis and PCR with *OC*-1 specific primers.

3.2.3.3.2 Primer Design

Nested primer sets were designed for the *OC*-1 gene with the use of the Primer 3 website (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). The primer sets (Table 3.1) were then submitted to the GenBank database so that a BLAST-P search could ensure that the primers were *OC*-1 specific (Altshul *et al.,* 1997) (http://www.ncbi.nlm.nih.gov).

Table 3.1: *OC*-1 primer characteristics illustrating the primer sequences, lengths in base pairs, melting temperature and guanine and cytosine contents in percentage.

Primer	Primer Sequence	Length	Tm	GC%
OC1L1	5'TCACCGAGCACAACAAGAAG3'	20 bp	60.02	50.00
OC1R1	5'CAAGAAGGCCAATTCTCTGC3'	20 bp	60.80	55.00
OC1L2	5'CGTAGCTGTCCGAACTGAGG3'	20 bp	59.96	50.00
OC1R2	5'GACCTCGAGGAACTCAGGTA3'	20 bp	59.80	50.00

3.2.3.3.3 The Polymerase Chain Reaction (PCR) protocol

PCRs were performed using a Perkin Elmer 9700 PCR machine and *OC*-1 (*oryzacystatin*-1) primers. The reactions were conducted in a 25 µl volume and the PCR master-mix consisted of 0.5 U Taq DNA polymerase (PROMEGA), 1X buffer, 1.5 mM $MgCl_2$, 20 µM of each dNTP, 0.2 µM primers and 30 µg/µl template DNA. The PCR cycle followed was 5 minutes at 94^{o}C, 30 cycles of 30 seconds at 94^{o}C, 1 minute at 57^{o}C and 1 minute at 72^{o}C, with the final extension of 7 minutes at 72^{o}C.

3.2.4 Further analysis of putative transformants

3.2.4.1 Determination of chlorophyll concentrations

Freshly picked *OC*-1 transformed peanut plant leaves and controls, the latter being transformed but without the *OC*-1 insert, were grinded using a mortar and pestle in an 85% (v/v) acetone solution until no colour was visible in the tissues. Calcium carbonate ($CaCO_3$) was added to the extract to prevent pheophytin formation before it was filtered through a Buchner funnel. The different extracts were made up to a known volume (20 ml) and stored in the refrigerator. Measurements of absorbance at different wavelengths were obtained using a spectrophotometer (Beckman) and concentrations of chlorophyll *a* and *b* were calculated given these formulae:

Total chlorophyll (mg/l) = $17.3\ A_{646} + 7.18\ A_{663}$

Chlorophyll *a* (mg/l) = $12.21\ A_{663} - 2.81\ A_{646}$

Chlorophyll *b* (mg/l) = $20.13\ A_{646} - 5.03\ A_{663}$

3.2.4.2 Protein extraction and concentration determination

Whole plants of each putative transformed plants and the control were collected, of which 1 g of each was used for the protein extraction. Protein extractions were performed using the method described by Hurkman and Tanaka (Hurkman and Tanaka, 1986). The protein samples were precipitated with acetone before calibration of total protein for each sample.

Different concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10 mg/ml) were prepared from the bovine serum albumin (BSA) stock solution (10 mg/ml) and used to draw a standard curve.

Averages from the sample extracts were plotted on the graph and compared with the standard curve (Bio-Rad Protein Assay, 1994).

All samples were diluted 80X by adding 395 µl H_2O to 5 µl of each sample and a blank, which served as a control, before adding 100 µl BioRad solution according to Bradford (1976). Protein concentrations were measured with the spectrophotometer (Beckman) at 595 nm wavelengths.

3.3 Results

The histochemical GUS assay done on the putative transformants showed positive results as shown below with the results of P3 (Figure 3.4).

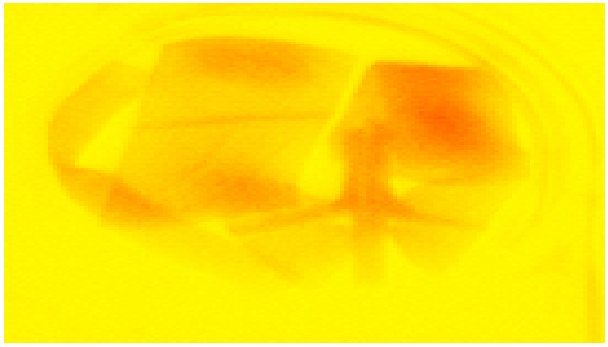

Figure: 3.4: Histochemical GUS assay results of P3

Three out of the originally 50 (6%) plants were transformed, two out of the 17 F_1 (12%) plants showed evidence of the presence of the *OC*-1 gene, and seven out of the 19 F_2 (37%) plants were selected as putative transformed plants while only three were selected for further studies. Of the three putative transformants, P3 was the shortest and had the least number of pods. P2 had the highest number of pods as well as highest number of double seeded pods (Table 3.2). P3 was found to be reasonably different from P1, P2 and the control in terms of plant height and number of pods. All four plants were different from each other in terms of plant height and number of single and double pods.

Table 3.2: Phenotypic characteristics of the three putative transformed plants (P1, P2 and P3) and a control.

Plant	Plant Height	No. of Pods	No. of Single (s) and Double (d) seeded pods
P1	28 cm	5	1s; 4d
P2	26 cm	11	2s; 9d
P3	13 cm	1	1d
Control	31 cm	15	6s; 9d

The three parents had up to five times higher MUG activity compared to progeny, with P3 showing the highest values. Of the F_1 generation plants, plant number 7 gave the highest MUG activity followed by plants number 9 and 12 (Figure 3.5) and all three plants were progeny of P3. Of the F_1 generation plants, 7 plants (plant number 1, 3, 7, 9, 12, 13 and 14) were selected for seed production for the F_2 generation plants.

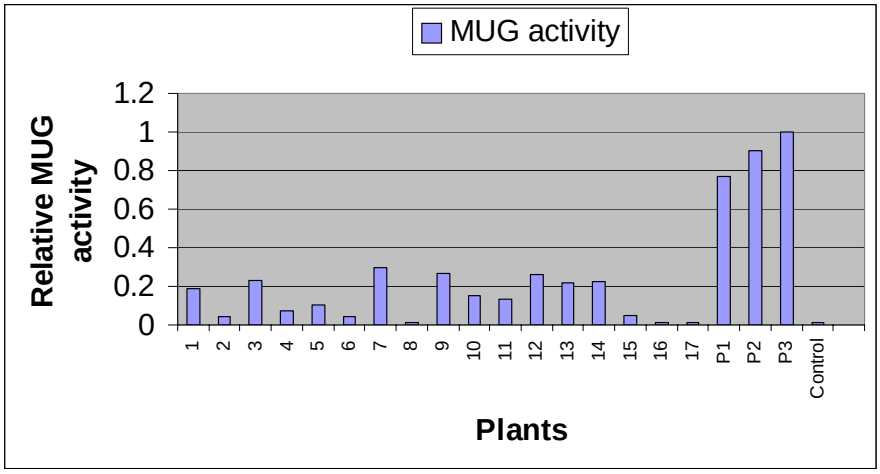

Figure: 3.5: The relative MUG activities as tested for the three putative transformants and the F_1 generation progeny

PCR analysis for the three parents and the F_1 generation plants (Figure 3.6) confirmed the MUG activity results (Figures 3.4 and 3.5). A 153 bp fragment was amplified with the *OC*-1 specific primers for the three parents as expected. Of the F_1 generation plants, only plants number 7 and 12 contained the *OC*-1 insert and this is shown by the presence of the 153 bp band. The transformed parental lines all showed a clear 153 bp band size.

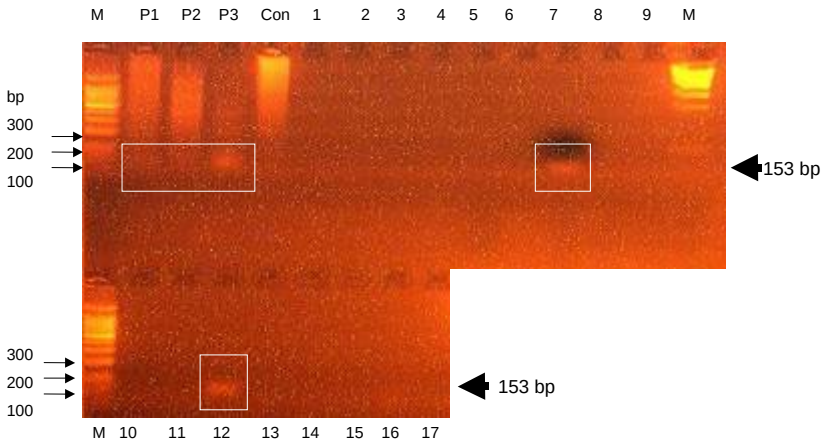

Figure 3.6 PCR analysis using *OC*–1 specific primer pair

All three putative transformants showed considerably higher concentrations of proteins per hour compared with the control plant (Figure 3.7). P3 gave the highest protein concentrations.

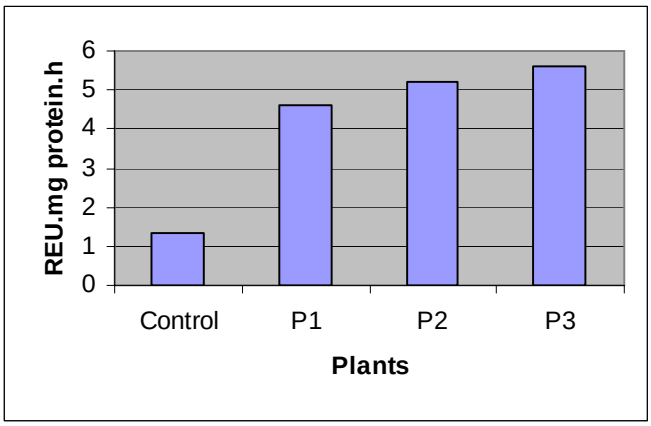

Figure 3.7: The amount of protein (mg) per hour of the putative transformants compared with the control

Due to the observed differences in leaf colour in P3 it was decided to measure the chlorophyll content of these putative transformed plants. The control showed the lowest amount of total chlorophyll, as well as chlorophylls *a* and *b*. The putative transformants (P1, P2 and P3) had most chlorophyll, and chl*a* and chl*b* (Figure 3.8). P1 had the highest level of chl*a* of all the plants. Interestingly P3 had an extraordinarily high amount of total chlorophyll and chl*b*, but normal quantity of chl*a* in comparison to the other plants. When statistical analysis was done, it was found that the three lines were significantly different from one another in terms of chlorophyll content, i.e. chl*a* and chl*b* (Jandel Scientific, Sigmastat, version 2.0).

