Development and application of SNP marker for low phytic acid gene (*lpa1-1*) with studies on the effect of low phytic acid on seed germination, vigour and yield in maize

by

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Thesis abstract

Maize grain contains high levels of phytic acid which chelates iron, zinc and other micronutrients as it passes through the digestive systems of monogastric consumers reducing their bioavailability. Breeding for low phytic acid (LPA) content to improve micronutrient bioavailability is hampered by a tedious and destructive colorimetric method on the grain, low yields compared to the wild-types and reduced seed germination and vigour of LPA mutants. Breeding for LPA therefore should also incorporate breeding for improved germination and vigour in the mutants. Molecular markers to speed up the selection process and studies on gene action and combining ability for germination, vigour and yield parameters of the LPA mutants in combinations with other different maize germplasm will speed up breeding for this trait. The objectives of this study were: to develop a molecular marker linked to the *lpa1-1* gene and apply this marker for foreground selection in a backcross breeding programme and to use amplified fragment length polymorphism (AFLP) markers for background selection to recover the recurrent parent genome to speed up the backcrossing process; to study gene action and combining ability for seed germination, vigour and yield from diallel crosses involving LPA mutants, QPM and normal endosperm maize inbred lines by replicated laboratory seed tests (standard germination test and accelerated aging test) and field evaluations in South Africa and Zimbabwe.

A co-dominant single nucleotide polymorphism (SNP) marker which detects the transition base change of C/T nucleotides was developed from the gene sequence to identify the *lpa1-1* trait. The 150 bp *lpa1-1* SNP marker was validated by forward and reverse DNA sequencing of the parental amplification products which confirmed the C to T base change resulting in the LPA phenotype. The *lpa1-1* SNP marker was used for foreground selection in 250 BC_2F_1 progenies of CM 32 (LPA) x P 16 as the recurrent parent. This SNP marker was used to genotype the lines into homozygous dominant (wild type) and homozygous recessive (LPA) genotypes by their melting profiles and heterozygous genotypes by the normalised difference plots using high resolution melt (HRM) analysis. Seventeen heterozygous and 11 homozygous recessive lines were identified for background selection by fingerprinting with AFLP markers to determine the amount of recurrent parent (P 16) genome present. There were six *Eco*RI/*Mse*I primer combinations tested with 277 data points scored (84% polymorphism rate). The amount of recurrent parent (P 16) genome recovered ranged from 62% to 92% with 13 lines showing greater than 83% of the recurrent parent genome.

The effects of diallel crosses generated between four LPA, three QPM and three normal endosperm maize lines were determined for seed germination and vigour using the standard germination and accelerated aging seed tests under laboratory conditions in accordance

with the procedures of the International Seed Testing Association. The specific combining ability (SCA) effects and general combining ability (GCA) effects were significant for the seed germination and vigour traits, indicating that genes with non-additive and additive effects were important in controlling these traits. However, the SCA effects were greater than GCA effects suggesting that genes with non-additive effects were predominant. The LPA parents showed reduced vigour compared to the normal and QPM inbred lines under both conditions, with LPA lines CM 31 and CM 32 showing stress tolerance. There were some combinations involving LPA lines, such as LPA x normal, LPA x QPM and LPA x LPA that retained high vigour and high germination rates under accelerated aging conditions, suggesting that they could be stress-tolerant..

A 10 x 10 diallel involving four LPA, three QPM and three Nm inbred lines was evaluated in replicated trials across six environments. Results show that both additive and non-additive gene effects were significant for resistance to northern corn leaf blight (NCLB), grey leaf spot (GLS) and *Phaeosphaeria* leaf spot (PLS) diseases. The additive gene effects were predominant for the yield and associated secondary traits such as days to mid-pollen shed (DMP), days to mid-silking (DMS), ear per plant (EPP) and grain moisture content (GMC) and grain yield. The LPA lines were early flowering and had quick grain dry down rate but all showed undesirable negative and significant GCA effects for yield. The yield of the LPA x LPA, LPA x Nm and LPA x QPM group of crosses was lower than the check hybrids by about 32 to 67% showing the need for yield improvement of the LPA combinations.

An eight x eight diallel involving two LPA and six normal endosperm lines was evaluated over two seasons in five locations with two replications for grain yield components and foliar diseases. There was significant additive and non-additive gene action for both seed germination and vigour traits with predominance of non-additive gene effects. Both additive and non-additive gene effects were significant for yield and associated traits such as anthesis dates and number of ears per plant. However, the additive gene action was predominant for yield and associated traits. Generally the LPA lines and their combinations showed lower germination and vigour. The LPA line, CM 32 showed stress tolerance under accelerated aging conditions. There were three LPA x Nm crosses that showed improvements to the means of seed germination and vigour and yield traits. Results indicated that there was not any significant correlation between yield and seed germination and between yield and vigour. Yield was, however, significantly and positively correlated with anthesis dates and GMC, indicating that higher yield was associated with longer growing cycles.

This study was able to successfully develop and apply the *lpa1-1* SNP marker for foreground selection and AFLP markers for background selection in a backcross breeding programme. Problems of low seed germination, seedling vigour and grain yield of LPA lines and their combinations were confirmed. However results also indicated some potential of combining the LPA and QPM traits in a single cultivar. In general, procedures such as reciprocal recurrent selection, that emphasise both GCA and SCA effects would be recommended to improve seed germination, seedling vigour and yield in developing varieties with LPA trait.

Declaration

This PhD study was carried out at the African Centre for Crop Improvement (ACCI) in the School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, Pietermaritzburg Campus, under the supervision of Professor P. Tongoona, Dr G. Watson, Dr. J. Derera and Professor M.D. Laing.

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List of abbreviations

A	adenine
AA	accelerated aging
ABC	ATP-binding cassette
AFLP	amplified fragment length polymorphism
BC	backcross
bp	base pair
C	cytosine
Ca	calcium
CAPS	cleavage amplified polymorphic sequence
DMP	days to mid-pollen shed
DMS	days to silk emergence
DNA	deoxyribonucleic acid
EDTA	Ethyl-dediaminetetra-acetic acid disodium salt
EMS	ethylmethane sulphonate
EPP	ears per plant
EST	expressed sequence tag
Fe	iron
G	guanine
GCA	general combining ability
GLS	grey leaf spot
GMC	grain moisture content
HIP	high inorganic phosphorus
HRM	high resolution melt
ISSR	inter-simple sequence repeat
K	potassium
KRC	Kadoma Research Centre
lpa/LPA	low phytic acid
MAS	marker-assisted selection
masl	meters above sea level
Mg	magnesium
mins	minutes
MIPS	myo-inositol-3-phosphate synthase
ml	millilitre
mM	millimoloar
MRP	multidrug resistance associated protein
NCLB	northern corn leaf blight
ng	nanogram
nM	nanomolar
Nm	normal
PCR	polymerase chain reaction
PLS	*Phaeosphaeria* leaf spot
QPM	quality protein maize
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RARS	Rattary Arnold Research Station
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcriptase polymerase chain reaction

SCA	specific combining ability
SCAR	sequence-characterised amplified region
sec	seconds
SNP	single nucleotide polymorphism
SSR	Micro-satellite or simple sequence repeat
Std	standard
STS-PCR	sequence-tagged-site-polymerase chain reaction
T	thymine
µg	microgram
µl	micro-litre
UV	ultra-violet
w/v	weight per volume
WT	wild-type
Zn	zinc

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Introduction to thesis

Importance of maize

Maize is the third most important food crop, after rice and wheat, in the world (Hoisington and Melchinger, 2005) and in Africa it is the second most important food crop after cassava (africancrops.net). The demand for maize is increasing as it is becoming more favoured as a major food and feed source due to its higher productivity, lower labour demands, easy processing, ease of digestibility and cheaper cost than other cereals. It is a valuable crop as every part of the plant has economic value to produce a large variety of food and non-food products. Maize is an important source of carbohydrate, protein, iron, vitamin B and minerals. It is the focus of many commercial plant breeding and biotechnology companies worldwide (Hoisington and Melchinger, 2005).

In Africa, 95% of maize is grown by small to medium scale farmers who cultivate 10ha or less with low yields, which barely exceed 1 t ha^{-1}. There are a number of constraints for maize grown by small scale farmers in Africa, including foliar diseases, drought, late maturity, limited use of fertilisers, declining soil fertility, *Striga* infestation, stem-borer attack, decreased protein content and lack of appropriate varieties.

Globally there are 1.09 billion people that are hungry and undernourished, of which 265 million are in Africa (FAO, 2009). This can be attributed to the global economic crisis which had the most adverse effects on the poorer people of the world. This has resulted in them eating fewer and less nutritious meals, reduction of their income spent on health and education expenses and selling assets to manage higher food prices, lower incomes and increasing unemployment (FAO, 2009). This is having further major detrimental effects on people in the developing world who subsist only on cereal crops for their daily nutritional requirements. The per capita consumption of maize is 147 calories per day per person in the world (FAO, 2009) and it is highest in eastern and southern Africa. There is a need to improve yield and nutritional aspects of staple crops such as maize to help alleviate hunger and malnourishment in the world.

Breeding for LPA in maize

Maize has high levels of phytic acid which is the main phosphorus-containing compound (75%) found in mature seed (Raboy, 1997). It is an anti-nutritional compound as it chelates essential minerals such as iron, zinc, potassium, magnesium and calcium as it passes through the digestive system of humans and other monogastric consumers. There is a considerable amount of evidence to support the fact that dietary phytate has a negative

effect on the bioavailability of dietary minerals in humans as the substitution of low phytic acid (LPA) grain in a maize based diet is associated with a substantial increase in zinc (Adams *et al.,* 2002; Hambidge *et al.,* 2004), iron (Mendoza *et al.,* 1998, 2001; Hurrell *et al.,* 2003), calcium (Hambidge *et al.,* 2005) and magnesium (Bohn *et al.,* 2004) absorption. This leads to increasing cases of iron and zinc deficiencies especially in communities that subsist on maize as their major daily food. Iron deficiency is considered to be the most common and widespread nutritional disorder in the world and zinc deficiency is estimated to affect billions of people (WHO, 2009). This research therefore aims to breed for LPA trait in the adapted tropical maize varieties to alleviate nutritional problems in sub-Saharan countries where maize is the major staple food.