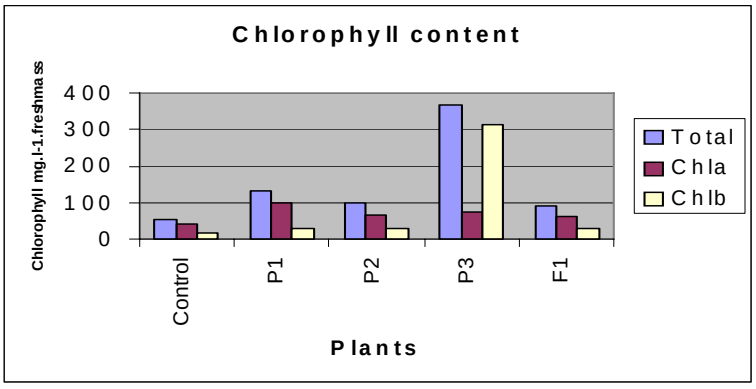

Figure 3.8: The amount of total chlorophyll (mg), chlorophyll *a* and *b* per fresh mass for the three putative transformants, the control and the average for the F_1 generation plants

P1 and P2 showed normal growth while P3 was stunted, shorter, less stems with less pods and seeds (Table 3.2 and Figure 3.9). P3 was darker in colour as compared to P1 and P2. The F_1 generation plants all showed normal growth. Other phenotypes were normal for all the plants.

P1 **P2** **P3**

Figure 3.9: Transformants showed phenotypes of normal to stunted growth while F_1 plants showed normal phenotypes

Nineteen F_2 generation plants were raised from seeds of the selected F_1 transformants, DNA extracted and screened for the presence of the *OC*-1 gene. Out of these, seven showed positive results with the newly designed *OC*-1 primers. The picture below shows two of the F_2 plants ran with a positive (Tobacco) and a negative (ddH_2O) control on a 1% Agarose gel at 100 V for twenty minutes (Figure 3.10).

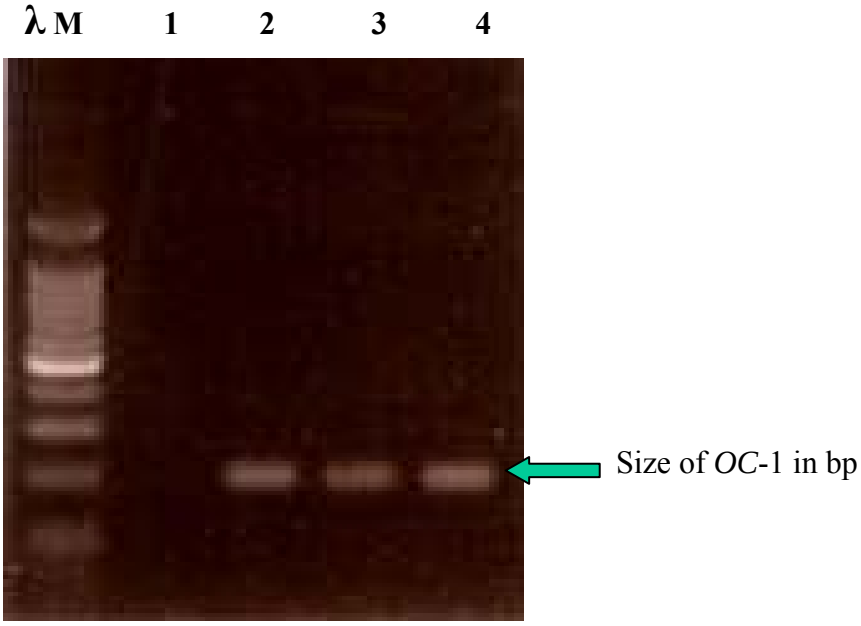

Figure 3.10: PCR with the newly designed *OC*-1 primers. Lane 1: 100 bp ladder. Lanes 2-4: Negative control (ddH_2O), Positive control (Tobacco), F_2 Peanut (26) and F_2 Peanut (36)

3.4 Discussion and conclusion

A total of three out of 50 (6%) plants were transformed, two out of the 17 F_1 (12%) plants showed evidence of the presence of the *OC*-1 gene and seven out of the 19 F_2 (37%) plants were selected as putative transformed plants. The three transformants showed phenotypes of normal to dwarf (Figure 3.9) and expressed GUS and MUG activities, which decreased considerably from parents to the F_1 generations. The histochemical GUS assay appeared as a coloured indigo dye, an indication of a positive transformation event (Figure 3.4).

In a very recent study where genetic transformation of peanut was done using *Agrobacterium* as a vector and cotyedonary node as explant, approximately 31% transformation frequency was accounted for (Anuradha *et al*., 2006). This finding came after it was discovered that the use of cotyledons as explants could give higher transformation frequency, more especially in the cultivar JL-24 (Sharma and Anjaiah 2000). In this study, leaf explants were used in an *Agrobacterium*-mediated transformation and up 37% transformation efficiency could be achieved.

Chl*a* is the form of chlorophyll that is most important in photosynthesis as it is directly involved in the light reaction as by converting solar to chemical energy. Chl*b* on the other hand is one of the accessory pigments that absorb light and transfer the energy to chl*a* (Campbell, 1993). All the transformants as well as the F_1 generation plants showed significantly high amounts of chl*a* and chl*b* compared to the control plant (Figure 3.8).

Chl*a* content was higher than chl*b* in the control, P1, P2 and the F_1 plants. Interestingly, one of the transformants (P3) revealed a higher chl*b* than chl*a* and it also showed a distinct phenotype with significant dwarfism and blue-green plant colour (Figure 3.9). Overall, the chlorophyll content correlated with the observed phenotypes.

It is finally concluded that the results showed stable inheritance of the gene in the progeny. Of the three transformed lines, P1, P2 and P3, only P3 could clearly be distinguished from P1 and P2 in terms of plant height and number of pods (Table 3.2).

Insect studies done did not give justifiable results. It is therefore recommended that further insect studies be done to investigate and evaluate the inheritance of the trait in the greenhouse as well as the tolerance levels of the putative transformants on insect feeding, particularly with *Aproaerema moducelia* (leave miner) and *Sitophilus zeamais* (maize weevil). This should be done to confirm the inhibitory effects that the gene is suppose to have on the pest infestations.

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Chapter IV

Genetic diversity of peanut lines as measured by molecular markers (i.e., AFLP and SSR) and phenotypic data

4.1 Introduction

4.1.1 Background and genealogy

Peanut cultivation in the Republic of South Africa started in the Natal province way back in 1843 and soon moved to the Transvaal province. Peanut breeding on the other hand comes a long way with the introduction of the 'Spanish' type peanut landrace, 'Natal Common', to the east coast of the country by Portuguese traders (Herselman, 2003). Today, 'Spanish' peanut types are still the most produced in the country with an annual production estimate of around 100 000 tons (Swanevelder, 2000). 'Natal Common' thus served as a parent to most of the country's peanut cultivars (Figure 4.1). A cross between 'Natal Common' and Namark, a landrace from Kenya, resulted in another cultivar called 'Sellie' (van der Merwe and Vermeulen, 1977). 'Sellie' has since become the most popular cultivar of all (Herselman, 2003). As a result of this common ancestral background, peanut cultivars in RSA have presented difficulties in the establishment of variation at genetic level even though they differ extensively in terms of morphology and phenotypic parameters. This phenomenon has been studied to a great extent in other parts of the world and many reports came forth (Hopkins *et al*., 1999; Lu and Pickersgill, 1993). Apart from the 'Spanish' types of peanut, 'Virginia', 'Valencia' and 'Runner' types are also cultivated in RSA today (Figure 4.1).

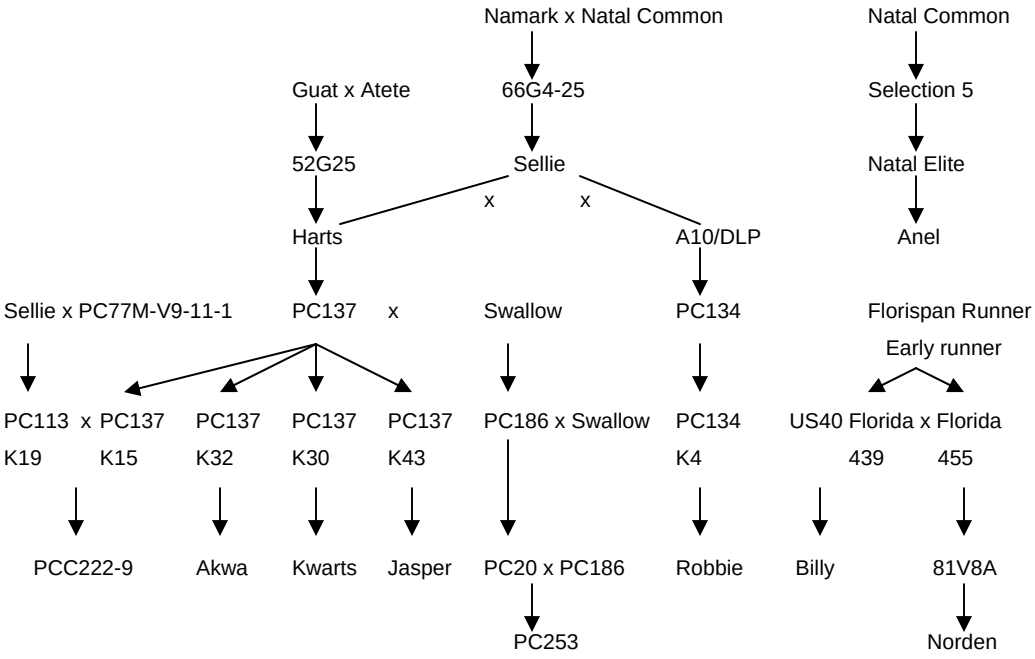

Figure 4.1 Schematic representation of the genetic origin of some selected genotypes, i.e. genealogy of RSA peanut cultivars (ADOPTED as published by Herselman, 2003).

4.1.2 Amplified Fragment Length Polymorphism

Today various molecular markers can be employed to allow for direct comparisons of the similarity of genotypes at DNA level (Lübberstedt *et al*., 2000). Restriction fragment length polymorphism (RFLP) is laborious, depends on the Southern blot analysis and only a few alleles can be detected at the end of the day (Prakash *et al*., 1996). Random amplified polymorhpic DNA's (RAPD's) on the other hand, is quite sensitive to reaction conditions and thus shows low reproducibility. Microsatellites provide high levels of polymorphism and require cloning and sequencing in order to obtain information on the flanking nucleotide sequences. The AFLP technique, which resulted from both the RFLP and RAPD techniques, can resolve some of the shortcomings that have been identified with the other techniques.

The AFLP technique produces DNA fragments as a result of the amplification process with the use of directed primers from restriction digested genomic DNA (Karp *et al*., 1997; Matthes *et al*., 1998). According to Vos *et al*. (1995), the technique is thus merely the detection of restriction fragments by PCR. The AFLP technique earned its popularity from detecting numerous polymorphic markers in a short space of time and above that, it detects a higher number of point mutations than RFLP and is reproducible (Prakash *et al*., 1996). The AFLP technique is applied in DNA fingerprinting, genetic diversity studies, local marker saturation, construction of genetic maps and quantitative trait loci (QTL) mapping in plants (Botha *et al*., 2003; Brugmans *et al*., 2003). The technique has been used extensively in the detection of the genetic diversity of many crops such as rice (Mackill *et al*., 1996), soybean (Powell *et al*., 1996), barley (Russel *et al*., 1997), sugar beet (De Riek *et al*., 2001) and potato (Jacoby *et al*., 2003).

The technique requires only a small amount of DNA and works on a variety of genomic DNA samples. Numerous restriction fragments can be amplified by only changing the nucleotide extensions on the primer sequences and in this way a number of markers can be generated, which will be of great use in breeding programs. The stringent PCR conditions are responsible for the high resolution while the high-throughput is due to the fact that AFLPs detect a high number of markers per reaction as well as the fact that the process can be automated using the Li-COR systems. One more important advantage is that no prior knowledge of the genomic sequence is needed for the technique (Myburg and Remington, 2000).

AFLP analysis on the Li-COR IR^2 automated sequencer uses infrared dye detection and thus radioactivity is eliminated (Myburg and Remington, 2000). Infrared dye-labelled primers are cheaper and much higher throughput, compared to conventional radiographic methods, can be obtained. Images are obtained in only several hours compared to two to four days with the conventional radiographic methods. Image scoring is faster with newly developed software. The distance between bands one base pair apart is much more constant throughout the whole length of the digital image. All fragments travel the same distance before passing through the scanner window (Myburg and Remington, 2000).

When the AFLP technique was applied in a study, in attempt to establish taxonomic relationships among *Arachis* species, 408 fragments were detected with only three primer combinations. A total of 406 of these fragments, an amount that amounts to 99%, were polymorphic and this showed the effectiveness of this technique in the establishment of genetic relationships among the peanut species. The study also showed that the grouping of the species based on AFLP data was in total agreement with the groupings using other types of data such as morphological, geographic distribution and cross-compatibility data (Gimenes *et al*., 2002). However, only six of the 94 fragments detected in the accessions of *A. hypogaea* were polymorphic. Gimenes and his co-workers concluded that the polymorphism detected with the AFLP technique using only three primer sets was too low to be utilised in consequent genetic studies in peanut species.

4.1.3 Microsatellite markers

Microsatellites examine a single locus at a time and reveal a great amount of polymorphism compared to other single loci markers such as RFLPs and isozymes (McCouch *et al*., 1997; Rossetto *et al*., 1999). Unlike AFLP markers, microsatellites are co-dominant markers and thus heterozygotes can be identified. The method is DNA based and therefore has very high-throughput and even dried leave material can be used successfully. After the identification of suitable primers and screening these across the population in question, it becomes fairly inexpensive and primers can amplify populations across-species (Robinson and Harris, 1999).

Microsatellites have been used to a great extent in diversity studies (Rossetto *et al*., 1999), which are important for taxonomy and conservation of genetic diversity, gene flow and mating systems (Chase *et al*., 1996) as well as paternity analysis (Streiff *et al*., 1999). The technique is very useful in the determination of gene flow between individuals, cultivar identification and paternity analysis by investigating combinations of loci (Hokanson *et al*., 1998). When used to compare the genetic diversity among maize inbred lines, SSR markers showed the highest polymorphism information content as compared to RFLP's, AFLP's and RAPD's (Yuan *et al*., 2000). SSR's were also used in determining the phylogenetic diversity and relationship between sorghum accessions and surfaced as most informative compared to RAPD's (Agrahama and Tuinstra, 2003). SSRs have been identified in many other plant genomes such as soybean (Akkaya *et al*., 1992; Morgante and Olivieri, 1993), rice (Wu and Tanksley, 1993), tomato (Broun and

Tanksley, 1996) and conifers (Tsumura *et al*., 1997). Hopkins and his co-workers (1999) discovered and characterized six polymorphic SSR's in cultivated peanut and revealed that markers could amplify a maximum of ten putative loci.

Previous studies showed that SSR primers designed for a particular taxon could amplify similar regions in closely related taxa (White and Powell, 1997). Six primer combinations were successfully used to detect polymorphism among a total of twenty-six cultivated and wild *Arachis* species (Hopkins *et al*., 1999). This was enough proof that microsattelites can be used to distinguish between cultivated peanut lines. In another study, microsatellites in peanut were successfully developed using the SSR enrichment procedure and revealed for the first time, a high number of alleles (He *et al*., 2003). The results of the study led to the assumption that microsatellites could be plentiful in peanuts as is the case with other legumes. Compared with the library screening method used by Hopkins and his colleagues in the identification of SSR markers in peanut, the enrichment procedure showed a higher percentage of markers that showed polymorphism, 19% versus 34% (He *et al*., 2003).

Ferguson and his colleagues supported these earlier findings when they identified and characterized 110 sequence tagged microsatellite (STMS) markers in peanut, a great improvement on previous reports (Ferguson *et al*., 2004). A more recent report reported the development of the first microsatellite based gene-rich linkage map using an F_2 generation from a cross between *A. duranensis* and *A. stenosperma* Karpov and W.C. Greg, the AA genome of *Arachis* (Fabaceae) (Moretzohn *et al*., 2005). SSR-enriched

genomic libraries together with expressed sequence tags (ESTs) and 'data mining' sequences from GenBank were used to develop 271 new microsatellite primers (Moretzohn *et al*., 2005).

4.2 Objectives

The objective of this chapter was to use molecular markers and phenotypic data to determine the degree the genetic diversity of 18 commercially cultivated South African lines and an additional transformed line.

4.3 Materials and Methods

4.3.1 Plant material

Eighteen commercially cultivated South African peanut lines (Chapter 2, Table 2.1) and an additional line, which was transformed with a cystatin gene from rice (Chapter 4), were selected for this study. These genotypes were selected in accordance with their commercial importance to the South African groundnut industry to date. All seeds were obtained from the Agricultural Research Council- Grain Crops Institute (ARC-GCI) at Potchefstroom, RSA. The seeds were sown in 60 litres capacity pots containing sandy-loamy soil and the plants were grown in the glasshouse for about three weeks after germination at 25^0C controlled temperature.

4.3.2 DNA extractions and clean-up

Five freshly picked leaves from five different plants of each genotype were wiped clean with 100% ethanol. The leaves were grounded in liquid nitrogen using a mortar and pestel. DNA was obtained using the DNeasy plant extraction kit from QIAGEN following the manufacturer's instructions. One mg of RNAse per 1 ml of DNA was added to all DNA samples to perform the RNAse treatment. Absorbencies were measured on the spectrophotometer at 260 nm and 280 nm to establish DNA quantity and quality of all samples respectively (Sambrook *et al*., 1989). Samples were further quantified using a 1% (w/v) agarose gel and lambda DNA as concentration standard. DNA samples for analysis were diluted to 50 ng/µl for AFLP analysis and 25 ng/µl for the microsatellite analysis. DNA extraction of the transformed line had been done with the DNA Extraction Buffer (DEB) method (Chapter 4).

4.3.3 AFLP analysis

AFLP template preparation was performed using AFLP template preparation kits from LI-COR Biosciences (LI-COR, Lincoln, NE, USA) according to the manufacturers' instructions, with the exception that 50 ng diluted genomic DNA per 30 µl reaction was digested in a 1X restriction/ligation (R/L) mix {R/L buffer [10 mM Tris HAc (pH 7.5), 10 mM MgAc, 50 mM KAc and 5 mM dithiothreitol (DTT)] with 1.2 U/µl *Eco*RI, a rare cutter, and 0.8 U/µl *Mse*I, a frequent cutter}, 2.0 mM MgCl2 and 1.0 U Taq polymerase were used in the preamplification step. Polymerase chain reactions (PCRs) were performed using a BIO-RAD iCycler (Version 3.021, BIO-RAD Laboratories, inc).

The preselective amplification cycle profile was as follows: incubation for 10 s at 72 ºC, followed by 30 cycles of denaturation for 10 s at 94 ºC, annealing for 30 s at 56 ºC, and extension for 1 min at 72 ºC with a 1 s per cycle increasing extension time. After amplification 5µl pre-selective amplification products were mixed with 2 µl loading dye (15% Ficol) and ran on a 1.2% agarose gel in a 1X TBE buffer and visualized under UV light.

Selective amplification was performed on 1:40 diluted (in SABAX water) preselective amplification products with the following cycling profile: 13 cycles of 2 min at 94 ºC, 30 s at 65 ºC (reduced by 0.7 ºC per cycle), and 1 min at 72 ºC; followed by 20 cycles of 10 s at 94 ºC, 30 s at 56 ºC, and 1 min (extended 1 s per cycle) at 72 ºC. In all reactions only the *Eco*RI primers were 5' labelled with infrared dyes (IRDye 700 or IRDye 800, LI-COR). The polymorphism rates and the total number of scorable fragments were

evaluated with 22 *Eco*RI/*MSe*I (+3/+3) primer combinations. Six primer combinations (Table 4.1) with the highest polymorphism rates and large numbers of clearly scorable fragments were selected to analyze the full set of 18 lines.

Three replications were done with each primer combination from the restriction digestion step in order to test the reproducibility of the AFLP technique.

Table 4.1: Selective nucleotides of six primer (*Eco*RI/ *Mse*I) combinations used for AFLP analysis of the 18 RSA commercially cultivated lines and the OC-1 transformed line

	***Eco*RI**	***Mse*I**
1	E-ACT	M-CGC
2	E-ACT	M-CCG
3	E-ACT	M-CCA
4	E-ACA	M-CCC
5	E-ACA	M-CCG
6	E-ACG	M-CCA

An equal volume of loading solution (LI-COR) was added to each selective amplification reaction. Samples were denatured at 95 ºC for 3 min and placed on ice for 10 min before loading. A volume of 0.8 µl was loaded with an 8-channel syringe (Hamilton, Reno, Nevada) onto 25-cm 8% Long Ranger gels (BMA, Rockland, ME, USA). Electrophoresis and detection of AFLP fragments were performed on LI-COR IR^2 (model 4200S) automated DNA analyzers. The electrophoresis parameters were set to 1500 V, 40 mA,

40 W, 50 ºC, and a scan speed of three. The run-time was set to 4 h and gel images were saved as TIF files for further analysis.

4.3.4 AFLP band scoring and data analysis

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Semi-automated scoring was performed with SAGAMX (Version 3.2, LI-COR) and followed by manual editing to make adjustments to the automated score where necessary. A locus was scored as polymorphic when the frequency of the most common allele (band present or absent) was less than 0.97 (absent or present in at least two individuals). Bands with the same mobility were considered as identical products (Waugh *et al.* 1997), receiving equal values regardless of their fluorescence intensity. Polymorphic and monomorphic fragments of 50-500 bp were analyzed using the SagaMX AFLP software (Version 3.2, Li-Cor). Bands were scored as present or absent (+ and - respectively) and a data matrix was generated with the NCSS 2004 computer program (http://www.ncss.com). The generated data matrixes were thereafter analyzed with the PAUP 4.0b10 (http://www.paup.csit.fsu.edu) program (Appendix 7). The program used the data matrixes to determine distances between different characters (lines) and thus their relatedness.

The polymorphic information content (PIC) and the marker indexes (MI) were calculated for all primer pairs using the following formulae:

PIC =1 - [f^2 + $(1-f)^2$], where 'f' is the frequency of the marker used in the data set (De Riek *et al*., 2001). The marker indexes were calculated with, MI = polymorphism (POL)

x PIC (Lübberstedt *et al*., 2000). A dendrogram was created using PAUP version 4.0b10 with the unweighted pair group method analysis (UPGMA; Sokal and Michener, 1958). Genetic similarity between accessions was calculated according to Nei and Li (1979).