Quality protein maize (QPM) lines were originally developed in late 1990s at CIMMYT, Mexico and contain higher levels of lysine (4.2 g/100 g protein) and tryptophan (0.9 g/100 g protein) compared to normal maize levels of lysine (2.6 g/100 g protein) and tryptophan (0.4g/100g protein) (Vivek *et al.,* 2008). There are calls to breed the LPA trait into QPM maize to boost nutrient content in a single variety. However, there are concerns that stacking the QPM and LPA traits in a single variety would lead to serious problems of low germination, low seedling vigour, and reduced grain yield with implications on food security especially in the smallholder sector where yield levels are already very low. Problems of low seedling vigour have already been reported in QPM (Modi and Asanzi, 2008). In addition, low grain yield potential and kernel weight were long ago reported to be associated with QPM (Poehlman and Sleper, 1995; Akande and Lamidi, 2006). However, there are counter reports that the QPM gene (*opaque2* gene) introduced into hybrids produced with the same yield potential as the wild-type hybrids (Gupta *et al.,* 2009) showing potential to improve yield. Therefore, it is prudent to test the levels of seed germination, yield and seedling vigour in crosses involving QPM, LPA and normal endosperm maize. The information would be crucial to the breeding programmes that seek to combine the QPM and LPA traits in a single variety.

There have been a number of maize inbred lines containing lower levels of phytic acid produced using ethylmethane sulphonate-induced (EMS) mutagenesis (Raboy, 2000). These maize LPA mutants are distinguished according to differences in inositol and inositol phosphate content. The maize *lpa1-1* mutant lines used in this study show a reduction in phytic acid accompanied by inorganic phosphate (Raboy, 2000). The gene from *lpa1-1* has been cloned and encodes a multidrug resistance associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi *et al.,* 2007). The *lpa1-1* mutation shows 66% reduction in phytic acid P (Raboy, 2000) and has been mapped onto the chromosome 1 distal region and

reported to be due to a single recessive mutation (Raboy *et al*., 2000). The LPA phenotype is thought to be due to a change of amino acids alanine to valine as a result of a cytosine to thymine base change (Shi *et al.,* 2007) which can be classified as a transition single nucleotide polymorphism (SNP).

Unfortunately, these LPA lines show reduced germination and vigour (Pilu *et al.,* 2003; Doria *et al.,* 2009) as well as yield reductions (Ertl *et al.,* 1998; Raboy *et al.,* 2000). Seed quality is usually associated with germination and purity but seed vigour also needs to be included. Vigour is a concept that describes several characteristics of the seed such as rate and uniformity of germination and growth, tolerance to environmental stresses after sowing and retention of performance after storage (Hrstková *et al.*, 2006). The low germination rates of LPA material are a serious concern during commercial seed production, and could result in serious yield reduction in the small-holder farmers if LPA varieties are deployed without addressing the problem of germination and vigour. It needs to be addressed to ensure that material with LPA trait is commercially viable. There is a need to determine germination and vigour levels of crosses between LPA lines and normal tropical (Nm) and quality protein maize (QPM), especially the LPA with QPM crosses for improvement of the nutritional aspects of maize.

Gene action study

In breeding programmes, the selection of parents and the determination of general and specific combining ability and gene action are important. To eliminate undesirable inbred lines and select the most desirable ones to constitute various hybrid combinations, the general and specific combining ability of the inbred lines and their crosses needs to be established. Strategies for breeding varieties with high plant vigour, high germination and yield are required. Information regarding the combining ability of these lines for yield and associated traits is useful in devising an appropriate breeding strategy to improve these traits. The LPA lines that are in a temperate background and show earliness in flowering, maturity and quick dry down of the grain at harvest are also desired in tropical environments. These advantageous traits need to be retained as well as the LPA trait during breeding to introgress into tropical material. The temperate germplasm is able to contribute genes for ultra-early flowering and early physiological maturity. Ultra-early germplasm is desired in managing drought as these varieties will flower and mature before the onset of drought which is favourable for farmers. Therefore the gene action determining these traits including anthesis dates, yield and resistance to foliar diseases in cross combinations that involve LPA, QPM and NM inbreds should be established.

Application of molecular markers to aid breeding for LPA in maize

The conventional method of detection for phytic acid is a destructive colorimetric assay of mature seed (Chen *et al.,* 1956). This method is long and comprises of an overnight incubation as well as requiring several hours of manual labour preparation for the assay (Lorenz *et al*., 2007). There is therefore a need for a non-destructive method of detecting the *lpa1-1* trait, particularly one that is reliable, quicker and less labour intensive. A solution to this would be a molecular marker that is able to detect the different LPA genotypes from vegetative tissue. Also to ensure wide-spread applicability of the marker in plant breeding programmes, it needs to be amenable to high throughput methods and more cost-effective than the conventional assay.

Markers are used routinely to track loci and genome regions in crop plants in many breeding programmes (Landridge and Chalmers, 2005). There are many markers and different types of markers available for maize which are used to map genes and traits of interest (Hoisington and Melchinger, 2005). Molecular markers can be used for detection of traits/phenotypes that are difficult to assess or for recessive genes. In the case of phytic acid, the trait is both difficult to phenotype as well as being a single recessive mutation; hence molecular markers can be applied to identify the *lpa1-1* trait with increased reliability and accuracy. Marker-assisted selection methods particularly those based on PCR are advantageous due to their objectivity and small amounts of sample tissue and results in accurate analysis of large numbers of individual plants early in the breeding programme.

Single nucleotide polymorphism (SNP) markers can occur naturally or through induced mutation techniques. The SNPs markers are stable, occur at high frequency in the genome and are of higher inheritance than most other markers. Marker techniques are usually based on detecting DNA sequence variation, with SNP markers detecting a single base change, insertion or deletion of many bases. SNP markers occur at varying frequencies depending on the species and the genome region being considered. There have been studies on maize with reports on varying frequencies of SNP occurrence (Gupta *et al.,* 2001; Tenaillon *et al.*, 2001; Ching *et al*., 2002; Barker and Edwards, 2009).

SNP marker development provides access to affordable and high-throughput genotype determination assays and automated data analyses that breeders require for MAS to be accepted (Mohler and Singrün, 2005). The choice of detection methods for SNP genotyping depend on many factors, including cost, throughput, equipment needed, difficulty of assay development and potential for multiplexing (Rafalski, 2002). SNPs are not commonly used in the plant sciences due to the high cost of developing SNPs detection assays, especially the

re-sequencing of DNA samples and the requirements for expensive instruments and complex procedures to detect SNPs (Batley *et al.,* 2003; Kim and Misra, 2007). HRM is the most inexpensive, simple and rapid of the technologies to detect SNPs (Gundry *et al.,* 2003; Wittwer *et al.,* 2003).

High resolution melt analysis (HRM) is an innovative approach for the simultaneous detection and differentiation of PCR products after PCR amplification. The PCR products are differentiated from each other by melting curve profiles (Ririe *et al.,* 1997) which allows heterozygous and homozygous genotypes to be identified (Gundry *et al.,* 2003, Wittwer *et al.,* 2003; Reed and Wittwer, 2004; Montgomery *et al.,* 2007). The melting curve is due to the plotting of fluorescence as a function of time as the thermal cycler heats through the dissociation temperature of the product (Ririe *et al.,* 1997). The fluorescence data is converted into melting curves by plotting the derivative of fluorescence with respect to temperature (dF/dt) over the temperature range in degrees Celsius. The shape and position of the melting curve are due to the GC/AT ratio, length and sequence and can be used to differentiate PCR products separated by less than 2°C in melting temperature (Ririe *et al.,* 1997). HRM analysis can differentiate PCR products of the same length but differ in GC/AT ratio and or GC content by having different melting curves, which is not possible in gel electrophoresis. Homozygous genotypes can be distinguished by shift in melting curves due to the difference in melting temperature (T_m) between the genotypes, while heterozygotes are distinguished by the altered curve shape not by T_m (Park *et al.*, 2009, Graham *et al*., 2005).

The advantage of HRM analysis is the elimination of post-PCR gel based or sequencing analyses, thereby reducing costs, time and labour (Ririe *et al.,* 1997; Zhou *et al.,* 2005). It is possible to combine HRM analysis with PCR amplification in one closed tube reaction (Montgomery *et al.,* 2007) depending on the type of equipment. Intercalating fluorescence dyes (Worm *et al.,* 2001; Liew *et al.,* 2004; Zhou *et al.,* 2004, 2005; Montgomery *et al.,* 2007; Park *et al.,* 2009) or fluorescence-labelled primers (Gundry *et al.,* 2003) can be used with this method. This advanced technology has been widely used in human genetics and is gaining more uses in animal and plant genetic studies especially in cases of SNP studies.