4.3.5 SSR Analysis

Hopkins and his co-workers (1999) designed the primers used for this study of which the sequences were submitted to Inqaba Biotechnology Industries (Pty) Ltd, Pretoria, RSA, for synthesis (Table 4.3). The extracted genomic DNA was diluted to a working concentration of 25 ng/µl and directly used in the touchdown PCR cycle (Hopkins *et al*., 1999).

The reactions were performed in 25 ul volumes containing 25-30 ng/ul genomic DNA, 1X PCR buffer (PROMEGA), 1.5 mM $MgCl_2$, 0.25 mM of each dNTPs, 0.5 U of Amplitaq DNA polymerase (PROMEGA) and 10 pmol of each primer (Table 4.3). The touchdown cycling conditions was done according to Mellersh and Sampson (1993) as cited by Hopkins and his co-workers (1999). The PCR cycling profile was as follows:
4 mins at 94^{o}C; followed by ten cycles of 30s at 94 ^{o}C; 30s at 65 ^{o}C and 1min at 72 ^{o}C; ten cycles at 30s at 94 ^{o}C; 30s at 65 ^{o}C; 1 min at 72 ^{o}C; 20 cycles at 15s at 94^{o}C; 30s at 55 ^{o}C and 1 min at 72 ^{o}C; and terminated for 10 min at 72 ^{o}C.

Table 4.2: Characteristics of primer combinations used in the SSR experiment

ID	Motif	Sequences	Expected size range (bp)	No. of expected fragments	Tm MIN/MAX
Ah4-4	$(GA)_{19}$	5'CGATTTCTTTACTGAGTGAG 3'(F) 5'ATTTTTTTGCTCCACACA 3'(R)	82-100	6	50.8/ 56.3
Ah4-26	$(GT)_{25}$	5'TGGAATCTATTGCTCATCGGCTCTG 3'(F) 5'CTCACCCATCATCATCGTCACATT 3'(R)	156-213	8	62.9/ 64.6
Lee-1	$(AT)_{18}$	5'CAAGCATCAACAACAACGA 3'(F) 5'GTCCGACCACATACAAGAGTT 3'(R)	218-281	14	55.9/ 60.6
Ah4-20	$(GA)_{19}$	5'ACCAAATAGGAGAGAGGGTTCT 3'(F) 5'CTCTCTTGCTGGTTCTTTATTAACTC 3'(R)	201-215	3	60.8/ 61. 4
Ah4-24	$(ATA)_{17}$	5'CTCACCCATCATCATCATCGTCACATT 3'(F) 5'CTCCTTAGCCACGGTTCT 3'(R)	403-418	5	58.5/ 59.9
Ah6-125	$(TTC)_{13}$	5'TCGTGTTCCCGATTGCC 3'(F) 5'GCTTTGAACATGAACATGCC 3'(R)	190-192	2	58.4/ 59.6

4.3.6 Agarose and polyacrylamide gel electrophoresis (PAGE)

The amplified products were first visualized on 3% Agarose gel, stained with Ethidium bromide under ultraviolet light. Products were also run on a Long Ranger (*CAMBREX*), [1X TBE, 1g/ 75 ml APS, 30 µll TEMED per 75 ml of gel solution] PAGE gel. A 25-well comb was carefully inserted between the casting plates and a 6% non-denaturing polyacrylamide gel was casted and left for an hour to polymerize. Two microlitres loading buffer (PROMEGA) and 2 µl SYBR Green 1 Nucleic Acid Gel Stain (1 µl/500 µl ddH_2O of 10 000x stock concentrate in DMSO) was added to 10 µl of each sample, loaded and ran on the gel for 4 hours at 170 V. Gels were visualized on the Biorad Versadoc System under ultraviolet light after which they were analyzed with the Quantity One, Biorad Versadoc System, version 4.4.

4.3.7 SSR band scoring and data analysis

Bands were scored with the Quantity One, Versadoc Biorad System, Version 4.4 as well as manually and bands were attributed in base pairs automatically on the system. The

number of fragments and fragment ranges for all primer pairs were recorded down for further analysis. Records of loci presence and absence were used to construct the UPGMA tree (Appendix 8).

4.3.8 Data matrix

A data matrix was compiled from the most important characteristics of the peanut lines, as well as the AFLP and SSR data sets (AFLP data as detailed in section 4.3.4 and Appendix A.4; SSR data as detailed in section 4.3.7 and Appendix A.5).

The agronomic characteristics of the 18 lines (Chapter 2, Appendix A.1.1-A.3.2) that was taken in account included:

1) Subspecies: where *fastigiata* spp. = 1 and *hypogaea* = 2.
2) Growth season: where <170 = 1 and ≥170 = 2.
3) Testa colour: where fleshy colour = 1 and others = 2.
4) Growth habit: where 'bunch' type = 1 and 'runner' types = 2.

4.4 Results

In this study, both the AFLP and SSR markers were used as molecular markers in evaluating the degree of polymorphism among 18 RSA peanut lines and an *OC*-1 transformed line.

Genomic DNA obtained after extraction using the DNeasy plant extraction kit from QIAGEN, contained high amounts of RNA (Figure 4.1, Lanes 1-19). An RNAse treatment was performed in order to have high quality DNA as starting material for both AFLPs and SSRs. The DEB method isolated DNA was RNA-free compared with the results from the DNeasy plant extraction kit (Figure 4.1, Lanes 20 and 21).

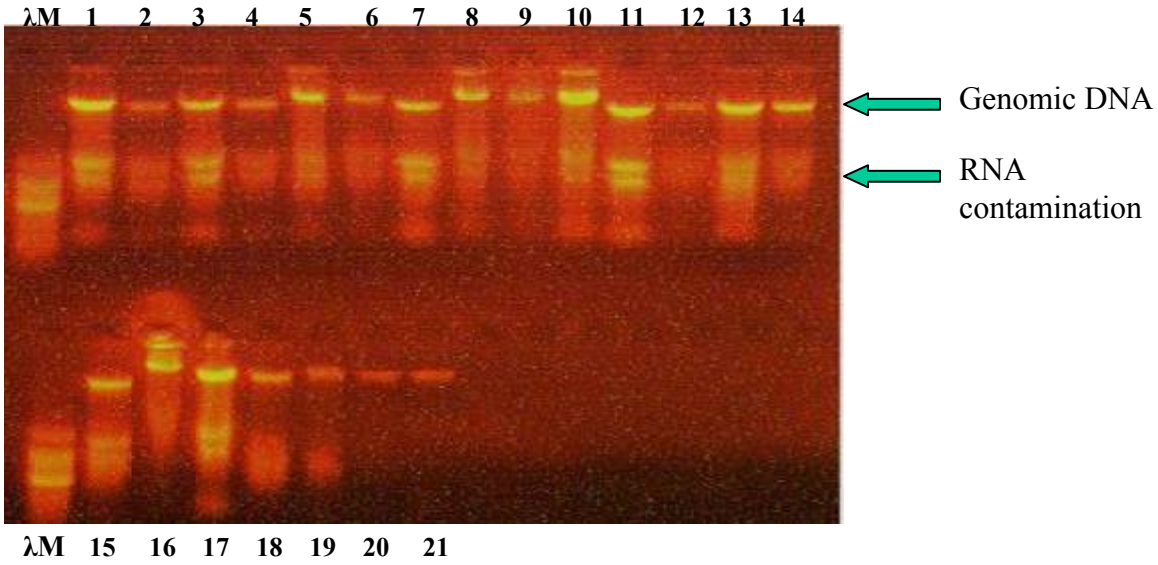

Figure 4.1: Isolated DNA on 1% Agarose gel. Lanes 1-19 = DNA from the different varieties; (1) 'Natal Common', (2) 'Harts', (3) ICGV-93415, (4) 'Billy', (5) 'Robbie', (6) 98Sel-3K1, (7) 'Jasper', (8) 98Sel-8K1, (9) 'Anel', (10) PC222-9, (11) 'Kwarts', (12) 'Sellie', (13) PC253K12, (14) JL24, (15) JL24, (16) 'Akwa', (17) 'Norden', (18) 98Sel-37K1, (19) 'Selmani'. Lanes 20 and 21 represents DNA of the transformed JL24 (*OC*-1), both extracted using the DNA Extraction Buffer (DEB) method. First lanes top and bottom shows lambda DNA marker III

After the RNAse treatment, the genomic DNA proved RNA-free and was then ready for use in the AFLP and SSR experiments (Figure 4.2). DNA quantity and quality obtained ranged from 56 ng/µl to 185 ng/µl and had A260/280 ratios at 1.7 to 1.97, respectively. The genomic DNA was diluted to final concentrations of 50 ng/µl and 25 ng/µl and stored at – 80 ^{0}C – 20 ^{0}C until further use.

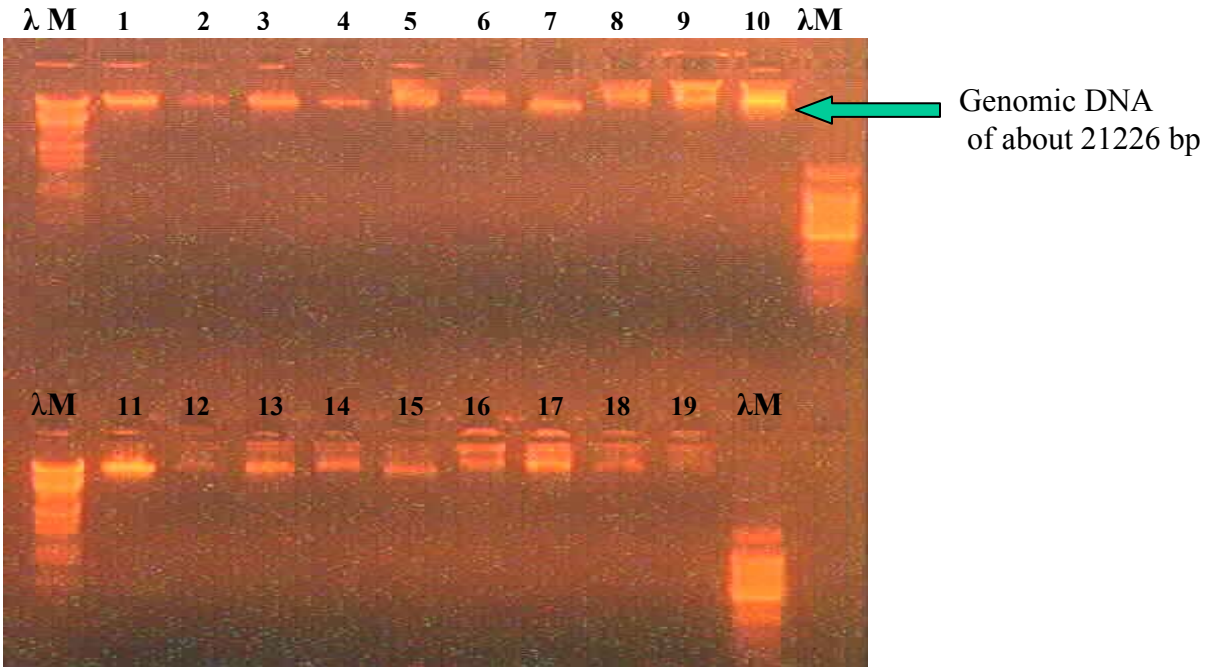

Figure 4.2: Genomic DNA ran on 1% Agarose gel. Lane 1-19 = DNA from the different varieties; (1) 'Natal Common', (2) 'Harts', (3) ICGV-93415, (4) 'Billy', (5) 'Robbie', (6) 98Sel-3K1, (7) 'Jasper', (8) 98Sel-8K1, (9) 'Anel', (10) PC222-9, (11) 'Kwarts', (12) 'Sellie', (13) PC253K12, (14) JL24, (15) JL24, (16) 'Akwa', (17) 'Norden', (18) 98Sel- 37K1, (19) 'Selmani'. Lanes 20 and 21 represents DNA of the transformed JL24 (*OC*-1), both extracted using the DNA Extraction Buffer (DEB) method. First lanes top and bottom shows lambda DNA marker III

4.4.1 AFLP analysis

In preparation for AFLP analysis, the 50 ng/µl genomic DNA was restricted with *Eco*RI and *Mse*I and ligated. After the restriction ligation step, a DNA smear was visible from

100 to 1000 base pairs, evidence of complete restriction digestion (Figure 4.4). The pre-amplifications were done in order to increase the amount of template to be used for the final selective amplification step and was visible as a smear between 100 and 1000 base pairs (Figure 4.5). Twenty-two *Eco*RI/*Mse*I (+3/+3) primer combinations were screened for AFLP analysis after which six polymorphic combinations were selected and used (Table 4.2).

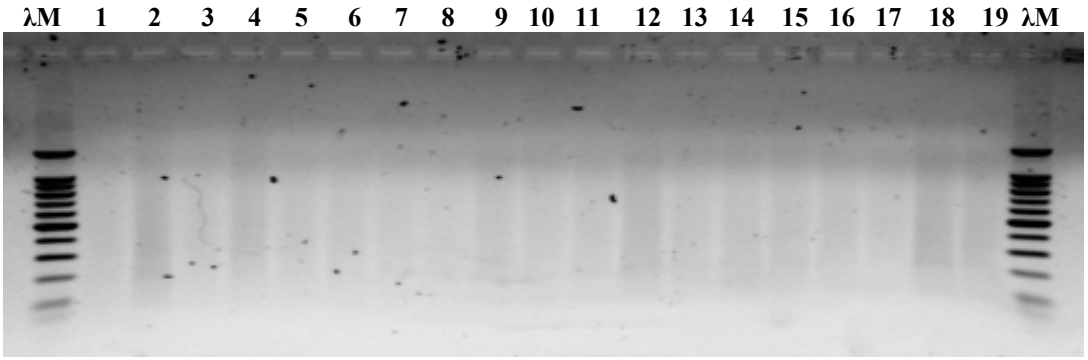

Figure 4.4: Restriction/ligation results ran on 1.2% agarose gel.Lanes 1-19 represent the 18 commercially cultivated RSA peanut lines and the *OC*-1 transformed line. The 100 base pair marker can be seen on first and last lanes

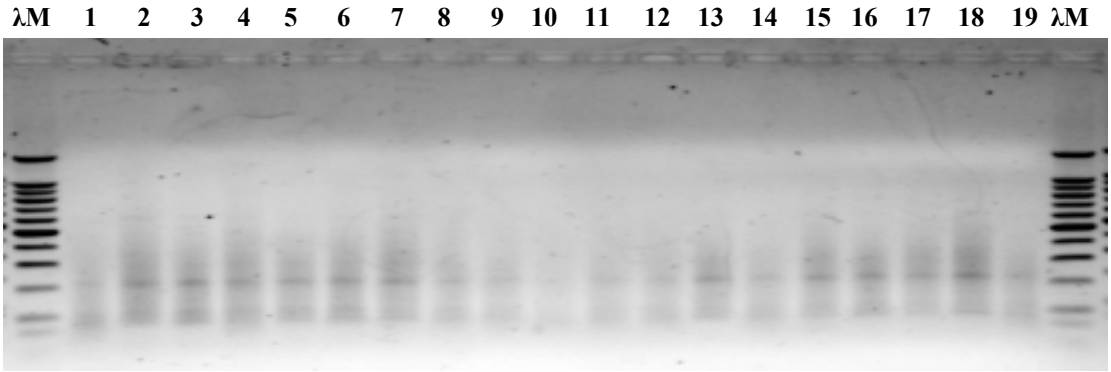

Figure 4.5: Pre-amplification results ran on a 1.2% agarose gel electrophoresis. Lanes 1-19 represent the 18 peanut lines and the *OC*-1 transformed line. The 100 base pair marker can be seen on first and last lanes

Six polymorphic combinations (Figure 4.6 and 4.7), of which the *Eco*RI primers were IRD700-labeled, were used for the final AFLP amplification in an attempt to establish genetic variation among 18 cultivated peanut lines and the additional transformed line (Figure 4.6).

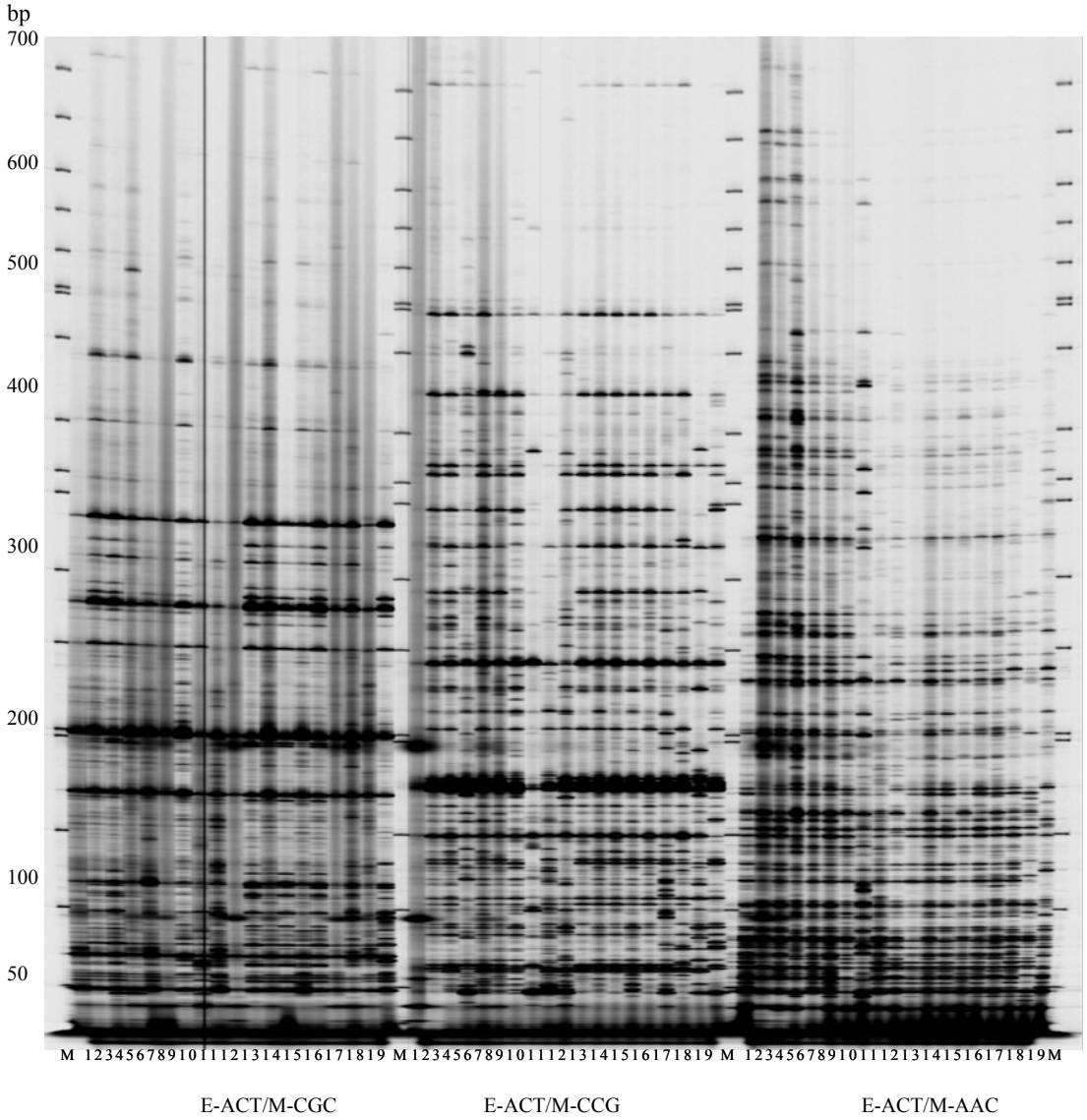

Figure 4.5: AFLP analysis of 18 peanut lines and a transformed line using primer combinations E-ACT/M-CGC, E-ACT/M-CCG and E-ACT/M-AAC respectively where where (l) 'Harts', (2) 'Kwarts', (3) 'Jasper', (4) 'Sellie', (5) 'Anel', (6) 'Natal Common', (7) JL24, (8) 'Norden', (9) 'Robbie', (10) 'Billy', (11) ICGV93415, (12) PC222-9, (13) 'Akwa', (14) PC253K12, (15) 98Sel-37K1l, (16) 98Sel-8K1, (17) 98Sel-3K1, (18) 'Selmani' and (19) JL24 (*OC*-1)

The AFLP fingerprinting images amplified with E-ACA/M-CCC, E-ACA/M-CCG and E-ACG/M-CCA primer combinations on separate occasions (A and B) produced similar images (Figure 4.7).