Markers can also be applied in backcross breeding programmes where loci are tracked to eliminate specific genetic defects in elite germplasm, for the introgression of recessive traits and in the selection of lines with a genome composition closest to the recurrent parent. In a backcross programme, markers can be used for indirect selection for favourable allele (Tanksley, 1983) and selection against the undesired genetic background of the donor genotype (Tanksley, 1989). For the introgression of a recessive trait (as for *lpa1-1*) in a

backcross breeding programme, there needs to be progeny tests at each generation to identify the homozygous and heterozygous genotypes to fix the heterozygous alleles. These progeny tests can be avoided until the last generation by the use of markers to track the trait of interest (Frisch, 2005). Background selection can reduce the number of generations required for gene introgression from six to three (Frisch *et al.,* 1999) thereby increasing the efficiency of the plant breeding programme. Although there are various options for molecular tools for background selection, including high density SNP genotyping and whole genome selection using other markers such as SSRs, due to the large costs associated with SNP genotyping and the availability and easy of assay, AFLPs were chosen for this study.

Summary of the research focus

Maize has high levels of phytic acid which inhibits absorption of essential minerals during digestion in monogastric consumers. This is especially detrimental for consumers that are dependent on a maize-based diet with little or no other food supplements. Temperate low phytic acid lines have been developed that contain lower levels of phytic acid but also show reduced seed germination and vigour and low yield.

The conventional method of determining phytic acid levels is a destructive colorimetric assay of the mature seed. This assay is not useful in breeding programmes where segregating material is being developed. The use of molecular markers can be highly effective in reducing the time required for extensive field screening, thereby aiding plant breeders in the selection processes of the breeding programme. The level of phytic acid can only be determined in the seed, whereas a molecular marker can potentially detect the level in leaf or vegetative material. Therefore, the application of molecular markers linked to *lpa1-1* gene to detect the level of phytic acid would prove to be very useful in reducing the time required in breeding programmes for low phytic acid. The use of markers for background selection to recover the recurrent parent genome in the breeding programme will aid in decreasing the amount of generations required for introgression of the trait of interest.

Due to the reduced germination, vigour and yield shown by LPA material, this study will estimate the genetic variance components by testing seed germination, vigour, and yield and associated traits. Also the knowledge of gene action, general and specific combining ability are important for selection of inbred lines and crosses between LPA, QPM and normal endosperm maize lines to adopt an effective breeding strategy for improvement of seed germination, vigour, and yield and associated traits.

Research objectives

This study addressed the following research objectives:

- To develop a SNP molecular marker for use at the early vegetative stage of the plant for the detection of the *lpa1-1* gene and to validate the SNP marker nucleotide change,
- to determine the amount of recurrent parent (P 16) genome present in each of the BC_2F_1 line, using the *lpa1-1* SNP marker for foreground selection, and AFLP molecular markers for background selection;
- To determine the type of gene action which controls seed germination, seedling vigour, grain yield, and resistance to foliar diseases in LPA x Normal and LPA x QPM maize crosses;
- To determine the level of grain yield, germination and vigour in hybrid combinations involving LPA and QPM maize inbred lines.

Research hypotheses

The following hypotheses were tested:

- The combination of LPA and QPM material in a single variety would result in compromised seed germination and vigour, as well as reduced grain yield; and
- The SNP molecular marker can be used to track the *lpa1-1* gene in a LPA x normal endosperm maize breeding population and molecular markers would be effective for use in background selection to recover the recurrent parent genome;
- Seed germination, seedling vigour and grain yield are controlled by genes with additive effects hence section procedure can be used to improve these traits in maize varieties.

Thesis structure

The above objectives and hypotheses were tested and reported in Chapters 2-6:

Chapter 1 Literature Review; Chapter 2 Development of a SNP marker for detection of the low phytic acid (*lpa1-1*) gene for use in maize breeding; Chapter 3 Marker-assisted selection for low phytic acid (*lpa1-1*) with SNP marker and AFLPs for background selection in a maize backcross breeding programme; Chapter 4 Seed germination and vigour analysis in diallel crosses among normal endosperm; low phytic acid (LPA) and quality protein maize (QPM) inbred lines under normal and accelerated aging conditions; Chapter 5 Combining ability

between temperate low phytic acid (LPA) and tropical normal endosperm inbred lines for seed vigour and grain yield components; Chapter 6 Grain yield and associated traits analysis in diallel crosses among normal endosperm, low phytic acid (LPA) and quality protein maize (QPM) inbred lines; Chapter 7 Overview

This thesis has been written in a chapter format and therefore there are overlaps either in context or literature citations.

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Chapter One

Literature Review

Introduction

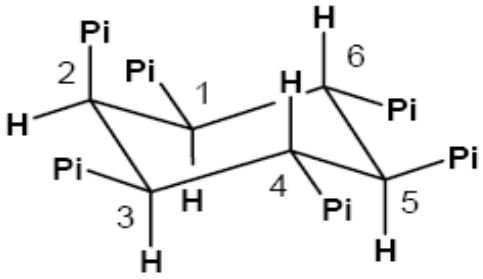
This chapter provides a context for the research by a) reviewing theory and literature on phytic acid, seed vigour and germination, molecular markers and marker-assisted backcross selection (MABC), b) reviewing literature on effects of phytic acid, maize low phytic acid mutant (*lpa1-1*), amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), high-resolution melt (HRM) analysis, foreground and background selection using markers in backcross breeding, and c) defining key technical terms of this study. Phytic acid is found in many cereal grains and inhibits absorption of essential minerals for monogastric consumers. Low phytic acid (LPA) mutant lines show reduced yield, field emergence, germination and vigour. The LPA trait needs to be introgressed into tropical material. Knowledge of the combining ability of maize inbreds is useful in devising an appropriate breeding strategy. A survey of the literature indicates that there is limited information regarding combining ability of *lpa1-1* lines, especially for seed germination and vigour.

Phytic acid

In the seed, phytate plays several roles including maturation, initiation of dormancy, providing a protected source of phosphorus and a source of cations for use during germination. Dietary phytate can have a negative global impact by contributing to mineral depletion and deficiency in populations who rely on whole grains and legume-based products as staple foods (Brown and Solomons, 1991). The advantages, however, are that it functions as an antioxidant and anticancer agent as well as having other beneficial effects on health. While phytic acid does show some beneficial effects, the limitations that have been shown in the uptake of minerals is a serious concern and needs to be addressed in order to increase the nutritional content of staple crops especially in the developing world. Studies should be aimed to achieve a significant decrease but not elimination of existing levels of phytic acid, if this happens, it would result in an increased uptake of the vital minerals and iron, thereby retaining the beneficial properties of phytic acid.

Definition and Importance

Phytic acid (Figure 1.1) is the amount and form of major phosphorus containing compound found in seeds. It is an anti-nutritional component found in many cereals and legumes. It is also a naturally occurring component of plant fibre.



$Pi = OPO_3H_2$

Figure 1.1: Structure of phytic acid (InsP6) (Chen and Li, 2003)

In mature seeds, 75% of the total phosphorus is found as phytic acid (Raboy, 1997), which represents a significant amount of all phosphorus removed from the soil by grain and legume crops. Phytate is a mixed cation salt of phytic acid (myo-inositol hexakis phosphoric acid). The three most important plant micronutrients that form phytate are P, K and Mg. It is able to bind with K^+ and Mg^{2+} and can also bind with Ca^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+} and Fe^{3+}. Once the phytic acid is digested, it binds to other seed-derived minerals, as well as other endogenous minerals that are present in the digestive tract, thereby inhibiting absorption of these minerals (Raboy, 2000).

One of the major nutritional problems in plant-based-diets of low income countries in the developing world is iron deficiency, mainly caused by poor iron content, low bioavailability of iron, or both (Brown and Solomons, 1991). Food components such as phytate, tannins and selected dietary fibres, which bind iron in the intestinal lumen, can impair iron absorption. Phytate has probably the greatest effect on iron status as there are a large variety of plants with high phytate content, which limits absorption of iron (Mendoza *et al.*, 2001). Dry cereal grains account for 77% of total phytic acid stored every year with maize seed producing 4.8 million metric tonnes of phytic acid annually around the world (Lott *et al*., 2000). Phytic acid occurs mostly in the embryonic tissues (O'Dell *et al.,* 1972) as well as other seed tissues such as the endosperm (Raboy, 2000).

Synthesis Pathway

The biosynthetic pathway to phytic acid is complex and can be summarized as consisting of two parts: *myo*-inositol (Ins) supply and the subsequent Ins polyphosphate synthesis (Figure 1.2C). The sole synthetic source of the Ins ring (Figure 1.2B) is the enzyme Ins (3) P^1 synthase (MIPS), that converts Glc-6-P to Ins (3) P^1. Phytic acid synthesis may also proceed in part via pathways typically associated with second messenger metabolism that involve phosphatidylinositol (PtdIns) phosphate intermediates and Ins (1,4,5) P^3 (Figure 1.2C). Depending on the position of mutations in the complex pathway, different LPA mutants with reduced levels of phytic acid have been found (Raboy *et al*., 2000; Pilu *et al*., 2003) (Table 1.1). The *lpa1-1* gene has been completely sequenced and comprises 11 exons and encodes MRP4, a multidrug resistance protein (MRP) ATP-binding cassette (ABC) transporter (Shi *et al.*, 2007). This transporter is expressed in embryos and to a lesser extent in immature endosperm, germinating seed and vegetative tissues (Shi *et al.,* 2007).

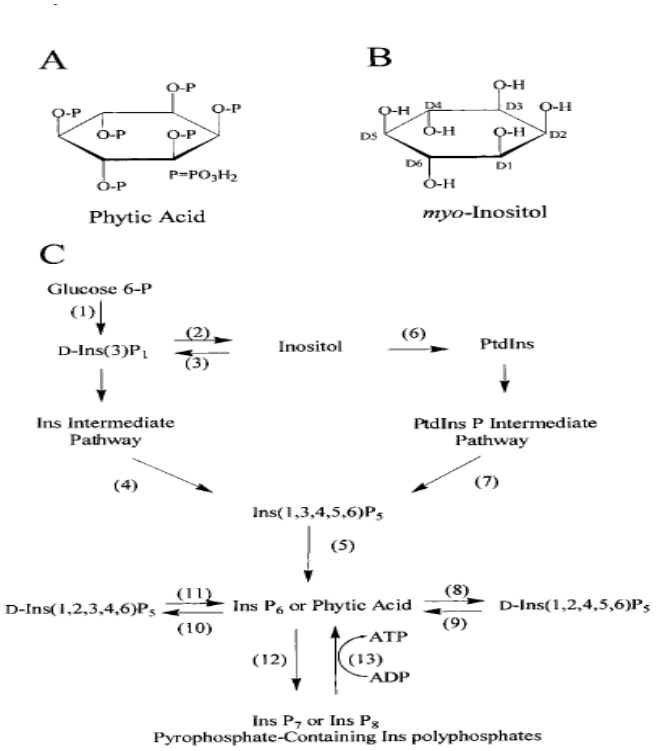


Figure 1.2: Biosynthetic pathways to phytic acid (Raboy *et al.,* 2000)

A: Structure of phytic acid; B: Structure of Ins; C: Biochemical pathways –
(1) D-Ins(3)-P1 synthase; (2) D-Ins 3-phosphatase; (3) D-Ins 3-kinase; (4) Ins P- or polyp kinases; (5) Ins (1,3,4,5,6) P5 2-kinase or phytic acid-ADP phosphotransferase; (6) PtdIns synthase; (7) PtdIns and PtdIns P kinases, followed by PtdIns P-specific phospholipase, and Ins P kinases; (8) D-Ins (1,2,3,4,5,6) P6 3-phosphatase; (9) D-Ins (1,2,4,5,6) P5 3-kinase; (10) D-Ins (1,2,3,4,5,6) P6 5-phosphatase; (11) D-Ins (1,2,3,4,6) P5 5-kinase; (12) pyrophosphate-forming Ins P6 kinases; (13) pyrophosphate-containing Ins PolyP-ADP phosphotransferases (Raboy *et al.,* 2000).

Impact on humans, animals and environment

There has been considerable progress in plant genetics leading to the identification and successful breeding of grains and legumes that are homozygous for allelic variants at a single gene that alters the phytate content of the grain or legume (Raboy *et al.*, 2000; Larson *et al.*, 2000; Adams *et al.*, 2002, Hambidge *et al.*, 2004; Dorsch *et al.*, 2003). In maize, the low phytate alleles have been identified and this can be used to facilitate measurements of the long term effects of dietary reduction on minerals' (zinc and iron) bioavailability in individuals with a high phytate diet (Adams *et al.*, 2002).

A low bioavailability of iron can result in iron deficiency, causing one of the major nutritional problems in the developing world. Phytate is able to bind iron in the intestinal lumen and inhibit iron absorption from many plant foods that have high phytate content. A possible solution to this problem is the use of low phytate crops that have been produced using genetic mutations that interfere with phytate synthesis. A long term strategy for reducing iron deficiency would include food fortification. A major drawback of phytic acid is its ability to bind some essential mineral nutrients in the digestive tract and can result in these minerals being excreted. This results in mineral deficiencies in humans and animals and can also lead to eutrophication of waterways.

There have been numerous studies comparing the effects of a LPA diet on humans (Agte *et al.*, 1999; Mendoza *et al.,* 2001; Adams *et al.*, 2002; Hurrell *et al.,* 2002, 2003; Ekholm *et al.,* 2003; Davidsson *et al.*, 2004; Hambigde *et al.,* 2004). There is a considerable amount of evidence to support the fact the dietary phytate has a negative effect on the bioavailability of dietary zinc in humans. The fractional absorption of zinc was compared in meals prepared from LPA maize to its matched wild type hybrid with a "normal" phytic acid content. It was concluded that substitution of LPA grain in a maize based diet is associated with a substantial increase in zinc absorption (Adams *et al.*, 2002). Hambigde *et al.* (2004) determined the relationship between fractional absorption of zinc and the phytate content and phytate:zinc molar ratios using maize tortillas prepared from hybrids containing different phytate contents. A negative relationship ($P < 0.001$) between fractional absorption of zinc and both dietary phytate and phytate: zinc molar ratio was found. It was therefore concluded that fractional absorption of zinc from maize tortillas is positively related to the extent of phytate reduction achieved with low-phytate hybrids. Zinc absorption was significantly greater after dephytinisation of soybean protein isolate (Davidsson *et al.*, 2004).

Phytate probably has the greatest effect on iron status due to the fact that most plant foods have high phytate contents that can severely inhibit iron absorption. Absorption of phytic acid

can be increased by the addition of fortificants or by the degradation or removal of phytic acid. There are different types of fortificants available and need to be assessed together with the type of diet, efficiency of dietary iron absorption and fortificant, and associated cost factors and sensory properties. Ekholm *et al.* (2003) compared three chelating agents, citric acid, malic acid and glucose. It was found that citric acid was the most efficient and that degradation reduced the influence of phytic acid. Mendoza *et al.* (2001) found that there was no significant effect on phytate content on iron absorption. However, iron was absorbed more efficiently when sodium iron EDTA was used as a fortificant, rather than ferrous sulphate, regardless of the type of maize (low phytate maize compared to unmodified wild type maize) (Mendoza *et al.,* 2001).

Phytic acid degradation can be achieved during home cooking and by industrial processing. Iron absorption was compared from industrially manufactured and home-cooked cereal foods by Hurrell *et al.* (2002). There was little or no difference in iron absorption observed, although amylase pre-treatment showed an increase in iron absorption. It was also concluded that iron absorption is only increased by those cooking procedures such as bread making, which extensively degrades phytic acid, or amylase pre-treatment, which substantially liquefies cereal porridges. Hurrell *et al.* (2003) measured the effect of phytic acid degradation on iron absorption from cereal porridges (rice, wheat, maize and sorghum). It was concluded that phytate degradation improved iron absorption from cereal porridges prepared with water and not with milk, except high-tannin sorghum. By using ion exchange chromatography to study the phytate degradation of 272 different traditional cooked meals, it was found that Indian vegetarian meals have higher total phytate content (1-2 g/kg diet) than Western and other Asian countries (Agte *et al.*, 1999).

Phosphorus is an essential nutrient found in animal feed. It is critical for growth, reproduction and the formation and maintenance of the skeletal system. Between 60 to 80% of phosphorus in maize is found as phytic acid and is largely unavailable to monogastric animals such as pigs and poultry. Phytic acid has very little bioavailability for monogastric animals due to their lack of phytase activity. A possible solution is the isolation of cereal mutants accumulating less phytic P and more free phosphate in the seed. A result of high phosphorus excretion by monogastric animals is the environmental pollution of water and soils.

Studies have been conducted to determine the effect and availability of phytic acid to pigs (Spencer *et al.,* 2000a,b; Veum *et al.*, 2001), chicks (Douglas *et al.,* 2000; Li *et al.,* 2000; Yan *et al.,* 2000; Peter and Baker, 2002), turkeys (Yan *et al.,* 2003) and rabbits (Marounek *et al.*, 2003). In particular, there were studies that used maize containing the *lpa1-1* mutant

(Spencer *et al.,* 2000a,b; Li *et al.,* 2000). In animal feeding studies using low phytate maize, there was an increase of 2-5 times the amount of bioavailable phosphorus observed (Douglas *et al.,* 2000; Li *et al.,* 2000; Spencer *et al.,* 2000a, b; Yan *et al.,* 2000, 2003; Veum *et al.,* 2001; Peter and Baker, 2002). It was found in rabbits that although inorganic phosphates hinder phytic acid hydrolysis, generally they were able to digest phytic acid fairly efficiently. Also, the addition of exogenous phytase to rabbit feeds could increase phytic acid hydrolysis in the upper part of the digestive tract and eliminate the need for inorganic phosphate supplements (Marounek *et al.*, 2003). Pigs that were fed low phytate maize, genetically modified maize naturally high in digestible phosphorus excreted less phosphorus, thereby reducing the potential for pollution from the swine industry. Veum *et al.* (2001) confirmed the increased availability of phosphorus in low phytate maize as compared to normal maize in a study with pigs. The reduction in phytic acid did not compromise the nutritional value of the low phytate maize, and confirmed the reduction of phosphorus excretion by feeding low phytate maize to pigs. Another benefit that was observed in the study was an increase in bone strength due to the replacement of normal maize with low phytate maize, with soybean meal as a supplement. The processing of low phytate maize in the manufacture of animal feed is exactly the same as normal maize.

These low phytate crops would be best suited for environments that are in sensitive areas of high soil test phosphorus levels and that are prone to serious soil erosion losses and or high concentrations of swine or poultry feeding operations. They can also be used to improve environmental sustainability when used in livestock farming. By improving nutrient digestibility, low phytate crops can help reduce unwanted phosphorus in livestock manure, which is a potential threat to water quality (Ertl *et al.*, 1998; Veum *et al.*, 2001).

Low phytate crops

Induced mutagenesis has become an important tool in locating genes on chromosomes, for studying gene structure, expression and regulation, and for exploring genomes, and for the plant breeders, using these radiation-induced mutations for changing plant traits. Over the last seventy years, there have been more than 2250 varieties (60% from 1985 onwards) that have been released derived either as direct mutants (70%) or from their progenies. Most of the varieties (75%) are in crops and the remainder in ornamental and decorative plants. Mutation-derived varieties have been released in 175 crop and plant species, with some having a major economic impact (Ahloowalia *et al*., 2004).