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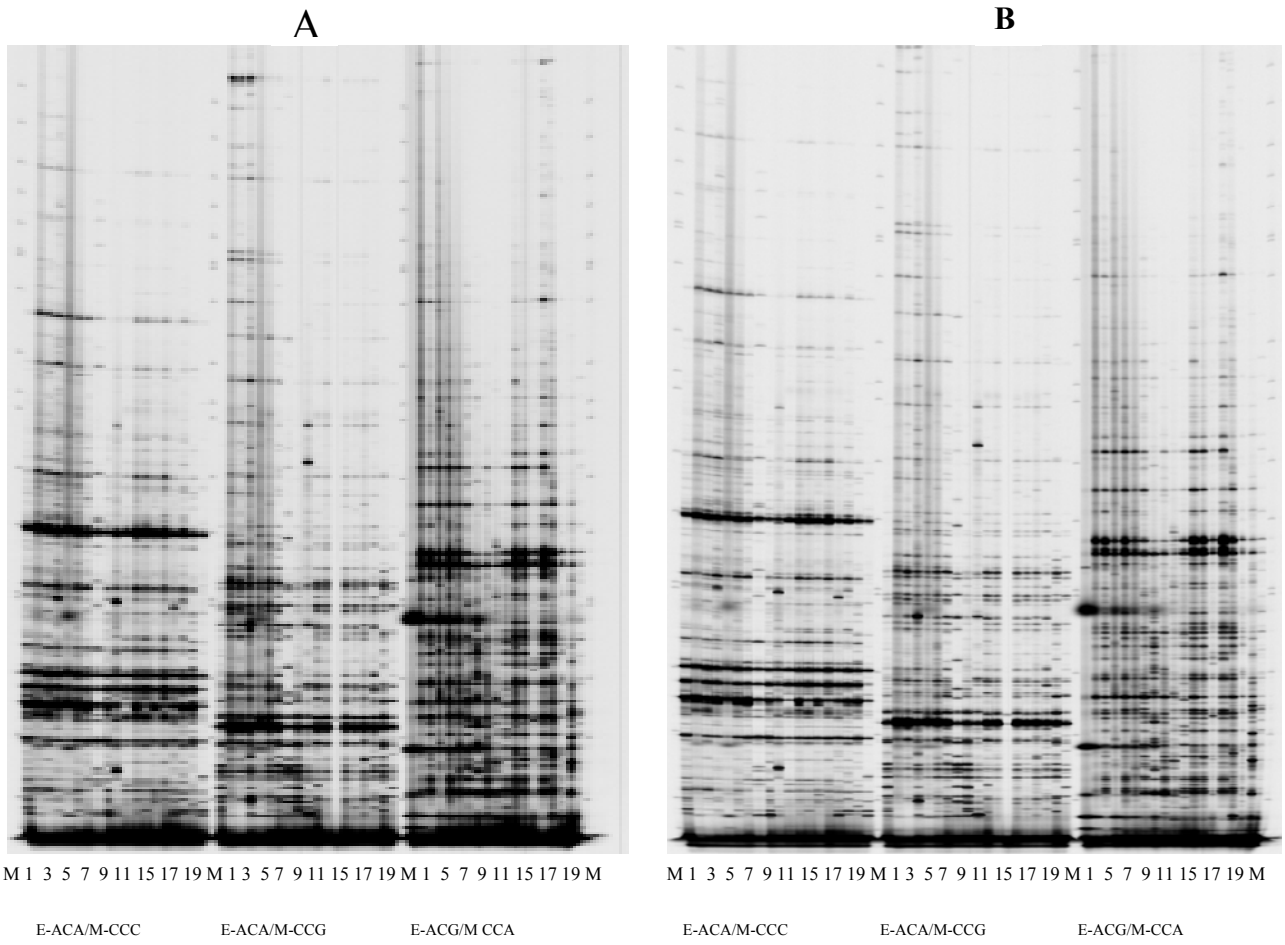

Figure 4.6: The 18 commercially cultivated RSA lines and a transformed line amplified with primer combinations E-ACA/M-CCC, E-ACA/M-CCG and E-ACG/M-CCA, where (l) 'Harts', (2) 'Kwarts', (3) 'Jasper', (4) 'Sellie', (5) 'Anel', (6) 'Natal Common', (7) JL24, (8) 'Norden', (9) 'Robbie', (10) 'Billy', (11) ICGV93415, (12) PC222-9, (13) 'Akwa', (14) PC253K12, (15) 98Sel-37K1l, (16) 98Sel-8K1, (17) 98Sel-3K1, (18) 'Selmani' and (19) JL24 (*OC*-1)

Ninety five percent of the total number of loci scored was present in both Figures 4.7 (A and B). Only 4.8% and 0.8% loci were only present in Figures 4.7 (A or B) respectively (Table 4.3).

Table 4.3: Comparisons of Figures 4.7 (A and B)

Figure	Total no. of loci	No. of loci present in A and B (%)	No. of loci present in A or B (%)
A	124	118 (95%)	6 (4.8%)
B	119	118 (95%)	1 (0.8%)

Table 4.4 illustrates the sequences, size ranges, minimum and maximum annealing temperatures (TM) values as well as the number of fragments obtained from the primer combinations.

Table 4.4: AFLP analysis table illustrating the number of fragments scored polymorphic and monomorphic) for the different primer pairs as well as the degree of polymorphism established for the individual pairs and collectively

AFLP Primers Combinations	No. Fragments Analyzed	No. Polymorphic fragments	No. Monomorphic Fragments	% Polymorphism (POL)	PIC	Marker Index (MI)
E-ACT/M-CGC	33	20	13	60.6	0.48	28.94
E-ACT/M-CCG	49	32	17	65.3	0.45	29.59
E-ACT/M-CCA	40	21	19	52.5	0.49	26.18
E-ACA/M-CCC	31	19	12	61.3	0.47	29.08
E-ACA/M-CCG	50	30	200	75.0	0.48	28.80
ACG/ CCA	43	26	17	60.5	0.48	28.91
TOTAL	246	148	88	NA	NA	NA
MEAN	41	24.7	16.3	60.0	0.48	28.58

NA=not applicable

A total of 246 fragments of which 148 were polymorphic and 88 were monomorphic fragments, were obtained after analysis with Li-Cor's SagaMX AFLP software (Table 4.4). Thus 60% of fragments analyzed were polymorphic and only 40% were

monomorphic. The polymorphic information content (PIC) and marker indexes (MI) for the primer pairs, giving information on how polymorphic they are in relation to one another, were also calculated. Primer E-ACT/M-CCG was the most informative (i.e., most polymorphic with a POL of 65.3%) and had a PIC of 0.45 and a MIC of 29.59.

4.4.2 SSR results

Six primer pairs were included in the study (Table 4.2). Amplification with the SSR primer pairs resulted in fragment sizes that varied between 60-520 bp. Primer pair Lee-1 resulted in the highest number of amplified loci (four) across the 19 peanut lines, while Ah4-4 only amplified one locus across the samples (see Appendix Table A.5). Figures 4.8 and 4.9 illustrate the results of two of the six SSR primer combinations used visualized on a 3 % agarose gel. The individual primers gave different banding patterns as can be seen in both Figures 4.8 and 4.9.

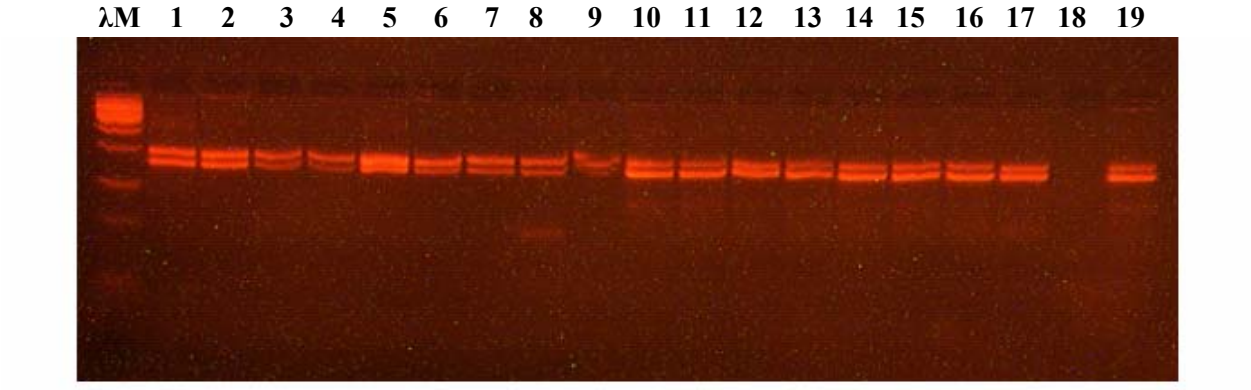

Figure 4.8: Lanes 1-19 represent the 19 peanut lines amplified with Ah4-26 SSR primer combination (Table 4.3). Samples were run on a 3% agarose gel in a 0.5x TBE buffer, where (l) 'Harts', (2) 'Kwarts', (3) 'Jasper', (4) 'Sellie', (5) 'Anel', (6) 'Natal Common', (7) JL24, (8) 'Norden', (9) 'Robbie', (10) 'Billy', (11) ICGV93415, (12) PC222-9, (13) 'Akwa', (14) PC253K12, (15) 98Sel-37K1l, (16) 98Sel-8K1, (17) 98Sel-3K1, (18) 'Selmani' and (19) JL24 (*OC*-1). λM = DNA Marker

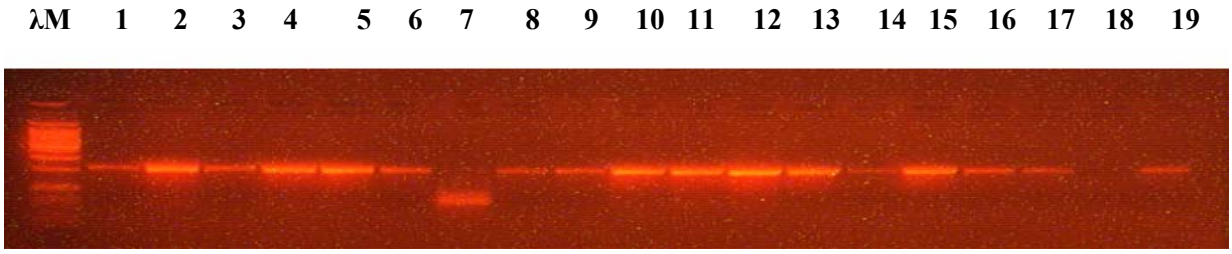

Figure 4.9: Lanes 1-19 represent the 19 peanut lines amplified with Ah4-20 SSR primer combination (see Table 4.2). Samples were run on a 3% agarose gel in a 0.5x TBE buffer where (l) 'Harts', (2) 'Kwarts', (3) 'Jasper', (4) 'Sellie', (5) 'Anel', (6) 'Natal Common', (7) JL24, (8) 'Norden', (9) 'Robbie', (10) 'Billy', (11) ICGV93415, (12) PC222-9, (13) 'Akwa', (14) PC253K12, (15) 98Sel-37K1l, (16) 98Sel-8K1, (17) 98Sel-3K1, (18) 'Selmani' and (19) JL24 (*OC*-1)

Products were then run on a 7.5% polyacrylamide non-denaturing gel for better resolution (Figure 4.10).