The main objective of mutation-based breeding is to upgrade the well-adapted plant varieties by altering one or two major traits, although selection of the desired genotype is important irrespective of the procedure used to create variation. The development of molecular probes offers an opportunity to select the desired mutants. Therefore, mutation induction, molecular marking of useful and selected mutations, sequencing of the mutated genes and the development of molecular probes can be vital in the continued and expanded use of induced mutations, mutants and cultivars in mutation breeding programmes.

Induced mutations can also play an important role in the creation of crop cultivars with traits such as enhanced uptake of specific minerals, deeper rooting system, tolerance to drought and salinity, and resistances to diseases and pests as a major component of the environmentally sustainable agriculture (Ahloowalia *et al*., 2004).

Low phytic acid mutants have been generated by chemical and physical mutagenesis in different crop species, such as maize (Raboy*,* 2000; Pilu *et al.,* 2003; Shi *et al.,* 2003, 2005, 2007), rice (Larson *et al.,* 2000; Liu *et al.,* 2007; Kim *et al.,* 2008), soybean (Wilcox *et al.* 2000; Hitz *et al.,* 2002; Yuan *et al.,* 2007), wheat (Guttieri *et al.,* 2004), beans (Campion *et al.,* 2009) and barley (Larson *et al.,* 1998; Rasmussen and Hatzack, 1998). The low phytate barley variety called Herald was released in 2006 (Bregitzer *et al.,* 2007). These mutants have substantial reductions in seed phytic acid P with molar-equivalent increases in inorganic P. These LPA mutants offer potential genetic resources to address the nutritional and environmental issues caused by poor availability of P in maize (Ertl *et al.,* 1998). These mutations do not affect the plant's ability to take up phosphorus and transport it to the developing seed. However, the mutations block the ability of a seed to synthesize phosphorus into phytic acid phosphorus (Raboy, 2002). Therefore, LPA mutants have higher amount of nutritionally available phosphorus and offer potential benefits in the sustainability of lands used to grow crops, the improved mineral nutrition of humans and animals, and reduction in pollution of waterways.

Low phytate maize and *lpa1-1* mutation

Raboy (2000) was involved in isolating more than 20 independent LPA mutants from maize (Table 1.1), barley and rice. Seeds homozygous for an LPA mutant contain normal levels of seed total phosphorus but greatly reduced levels of phytic acid phosphorus. Substantial variation in the concentration of seed phytic acid was typically observed. However under standard production conditions, the crops showed that the proportion of seed total phosphorus and phytic acid phosphorus remained at constant levels. Mutants were therefore

selected whose seeds contained normal levels of total phosphorus with greatly reduced levels of phytic acid phosphorus (Raboy, 2000).

Low phytate maize has a phosphorus utilization of 96% versus standard maize of about 30% (Raboy, 2000). The first step to obtain maize LPA mutants was to generate a population of ethylmethane sulphonate-induced (EMS) mutants, followed by screening for mutants whose seed contain substantial reductions in phytic acid phosphorus. The level of phytic acid phosphorus was reduced and matched by an equal increase in inorganic phosphorus. The LPA seeds have inorganic phosphorus representing 50% of the total seed phosphorus (Raboy, 2000).

This trait is being introduced into a variety of maize types, including yellow and white, and temperate and tropical types (Raboy, 2000). An important part of the breeding programme is to include breeding for adequate and stable yield in an LPA background in a wide variety of environments. It is essential that seeds homozygous for the mutation germinate well and produce adequately productive plants (Ertl *et al.*, 1998). Low phytic acid mutants were used to determine the effect of different *myo*-inositol on raffinose family oligosaccharides, due to these seeds requiring less *myo*-inositol for the synthesis of phytic acid and may therefore contain elevated levels of *myo*-inositol (Karner *et al.*, 2004).

The maize LPA mutants produced by Raboy (2000) are distinguished according to differences in inositol and inositol phosphate content. The maize *lpa1-1* mutants show a reduction in phytic acid accompanied by inorganic phosphate, *lpa2* mutants show significant increases in inositol phosphate intermediates, *lpa3* mutants accumulate inorganic phosphate and myo-inositol but not inositol phosphate intermediates and *lpa241* show increased inorganic phosphate and decrease of phytic acid (Raboy *et al.,* 2000; Pilu *et al.,* 2003; Shi *et al.,* 2005). The genes from *lpa1-1*, *lpa2* and *lpa3* have all been cloned (Shi *et al.,* 2003, 2005, 2007). The *lpa1-1* gene encodes a multidrug resistance associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi *et al.,* 2007) while *lpa2* gene encodes an inositol phosphate kinase (IPK) belonging to the Ins (1, 3, 4) P3 5/6 kinase gene family (Shi *et al.,* 2003), *lpa3* gene encodes a myo-inositol kinase (MIK) gene (Shi *et al.,* 2005). The LPA mutant line, *lpa241* is allelic to *lpa1-1* (Pilu *et al.,* 2005) and shows a decrease in expression of the first enzyme of phytic acid pathway, myo-inositol-3-phosphate synthase (MIPS) (Pilu *et al.,* 2003).

The two mutants of maize (*lpa1-1*, *lpa2*) have been mapped onto the maize chromosome map (Figure 1.3). The *lpa1-1* mutants have 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g P_i (66% of total P) compared to the normal maize of 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g P_i (7% of total P) (Raboy *et al.,* 2000). The *lpa1-1* gene was chosen to

introgress the LPA trait into tropical adapted maize line (P16) due to its high percentage of reduction of phytic acid (66%), available gene sequence and the exact type and position of the mutation identified. However, due to its recessive nature of the *lpa1-1* trait, marker-assisted selection is required to detect the various genotypes.

It was found that *lpa1-1* is a good candidate for use in breeding. These inbred lines were used to produce at least 14 pairs of near-isogenic hybrids, which were used for studies on the effect of *lpa1-1* on germination, yield, and other agronomic characteristics (Ertl *et al.*, 1998). Different studies have found different agronomic results for the LPA mutants. Ertl *et al.* (1998) found that there was little or no difference in germination, stand establishment, stalk lodging, plant height, ear height, flowering date, or “stay green” score observed between normal (non-mutant) and mutant isolines of a given hybrid.

Other studies have found reductions for the LPA lines. The yields of the maize LPA lines were found to have been reduced to between 5 and 15% compared to the highest yielding commercial varieties (Raboy, 2000). On average, a 6% yield loss was observed in the *lpa1-1* hybrids as compared with the normal hybrids (Ertl *et al.*, 1998). The maize *lpa1-1* mutant lines have shown yield reductions compared to the WT parent (Ertl *et al.,* 1998), which is also observed in *lpa1* barley mutants (Bregitzer and Raboy, 2006). In rice, the LPA mutations were found to most likely be the causative factor of grain yield reduction (12.5-25.6%) (Zhao *et al.,* 2008). The wheat LPA mutants had delayed development and reduced grain yield (8-25%) partly due to reduced kernel size in a high yielding environment (Guttieri *et al.,* 2006).

The use of LPA mutants in plant breeding has been limited due to non-germination of genotypes with homozygous *lpa1* alleles (Raboy, 2000), reduced seed weight of *lpa1-1* (Raboy *et al.,* 2000), and the lower vegetative growth rate and impaired seed development due to the *lpa241* mutation (Pilu *et al.,* 2005). These LPA mutants have inferior agronomic and seed viability than their wild-type (WT) parents, leading to yield reduction (Raboy *et al.,* 2000) due to reduced seed weights and low vegetative growth.

The *lpa1-1* mutation shows 66% reduction in phytic acid P (Raboy, 2000). Genetic mapping has shown the mutant phenotype and the INS (3) P_1 synthase (MIPS) gene found in the phytic acid biosynthetic pathway to map to the same chromosomal region in maize. The MIPS genomic sequence showed 10 exons and 9 introns, with the reduction in MIPS gene activity and LPA content due to a mutation affecting the promoter or transcriptional factor controlling MIPS expression (Shukla *et al.*, 2004). The *lpa1-1* gene has been mapped onto the chromosome 1 distal region and reported to be due to a single recessive mutation

(Raboy *et al*., 2000). The gene was completely sequenced (5149 bp) and the *lpa1-1* phenotype reported to be due to a change of amino acids alanine to valine as a result of a cytosine to thymine base change. The amino acid is conserved in MRP proteins and located in the second ATP-binding domain and is likely to account for the *lpa1-1* phenotype (Shi *et al.,* 2007).

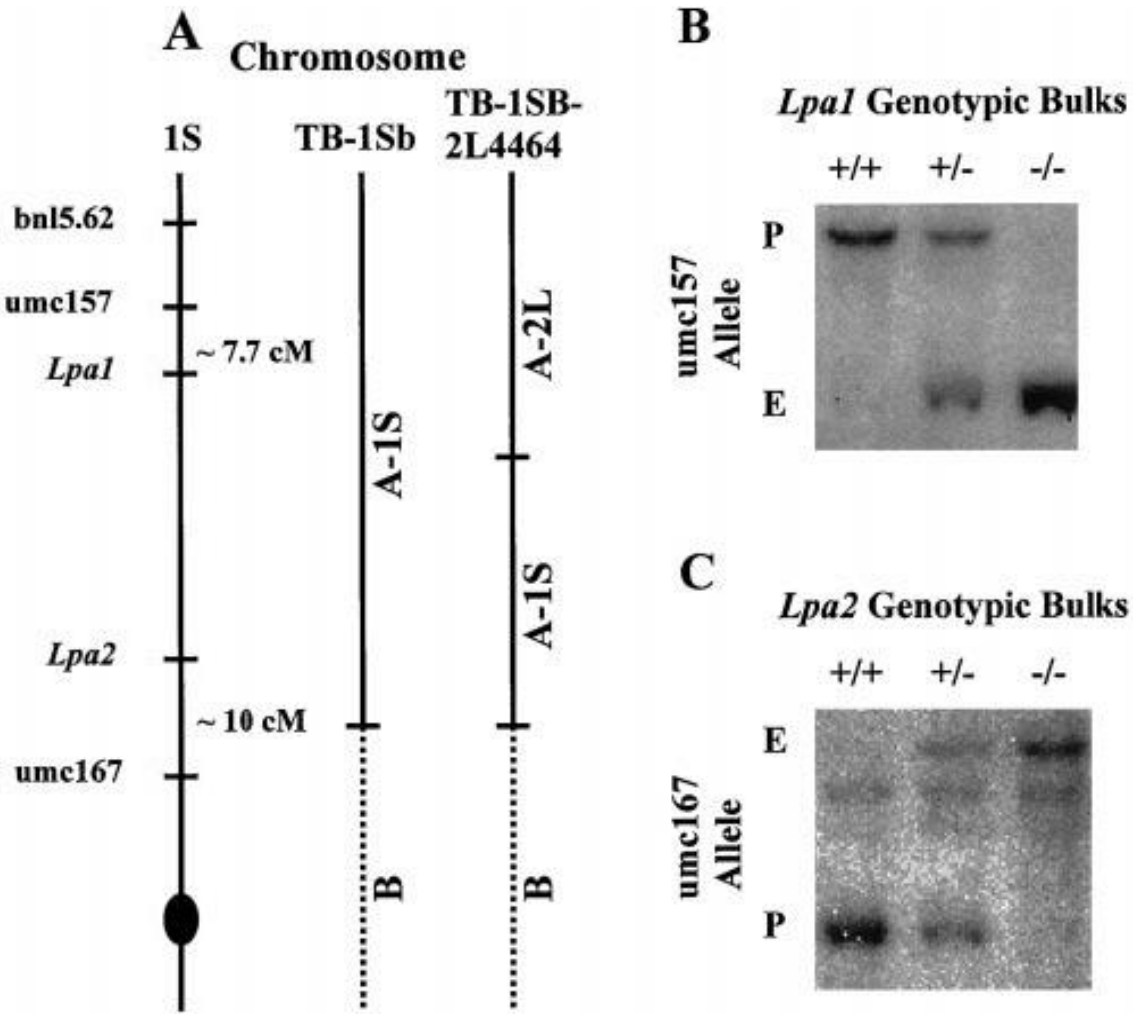


Figure 1.3: Chromosome mapping of maize *lpa1-1* and *lpa2-1*

A: Approximate map positions of *lpa* loci and markers on chromosome 1S and their relation to two chromosome 1S B-A translocations. Approximate distance (cM) of *lpa1* to umc157 and *lpa2* to umc 167. B and C, RFLP mapping of *lpa* loci using bulked segregant analyses. A genotypic bulk DNA was prepared to represent the three *lpa1* or *lpa2* F2-mapping population segregant classes: +/+, homozygous normal, +/-, heterozygous, -/-, homozygous mutant. DNA was isolated from each of the individuals representing each class and combined so that each individual contributed equally to the bulk. Bulk DNA was digested with *Eco*RV*,* fractionated, and probed with the indicated RFLP marker. P and E are the parental Pioneer Hi-Bred inbred and Early-ACR alleles, respectively (Raboy *et al.*, 2000).

Table 1.1: Summary of LPA mutants in maize

Name of mutation	**Type of mutation**	**Seed phytic acid**	**Total inositol P (mg/g) (% of total P)**	**Inorganic P (mg/g) (% of total P)**	**Position of mutation and effect on pathway**	**Reference**
wild type		75-80% of total P	3.4 (76%)	0.3 (7%)		Raboy *et al*., 2000
lpa1-1	single EMS pollen grain	66% reduction	1.1 (23%)	3.1 (66%)	distal region of 1S affecting Ins supply part	
lpa2-1	seed specific spontaneous effect	50% reduction	2.6 (57%)	1.3 (28%)	proximal half of 1S affecting Ins phosphate metabolism part, mutation of ins P kinase gene	
lpa3	n/a*	50% reduction	1.3 (n/a*)	0.7 (n/a*)	near the adh1 locus on chromosome 1	Shi *et al.,* 2005
lpa241	chemical mutagenesis- EMS	90% reduction	0.4 (9%)	3.3 (72%)	distal region of 1S (bin 1.02) affecting MIPS1S gene coding for 1st enzyme in PA pathway	Pilu *et al*., 2003

n/a*: not available

Methods of assessment

Phytate can be isolated and measured by many methods, including ferric precipitation, paper chromatography, thin layer chromatography, gel chromatography, gas liquid chromatography, ion exchange chromatography and ^{31}P-NMR. Some methods are unable to distinguish phytic acid from other inositol phosphates. This can lead to inaccurate assessments of total levels of phytate. A method that can be used to measure % total P is dry ashing followed by a molybdate blue spectrophotometric assay (Lott *et al*, 2000).

Phytate salt forms one to several per cent of the dry weight of many seeds and in many cases accounts for 50-80% of the total P in seeds. It is found in most plant tissues. It is more reliable to use estimates obtained from field grown plants as they contain a lower P concentration as compared to greenhouse plants. Other factors such as type of cultivar, soil conditions, fertilizer application, moisture and climatic factors also play a role in the variation of phytic acid and P concentrations.

Normal seeds have consistently low levels of inorganic phosphorus at maturity, typically < 0.5 mg P/g, whereas LPA seeds typically contain > 1.0 mg/g. This high inorganic phosphorus (HIP) phenotype of LPA seeds provides the basis for a quick, sensitive, inexpensive and straightforward test for the trait (Figure 1.4). The procedure is as follows: single seeds are individually crushed and extracted overnight in 0.4 M HCl (10v/w). Ten µl of extract is then assayed for inorganic phosphorus in microtitre plate's wells. Reagent inorganic phosphorus is used as colorimetric standards. Reagent inorganic phosphorus is added to five standard wells to give: 0.0 µg P; 0.15 µg P; 0.46 µg P; 0.93 µg P; and 1.39 µg P (Chen *et al.,* 1956). Using this method, normal seeds usually produce colour development less than 0.46 µg P; whereas LPA seeds usually produce colour development greater than 0.46 µg P (Raboy, 2002). Traditional backcrossing breeding methods have been used the HIP test to identify LPA mutants.

Since phytic acid is mainly found in the embryo of mature seed (O'Dell *et al.,* 1972), the method of detection has been a colorimetric assay of the crushed seed which effectively destroys the seed (Chen *et al.,* 1956). This method is detrimental in breeding programmes especially in cases of segregating material when screening for the LPA trait as once the seed has been crushed for the assay; it cannot be used to generate a seedling. The colorimetric method is long (overnight incubation), has low repeatability and requires 11-12 hours of human labour to analyse 100 samples of which the majority is manual preparation of samples into plates (Lorenz *et al*., 2007). There is therefore a need for a non-destructive method of detecting the *lpa1-1* trait, particularly one that is reliable, quicker, less labour intensive and that can be amenable to high throughput methods. An alternative assay which

is more reliable and less labour intensive is required to determine the presence of the LPA trait. Selection efficiency in breeding programmes would be greatly increased if the leaf or vegetative stages can be used for the assay instead of the mature seed. An ideal solution would be a co-dominant molecular marker that is able to distinguish between both heterozygous and homozygous individuals in the breeding programme.

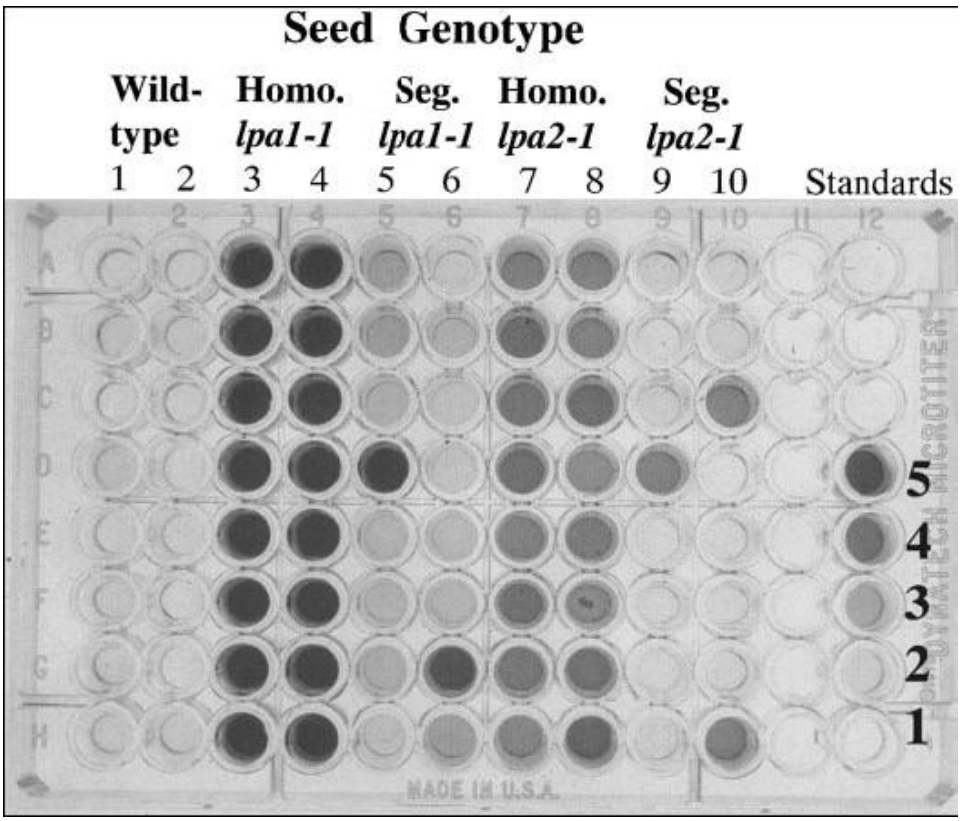


Figure 1.4: An assay for the HIP phenotypes of LPA seed

Shown are tests of 20 single seeds sampled from ears of maize that were either wild-type (columns 1 and 2), homozygous *lpa1-1* (columns 3 and 4), segregating for *lpa1-1* (columns 5 and 6), homozygous *lpa2-1* (columns 7 and 8) or segregating for *lpa2-1* (columns 9 and 10). The five standard wells represent 1) 0.0 µg P; 2) 0.15 µg P; 3) 0.46 µg P; 4) 0.93 µg P; and 5) 1.39 µg P (Raboy, 2002).