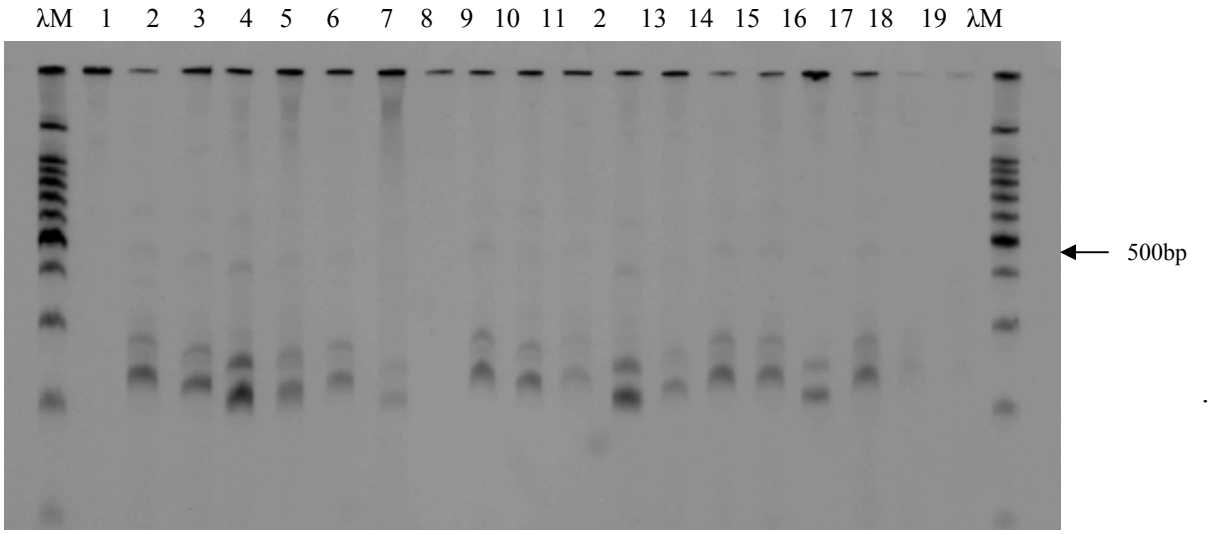

Figure 4.10: A polyacrylamide non-denaturing gel showing the 19 peanut lines amplified with the SSR primer pair Ah4-20. Lanes 1-19 represent the 19 lines while the first and last lanes are the 100 base pair markers. (l) 'Harts', (2) 'Kwarts', (3) 'Jasper', (4) 'Sellie', (5) 'Anel', (6) 'Natal Common', (7) JL24, (8) 'Norden', (9) 'Robbie', (10) 'Billy', (11) ICGV93415, (12) PC222-9, (13) 'Akwa', (14) PC253K12, (15) 98Sel-37K1l, (16) 98Sel-8K1, (17) 98Sel-3K1, (18) 'Selmani' and (19) JL24 (*OC*-1)

The amplification products obtained after amplifying with SSR primer pair Ah4-20 are presented in Figure 4.10. Two loci were amplified in this case with fragment ranges of between 200-270bp. Both loci were not visible in lines one and eight as can be seen on the gel but with increased sensitivity, the loci could be picked up by the system.

4.4.3 Combined analysis

After compiling the data matrix including all the data, a dendrogram was constructed using PAUP version 4.0b10 with UPGMA (Figure 4.11). Two main subgroups were exposed at 0.6 cut-off points indicating the two subspecies of peanut namely ssp. *hypogaea* and ssp. *fastigiata*. Of all the RSA peanut lines, the 'Spanish' types clustered together (i.e. 'Kwarts', PC222-9, 'Jasper', PC253K12, 'Anel', 'Robbie', 'Akwa', 'Sellie' and 'Natal Common'). JL24, which was bred and provided by ARC-Potschefstroom, grouped together with the 'Spanish' types. The 'Virginia' peanut lines grouped together (i.e. 'Billy', 98Sel-8K1, 98Sel-3K1 and 98Sel-37K1) with ICGV-93415, a 'Spanish' type also found in this group. The two 'Runner' types, 'Norden' and 'Selmani' were outliers together with the only 'Valencia' type namely 'Harts'. JL24 (*OC*-1), the transformed groundnut line, was also an outlier and this can potentially be attributed to the different DNA extraction method (DEB) used during the isolation of the DNA and/or the transformation event.

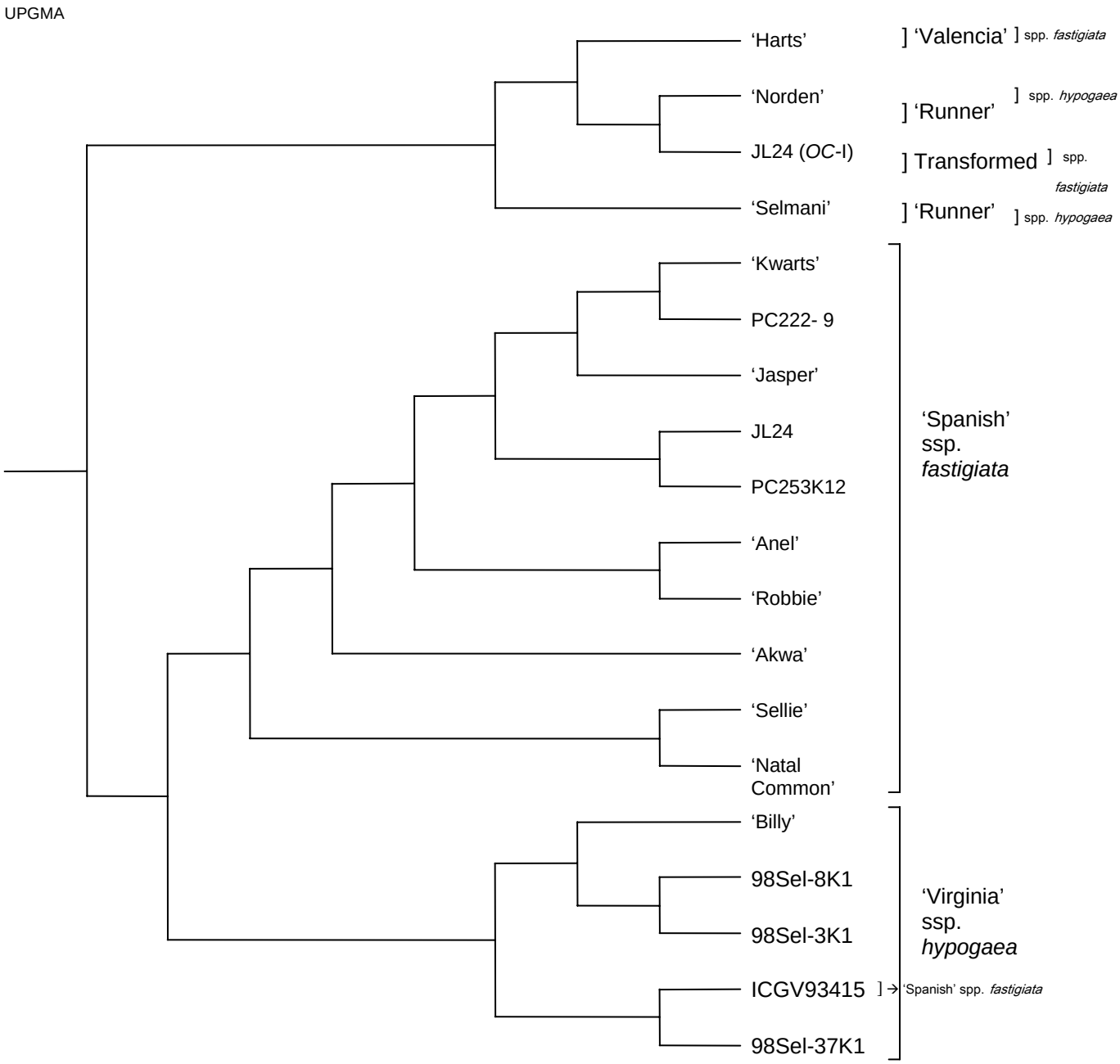

Figure 4.11: A dendrogram derived from molecular markers data and phenotypic data using UPGMA on PAUP version 4.0b10

4.5 Discussion

A total of 246 AFLP fragments was analyzed during this study, of which 60% were polymorphic and only 40% monomorphic (Table 4.4). The polymorphic information content (PIC) and marker index (MI) for the different primer pairs were also calculated. The number of loci amplified as well as the fragment size ranges differed from results reported by Hopkins and co-workers (1999) using the same primer pairs (see Appendix Table A.5).

The AFLP primer combination E-ACT/M-CCG appeared to be the most variable, followed by E-ACA/M-CCG and E-ACT/M-CGC while the primer combination E-ACA/M-CCC had the least number of polymorphic fragments (POL of 52.5%). The average PIC (Table 4.4) for the markers appeared to be low but this can be explained by the inclusion of the monomorphic (f >0.5) fragments in the calculations in order to overrule the absence of a particular marker in a specific line. Despite the many previous reports that revealed low levels polymorphism in *A. hypogaea*, AFLPs revealed enough evidence of genetic diversity to distinguish between the 19 lines used in the study. Sixty three percent polymorphism was accounted for with AFLPs, which is considerably higher when compared to previous studies along the same line and which mark the first time that closely related peanut lines exposed so much polymorphism. An early study on the same lines which excluding only 'Selmani', JL24 and the transformed JL24 (*OC*-1) revealed only 2.78% polymorphism (Herselman, 2003).

Peanut is generally a self-pollinating plan and many cultivars are closely related. It is very clear from the UPGMA tree that the majority peanut lines in are from the ‘Spanish’ types and this relates very well with the available literature (Swanevelder, 2000; Herselman, 2003). The grouping of JL24 with the ‘Spanish’ types came as no surprise as the ‘Spanish type’, ‘Natal Common’ served as a parent for most of the cultivars bred at ARC-Potschefstroom over the year (Swanevelder, 2000; Herselman, 2003). ICGV-93415, a ‘Spanish’ type, grouped with the ‘Virginia’ types, stressing the inbreeding in the RSA peanut industry. Most interestingly, ‘Sellie’, which replaced ‘Natal Common’ in terms of popularity amongst farmers and which, is also an offspring of the Namark X ‘Natal Common’ cross (van der Merwe and Vermeulen, 1977), clustered together with it in a separate group. In conclusion, a reasonable correlation between collected data and the pedigree was obtained.

4.6 Acknowledgements

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Chapter V

Summary

Peanut cultivation in the Republic of South Africa (RSA) is important for both commercial and communal farmers for the provision of plant protein and oil. Peanut production in RSA varies between 80 000 to 250 000 tons per annum, with most production coming from the commercial sector and mainly in the Northern Cape, North West and Free State Provinces. Apart from the 'Spanish' types of peanut, 'Virginia', 'Valencia' and 'Runner' types are also cultivated in RSA today. All RSA peanut cultivars have a common ancestral background (i.e., 'Natal Common') and thus, peanut cultivars have presented difficulties in the establishment of variation at genetic level even though they differ extensively in terms of morphology and phenotypic parameters. The objective of this study was firstly to evaluate the agronomic performance of 18 South African peanut lines. Not much variation was observed among the lines in terms of growth habits, while lines were considerably different with regard to growth seasons. Compared to varieties cultivated in Minnesota, USA, the study found that South African peanut lines required longer growth seasons. In terms of testa colour, 80% of the lines were flesh coloured, which is most popular amongst consumers. When the lines were selected for oil percentage, there were significant differences observed in oil percentage and high oleic acid content while oleic acid/linoleic acid ratios were low. It was also attempted to transform the wild-type JL24 peanut line with an exogenous cysteine proteinase inhibitor gene for rice (*OC*-1) in order to confer resistance against peanut storage pests and to evaluate the transformation efficiency of JL24 in the greenhouse. The study revealed 6% transformation efficiency with *Agrobacterium tumefaciens* strain C58p MP90 and the transformed plants showed clear GUS and MUG activities. When tested for their chlorophyll contents, the transformed plants displayed high chl*a* and chl*b* compared with

the control. The presence of the gene in the F_2 generation plants was evident after confirmation with newly developed *OC*-1 specific primers. Lastly, the study aimed to establish the genetic diversity amongst different South African commercial peanut varieties, including the genetically enhanced variety JL24 (*OC*-1), using amplified fragment length polymorphism (AFLP) and microsatellite (simple sequence repeat, SSR) markers. The information revealed that collectively (i.e., the use of the AFLP and SSR data matrixes combined with phenotypic data) the obtained results could easily be related to the known peanut pedigree data. In conclusion, all objectives set in the study were met, but it is however recommended that further studies and tests be done on the transformed plants to evaluate their ability to confer resistance against storage pests.