Molecular marker technology

Due to the advent of a wide range of analytical tools for deoxyribonucleic acid (DNA) analysis, there are various types of molecular markers available to plant breeders, geneticists, and germplasm scientists. Molecular markers are well established tools in plant breeding and genetics that improve the efficiency and sophistication of breeding. They have played a key role in improving the understanding of the genome organization, structure and behaviour for many major crops.

There are three marker types: morphological (traits), biochemical (seed storage proteins and isozymes), and molecular or DNA-based polymorphisms that can be used to establish linkages with traits of economic importance. Polymerase chain reaction (PCR) based molecular markers have the greatest application for marker-assisted selection (MAS) (Kelly and Miklas, 1999). There are three classes of molecular markers: the first generation markers include random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphisms (RFLPs) and their modifications; second generation markers include SSRs, AFLPs and their modifications while the third generation markers include single nucleotide polymorphisms (SNPs) and expressed sequence tags (ESTs) (Gupta *et al.,* 2001). ESTs have been used in functional genomics studies while SNPs are the new generation of molecular markers that can be used for MAS in plants.

Markers have been identified, such as amplified fragment length polymorphisms (AFLPs) that combine restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) technologies (Vos *et al*., 1995), and single nucleotide polymorphisms (SNPs). These newer markers have gained popularity in higher plants through comparative mapping and DNA fingerprinting applications (Mohan *et al*., 1997; Staub *et al*., 1996). Scientists saw the potential for these highly abundant markers and used them extensively to expand the genetic linkage maps for most commodities. Many of these maps had previously been limited to either morphological traits or protein markers (Bassett, 1991; Gepts, 1988). Maps proved valuable in positioning traits in relation to each other but were limited in the traits segregating in the mapping population (Kelly and Miklas, 1999).

Molecular markers that have a wide coverage of the genome and that are highly polymorphic are preferred. Markers are based on detecting sequence variation in DNA and AFLP markers in particular target the repetitive regions of the genome. The choice of marker system to use often depends on the species, objective of the work, genetic resolution and financial and technological resources available. They differ in information content, number of scorable polymorphisms per reaction, and degree of automation (Mohler and Schwarz, 2005).

Amplified Fragment Length Polymorphisms (AFLPs)

AFLPs are a universal, multi-locus marker technique that can be applied to genomes of any source or complexity. It is a reproducible and reliable technique within and among mapping populations (Mohler and Schwarz, 2005). No prior sequence information is required and highly informative fingerprints are produced due to the large number of bands produced per primer combination. AFLP is an ingenious combination of RFLPs and PCR designed by Vos *et al*. (1995). It has become very popular and is a powerful approach to identify DNA polymorphisms. AFLP marker bands are mainly dominant but in some cases co-dominance can be detected.

Applications of the AFLP technique include assessing genetic variation, assigning inbred lines into heterotic groups, fingerprinting lines, predicting single cross hybrid performance, identifying QTLs/genes for disease resistance, grain yield and grain-associated traits, phosphorus efficiency, drought tolerance and other important traits of maize. It is a reliable and effective method with a variety of applications in maize and other food crops.

AFLPs have been used extensively to assess genetic diversity in maize (Ajmone-Marsan *et al.,* 1998; Pejic *et al.,* 1998; Lübberstedt *et al.,* 2000; Heckenberger *et al*., 2003; Adawy *et al.,* 2004; Beyene *et al.,* 2005, 2006; Legesse *et al.,* 2007; Hartings *et al.,* 2008) and other crops such as soybean (Maughan *et al.,* 1996; Powell *et al.,* 1996; VanToai *et al.,* 1996), potato (McGregor *et al.,* 2000; Spooner *et al.,* 2005), barley (Russell *et al.,* 1997; Varshey *et al.,* 2007), wheat (Bohn *et al.,* 1999; Maccaferri *et al.,* 2007), rice (Saini *et al.,* 2004; Jeung *et al.,* 2005), groundnuts (Herselman, 2003), sugarcane (Lima *et al.,* 2002), lentils (Sharma *et al.,* 1996) and black pepper (Joy *et al.,* 2007).

The AFLP technique was successful in detecting genetic diversity in all studies with high levels of polymorphism, effective multiplex ratio, marker index and genotype index being observed. The technique was useful in assigning inbreds into heterotic groups, revealing pedigree relationships among lines, showing relationships between morphological and or phenotypic traits and AFLP marker data. It was concluded that SNP markers are better suited for characterizing and conserving genebank materials with AFLP markers being more suitable for diversity analysis, fingerprinting (Varshey *et al.,* 2007), cultivar identification, phylogenetic studies (Joy *et al*.,2007). In general AFLP markers are the most appropriate for various aspects of germplasm analysis (Lübberstedt *et al.,* 2000).

Due to the advancements in genome research, molecular markers are being used to predict hybrid performance in crop breeding programmes. This method allows the maize breeder to predict combinations of lines that would result in high-yielding single cross hybrids. There

have been various studies on maize that have used AFLPs and found the genetic distances based on AFLP data were significantly correlated with F_1 yield and was also significantly correlated with specific combining ability (Wu, 2000). Other studies on maize on the relationship between hybrid performance and AFLP-based genetic distances found AFLPs to be practical to predict hybrid performance but with varying rates of correlations with different traits (Ajmone-Marsan *et al.,* 1998; Schrag *et al.,* 2006, 2007; Legesse *et al.,* 2008). Linkage disequilibrium was compared in elite maize lines using AFLP and SSR markers and found both marker types suitable for genome wide association mapping, however the ratio of linked to unlinked loci pairs in linkage disequilibrium was higher for AFLPs than SSRs, with the recommendation of AFLPs for use in populations with a long history of recombination (Stich *et al.,* 2006).

A study on advanced backcross QTL analysis to improve hybrid yield identified and manipulated useful QTLs in heterotic inbreds of maize using AFLP markers (Ho *et al.,* 2002). There have been studies on maize identifying QTLs to improve phosphorus efficiency (Chen *et al.,* 2008;2009), drought (Sari-Gorla *et al*., 1999) and grain yield and grain related traits (Ajmone-Marsan *et al.,* 2001). AFLP markers have been used to generate high density maps in maize (Castiglioni *et al.,* 1999, Vuylsteke *et al.,* 1999) and to map genes/QTLS linked to important maize diseases such as southern corn leaf blight (Cai *et al.,* 2003, Chen *et al.,* 2004), grey leaf spot (Lehmensiek *et al.,* 2001) and sugarcane mosaic virus (Yuan *et al.,* 2004). Marker-assisted selection and map-based cloning of S cytoplasmic male sterility in maize has been successful using AFLP markers (Tie *et al.,* 2006, Zhang *et al.,* 2006). AFLP markers were successfully linked to all individual mutant alleles from ten mutations resulting in the appearance of defective, miniature or collapsed endosperm effects in maize seeds (Pasini *et al.,* 2008).

There are many studies on DNA fingerprinting of inbred lines in maize and other crops; however there are few studies on the use of AFLPs in backcross plant breeding programmes to determine the percentage of recurrent parent genome. These studies were on crops such as cotton (Zhong *et al.,* 2002); rice (Chen *et al.,* 2000, 2001; Zhou *et al.*, 2003; Gopalkrishan *et al.*, 2008); soybean (VanToai *et al.,* 1996) and beans (Mũnoz *et al.,* 2004).

A study on the reproducibility of RAPDs, SSRs and AFLPs found the AFLP technique difficult to perform initially but with greater familiarity this problem was resolved and the AFLP profiles showed extremely high reproducibility (Jones *et al.,* 1997). Statistical analysis of AFLPs can be of two kinds: band-based methods (direct study of band presences/absences in AFLP profiles) or allele frequency-based methods (application is contingent on estimation of allele frequencies within populations, therefore population-centered) (Bonin *et al.,* 2007).

There are numerous advantages of AFLPs including versatility as PCR with random primers. No prior sequence information is required, high stringency is applied during PCR to ensure robustness and high reproducibility, a limited set of AFLP primers can yield a large set of primer combinations, each with its own unique set of amplified fragments, the multiplex ratio is high and can be increased by altering the length of the 3'-nucleotide extensions and/or choice of enzyme. These advantages make AFLPs a very useful tool for numerous molecular applications. The pattern complexity can be in the range of 20 and 60 bands per primer combination in the fragment range of 50 to 500 bp, depending on the species. There are some disadvantages that include dominance of markers, clustering of markers (depending on the restriction enzyme choice), limited levels of polymorphisms in some species, and the requirement for good quality and high amounts of DNA as compared to other markers. This is very species-dependent as some species are highly polymorphic while others are not depending on the size of the genetic bases in these species.