Appendixes

Table A.1.1: Botanical type, growth season, growth habit and testa colour of the 18 peanut lines used during the study.

NO		Cultivar type (Genealogy)	Growth season	Growth Habit	T Color
1	'Sellie'	1	151	4.5	1
2	'Natal Common'	1	151	4.5	1
3	'Selmani'	1	180	9.0	1
4	'Harts'	1	120	4.5	2
5	JL 24	1	170	4.5	1
6	'Norden'	2	170	9.0	1
7	'Akwa'	1	150	4.5	1
8	'Kwarts'	1	150	4.5	1
9	ICGV93415	1	150	4.5	1
10	'Billy'	2	170	4.5	7
11	'Robbie'	1	150	4.5	1
12	98Sel-3K1	2	170	4.5	2
13	98Sel-37K1	2	170	4.5	1
14	98Sel-8K1	2	170	4.5	1
15	'Anel'	1	150	4.5	1
16	PC222-9	1	150	4.5	1
17	PC253KI2	1	150	4.5	1
18	'Jasper'	1	150	4.5	1

* Obtained from Cilliers *et al*. (2001). Catalogue of Groundnut Germplasm. ARC-Grain Crops Institute Potchefstroom.

Table A.1.2: Statistical analysis results of peanut growth season.

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	17	10125.333	595.608	2.74E+14	<0.001
Residual	36	7.82E-11	2.17E-12		
Total	53	10125.333			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Table A.2.1: Kernel sizes determined with different grades

Cultivar	Grade 8.3 mm*	Grade 7.6 mm*	Grade 6.7 mm*	Round*
'Sellie'	9	38	28	98
'Natal Common'	2	15	38	90
'Norden'	0	0	0	0
'Harts'	0	0	0	0
JL24	0	0	0	0

* Obtained from Cilliers *et al*. (2001). Catalogue of Groundnut Germplasm. ARC-Grain Crops Institute Potchefstroom.

Table A.2.2: Statistical analysis of kernel size grading results

One Way Analysis of Variance Tuesday, February 27, 2007, 16:48:01

Data source: Data 1 in Notebook

Normality Test: Passed (P = 1.000)

Equal Variance Test: Passed (P = 1.000)

Group	N	Missing
Sellie	3	0
Natal Common	3	0
Norden	3	0
Harts	3	0
JL24	3	0

Group	Mean	Std Dev	SEM
Sellie	9	0	0
Natal Common	2	0	0
Norden	0	0	0
Harts	0	0	0
JL24	0	0	0

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	4	182.4	45.6	>1e20	<0.001
Residual	10	0	0		
Total	14	182.4			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Table A.3.1: Total oil percentage and oleic to linoleic acid ration of selected peanut lines

Cultivar	Oil%*	C 16:0%*	C 18:0%*	C 18:1%*	C 18:2%*	O:L ratio*
'Sellie'	52.8	12.1	3.2	39.9	34.8	1.1
'Norden'	47.4	11.4	2.3	43.5	36.3	1.2
'Selmani'	44.4	10.9	2.1	38.8	40.5	0.9

* Obtained from Cilliers *et al*. (2001). Catalogue of Groundnut Germplasm. ARC-Grain Crops Institute, Potchefstroom.

Table A.3.2: Peanut oil percentage

One Way Analysis of Variance Tuesday, February 27, 2007, 16:59:36

Data source: Data 1 in Notebook

Normality Test: Passed (P = 1.000)

Equal Variance Test: Passed (P = 1.000)

Group	N	Missing
Sellie	3	0
Norden	3	0
Selmani	3	0

Group	Mean	Std Dev	SEM
Sellie	52.8	0	0
Norden	47.4	0	0
Selmani	44.4	0	0

Power of performed test with alpha = 0.050: 1.000

Source of Variation	DF	SS	MS	F	P
Between Treatments	2	108.72	54.36	7.70E+13	<0.001
Residual	6	4.24E-12	7.06E-13		
Total	8	108.72			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: Cultivar

Comparison	Diff of Means	p	q	P<0.05
Sellie vs. Selmani	8.4	3	17317983	Yes
Sellie vs. Norden	5.4	3	11132989	Yes
Norden vs. Selmani	3	3	6184994	Yes

Table A.4: AFLP Data Matrix used to draw the UPGMA tree.

An example of the first 33 characters obtained from the first primer combinations.

#NEXUS

BEGIN DATA;

Dimensions ntax=21 nchr= 150 for first primer set, 162 for second primer set]

Format missing=? Symbols “1 2”interleave;

[Saga Scoring Characters are defined as: + = Present, H = Heterozygote, - = Not Present, ? = Unknown,

MATRIX

d1	1	1	1	1	1	2	1	2	1	1	2	2	1	1	2	1	1	2	2	2	2	2	1	2	2	1	2	2	2	2	2	2	2
d2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	1	2	2	2	2	2	2	1	2	1	2	2	2	1	1	2	2
d3	2	2	2	2	1	2	2	2	1	2	2	2	2	2	2	2	1	1	2	2	2	2	2	2	2	1	2	2	1	2	2	2	2
d4	2	2	2	1	1	2	2	2	1	2	2	2	2	2	2	1	2	1	2	2	2	2	2	2	2	2	2	2	2	1	1	2	2
d5	1	1	1	1	1	2	1	2	1	2	2	2	2	2	2	1	2	1	2	2	2	2	2	2	2	1	2	2	2	1	1	2	2
d6	1	2	2	2	2	2	2	2	2	1	2	2	2	2	2	1	2	1	2	2	2	2	2	2	2	1	2	2	2	1	1	1	2
d7	2	2	1	1	1	2	2	2	1	2	2	2	2	2	2	2	1	2	2	2	2	2	1	2	2	1	2	2	2	1	2	1	2
d8	1	1	1	1	1	2	1	2	2	1	2	2	1	1	2	1	2	1	2	2	2	2	2	2	2	1	2	2	2	2	1	1	2
d9	2	1	1	1	1	2	1	2	2	2	2	2	1	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
d10	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	1	2	2	2	2	1	2	2	1	2	1	2	2	2	1	1	1	1
d11	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	1	2	2	2	1	2	2	2	2	2	1	2	2	2
d12	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	1	2	2	2	1	1	2	2
d13	1	2	1	1	1	2	2	2	1	2	2	2	2	2	2	1	1	1	2	1	1	2	2	1	2	1	2	2	1	2	2	2	2
d14	2	2	1	1	1	2	2	2	1	2	2	2	2	2	2	2	1	2	2	1	2	2	1	2	2	1	2	2	2	1	1	2	2
d15	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	2	1	2	2	2	1	2	1	2	2	2	1	2	2	2
d16	1	2	2	2	2	2	1	2	2	2	2	2	2	1	2	2	2	1	2	2	2	2	2	2	2	1	2	2	1	1	1	1	2
d17	2	2	2	2	2	2	2	2	2	1	2	2	2	1	2	2	2	1	2	2	1	2	2	2	2	1	2	2	2	2	2	1	1
d18	1	1	2	2	2	2	1	2	2	1	2	2	2	1	2	1	2	2	2	2	1	2	2	2	2	2	2	2	1	2	2	1	2
d19	1	1	2	2	2	2	2	2	2	1	2	2	2	2	2	1	1	1	1	1	1	2	1	2	2	1	2	2	2	2	1	1	2

;

begin assumptions;

CHARSET firstprimerset = 1-122;

CHARSET secondprimerset = 123-236; END;

Table A.5: SSR data matrix from the Biorad-Versadoc system also used to draw the UPGMA tree (Quantity One 1-D Analysis Software Version 4.4 Results)

Primer	Ah4-4	Loci	Ah4-26	Loci	Lee-13	Loci	Ah4-20	Loci	Ah4-24	Loci	Ah6-125	Loci
1	'Harts'	**1**	'Harts'	**3**	'Harts'	**0**	'Harts'	**0**	'Harts'	**0**	'Harts'	**0**
2	'Kwarts'	**0**	'Kwarts'	**3**	'Kwarts'	**0**	'Kwarts'	**2**	'Kwarts'	**0**	'Kwarts'	**1**
3	'Jasper'	**0**	'Jasper'	**3**	'Jasper'	**0**	'Jasper'	**2**	'Jasper'	**0**	'Jasper'	**1**
4	'Sellie'	**1**	'Sellie'	**2**	'Sellie'	**1**	'Sellie'	**2**	'Sellie'	**0**	'Sellie'	**0**
5	'Anel'	**0**	'Anel'	**3**	'Anel'	**0**	'Anel'	**2**	'Anel'	**1**	'Anel'	**1**
6	'Natal Common'	**1**	'Natal Common'	**3**	'Natal Common'	**0**	'Natal Common'	**2**	'Natal Common'	**1**	'Natal Common'	**0**
7	JL24	**0**	JL24	**3**	JL24	**0**	JL24	**2**	JL24	**1**	JL24	**1**
8	'Norden'	**0**	'Norden'	**3**	'Norden'	**4**	'Norden'	**0**	'Norden'	**0**	'Norden'	**0**
9	'Robbie'	**0**	'Robbie'	**2**	'Robbie'	**0**	'Robbie'	**2**	'Robbie'	**0**	'Robbie'	**2**
10	'Billy'	**0**	'Billy'	**3**	'Billy'	**0**	'Billy'	**2**	'Billy'	**1**	'Billy'	**1**
11	ICGV93415	**0**	ICGV93415	**2**	ICGV93415	**0**	ICGV93415	**2**	ICGV93415	**1**	ICGV93415	**0**
12	PC222-9	**0**	PC222-9	**3**	PC222-9	**0**	PC222-9	**2**	PC222-9	**1**	PC222-9	**1**
13	'Akwa'	**0**	'Akwa'	**3**	'Akwa'	**3**	'Akwa'	**2**	'Akwa'	**1**	'Akwa'	**1**
14	PC253K12	**0**	PC253K12	**3**	PC253K12	**1**	PC253K12	**2**	PC253K12	**1**	PC253K12	**1**
15	98Sel-37K1	**1**	98Sel-37K1	**3**	98Sel-37K1	**1**	98Sel-37K1	**2**	98Sel-37K1	**0**	98Sel-37K1	**1**
16	98Sel-8K1	**0**	98Sel-8K1	**3**	98Sel-8K1	**3**	98Sel-8K1	**2**	98Sel-8K1	**0**	98Sel-8K1	**1**
17	98Sel-3K1	**0**	98Sel-3K1	**3**	98Sel-3K1	**3**	98Sel-3K1	**2**	98Sel-3K1	**0**	98Sel-3K1	**1**
18	'Selmani'	**0**	'Selmani'	**3**	'Selmani'	**1**	'Selmani'	**2**	'Selmani'	**0**	'Selmani'	**0**
19	JL24 (*OC*-1)	**0**	JL24 (*OC*-1)	**3**	JL24 (*OC*-1)	**0**	JL24 (*OC*-1)	**2**	JL24 (*OC*-1)	**0**	JL24 (*OC*-1)	**1**
NO: LOCI:		**1**	**3**		**4**		**2**		**1**		**2**	
LOCI SIZES:	**60 bp**		**218,241-255, 318 bp**		**270,317,380,520 bp**		**210-230, 245-270 bp**		**450 bp**		**153, 226 bp**	