For screening large breeding populations with AFLP markers at low cost, there needs to be a conversion of AFLP markers into sequence specific PCR markers (eg. SCAR markers) (Dussle *et al.,* 2002). The AFLP technique was chosen for this study as it is reliable and is highly reproducible across laboratories. It also detects a higher number of polymorphisms in one reaction compared to the other DNA based techniques. No prior knowledge of sequence of the organism needs to be known before the technique is attempted. The technique was chosen due to the availability of the equipment and technology, cost benefits and familiarity of the technique.

Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are defined as single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater (Brookes, 1999). They occur naturally on the genome or can arise due to induced mutation techniques. These markers are stable, abundant in the genome, can have gel and non-gel based assays and have a higher inheritance than other markers.

There are two types of SNPs: substitution of one nucleotide for another (transitions/transversions) or an insertion/deletion (indel) of a nucleotide. A transition is a change of pyrimidine to pyrimidine (C/T) or purine to purine (G/A) bases, while transversions can be pyrimidine to purine (C/G, A/T, C/A or T/G) base change (Batley *et al*., 2003). It can either directly contribute to a phenotype or due to linkage disequilibrium can be associated

with a phenotype. The natural SNPs can be used as simple genetic markers due to their polymorphisms and they can occur as flanking markers or within the gene of interest depending on their position on the genome. Due to the high level of polymorphism found in many plant species, it is conservatively estimated in maize there are 20 million polymorphisms for analysis (Rafalski, 2002a) therefore this marker technology holds great promise for MAS and plant breeding.

SNPs markers are biallelic and occur throughout the genome in all coding and non coding regions, and are at a higher density in the genome than microsatellites (Batley *et al.,* 2003) and are present in most animal and plant marker systems (Gupta *et al*., 2001). They are direct markers as their sequence information shows exactly the allelic nature of the individual due to a high level of intraspecific nucleotide diversity. Ideally, it would be optimal to have several (two to four) SNPs located close to the gene/trait of interest to completely define haplotypes. However if the SNP is targeting the exact nucleotide change that causes the trait, it is a direct marker. SNPs have been used extensively both in animal genetics (Vignal *et al*., 2002) and in human genetics (Picoult-Newberg *et al*., 1999).

SNP discovery can be relatively easy due to the availability of many genes and expressed sequence tag (EST) sequences. In humans, the EST database was used to discover SNPs that are biologically useful and that can be used for the diagnosis of diseases (Picoult-Newberg *et al*., 1999). Previously, SNPs were detected using RFLPs or creating restriction sites through PCR primer design, oligonucleotide probing or direct sequencing (Schork *et al.,* 2000). The assays for SNPs that do not require DNA separation by size can be easily automated and are easier to locate in single copy regions of the genome (Rafalski, 2002a). Due to its binary nature and stability, SNP markers are amendable to automated high-throughput genotyping and are therefore an attractive tool for MAS in plant breeding programmes. SNP genotypes are determined by the basic method of Sanger dideoxysequencing and this can miss SNPs when the DNA template is heterozygous, therefore time-consuming and expensive (Mohler and Schwarz, 2005). Newer technologies with high throughput-methods lead to lower cost and higher volume, repeatability and accuracy, like high resolution melt analysis (HRM), micro-arrays and high density SNP genotyping, leading to future increased use and applicability of SNPs as molecular markers.

A study to compare SNPs to SSRs using 58 inbred lines and four hybrids of maize resulted in SNPs showing lower level of missing data, higher repeatability and an estimated lower cost of <$0.25/sample data point (five to ten times lower than SSRs) than SSRs (Jones *et al.,* 2007). However in a larger study by Hamblin *et al*. (2007) to determine diversity and relatedness of maize showed SSRs having better clustering and more resolution in measuring genetic distance than SNPs. This could be attributed to SNPs being biallelic and

having a maximum heterozygosity of 0.5 while SSRs has multiple alleles and their maximum heterozygosity can approach 1.0. SSRs are better utilised for applications such as germplasm identification where the multiallellic nature is an advantage (Rafalski, 2002b). A study on barley comparing AFLPs, SSRs and SNPs that found SNP markers are best suited for the characterisation and conservation of genetic material while the AFLP and SSR markers are better for diversity analysis and fingerprinting (Varshey *et al*., 2007).

SNPs are widely used in genetic epidemiology studies in humans to study complex traits and diseases (Schork *et al*., 2000) due to over one million SNPs being catalogued (Rafalski, 2002a) In plants, there has been a few extensive studies with their uses being genome mapping, high resolution genetic mapping of traits and association studies. A study by Barker and Edwards (2009) on the comparison of SNPs over the major cereal crops of the world included wheat, barley, maize, sorghum and rice found as the ploidy of the cereal became more complex, the number of SNPs per kb increased, which was shown by wheat showing the highest number of 16.5 SNPs/kb (Barker and Edwards, 2009). There are reports on SNP markers associated with economic value genes in rice (waxy gene controlling amylose content, dwarfing gene), onion (male sterility), soybean (cyst nematode resistance), and SNPs for cultivar identification and diversity evaluation in barley, tree species, wheat and maize (Gupta *et al.,* 2001)

Multiple nucleotide changes and various lengths of insertions/deletions have been identified in maize due to the high frequency of polymorphism which can be used potentially as genetic markers. There are reports of 8.9 SNPs/kb which covered 41.3% of the genome (Barker and Edwards, 2009), 1 SNP per 104 bp on chromosome 1 between two randomly sampled sequences (Tenaillon *et al.*, 2001) and 1 SNP per 124 bp (coding regions), 1 SNP per 31 bp (non coding regions) in 36 inbred lines using 18 maize genes (Ching *et al*., 2002) and 1 SNP per 70 bp and 1 indel per 160 bp (Gupta *et al.,* 2001). Single nucleotide changes occur on average every 60.8 bp and most of these changes that occur in the protein coding regions are silent, with only five of eighteen changes resulting in an amino acid substitution (Ching *et al.,* 2002). Batley *et al.* (2003) developed a computer-based method using over 102 551 maize ESTs to identify 14 832 candidate SNP polymorphisms of which 13 122 were nucleotide substitutions and 264 SNPs from 27 loci were validated. There are a number of public databases for SNPs available, Panzea is selectively for maize SNPs (Zhao *et al.,* 2006) with a geographical map tool to visualise distribution of SNPs, SSRs and isozyme alleles as well as a graphical view of the placement of Panzea markers and genes/loci on genetic and physical maps (Canaran *et al*., 2008). There is also AutoSNPdb database available for crops such as rice, barley and Brassica (Duran *et al*., 2008).

Although indirect SNP assays are used widely in plant breeding, direct SNP assays are rare. The direct SNP assay is useful when the trait is a result of either a transition or transversion change. In these cases the direct SNP is the ideal marker as it targets the exact position of the polymorphism causing the change of the trait. In a study on mining for maize SNPs using EST sequences it was found there were 6 640/13122 (51%) transitions of which 3277/6640 (49%) were C/T transitions (Batley *et al*., 2003) showing the relative high frequency of C/T transition changes. It was reported that there is a relative increase in the proportion of transitions over transversions (Batley *et al.,* 2003) with a ten fold increase in frequency of C to T mutation than average transitions found after methylation (Coulondre *et al*., 1978).

The *lpa1-1* gene is due to a single recessive mutation (Raboy *et al.,* 2000) with an alanine to valine amino acid change identified to be probably responsible the LPA phenotype (Shi *et al*., 2007). This amino acid change results in a C/T nucleotide base change, therefore making a SNP marker is an ideal marker for detecting this type of mutation. There have been direct SNP markers (C/T transition type) used in rice studies to identify the fragrance gene (*fgr*) (Jin *et al*., 2003), semi-dwarfing gene (*sd-1*) (Sasaki *et al*., 2002) and blast resistance gene (Bryan *et al.*, 2000).

High resolution melt (HRM) analysis

High resolution melt analysis (HRM) provides an innovative approach for the simultaneous detection and differentiation of PCR products by melt curve analysis after PCR amplification. This technique has been used for SNP and SSR markers with promising results. SNPs are most easily detected since the result is limited to only one or two bases at a given position. These markers can usually be identified by hybridization to probes or direct DNA sequencing. However sequencing requires multiple steps after PCR, i.e. cycle sequencing and gel electrophoresis. However to simplify the process of SNP detection, an automated method is required that would not require PCR product separation on gels or columns.

HRM analysis can be performed immediately following PCR amplification so that PCR product analysis can be performed in one closed tube reaction depending on the instrument used. The use of intercalating fluorescence dyes or fluorescence-labelled primers is necessary with no added purification steps after PCR. This advanced technology has been widely used in human genetics and is gaining more uses in animal and plant genetic studies especially in cases of SNP studies. It eliminates the post-PCR analyses such as gel-based or sequencing analyses, thereby increasing the efficiency of marker system as well as reducing costs, time and labour. PCR products are differentiated from each other by melting

curve profiles. The factors determining the melting curve profile include mostly the GC content, and the length and sequence of the product (Ririe *et al*., 1997). A major advantage of HRM over gel electrophoresis is the differentiation of PCR products of the same length but differ in GC/AT ratio and or GC content having different melting curves. The position and width of the melting curves are affected by dye concentration and temperature transition rates (Ririe *et al*., 1997).

Fluorescence emittance of the dsDNA dyes is measured during an increase in temperature at specific intervals and product denaturation is observed as a rapid loss of fluorescence near the denaturation temperature. The fluorescence data is converted into melting curves by plotting the derivative of fluorescence with respect to temperature (dF/dt) over the temperature range in degrees Celsius. PCR products melt at varied temperatures with characteristic melt curves thus allowing product differentiation. This technology is able to differentiate between alleles of homozygote recessive, homozygote dominant and heterozygotes. Homozygous genotypes can be distinguished by shift in melting curves due to the difference in melting temperature (T_m) between the genotypes, while heterozygotes are distinguished by the altered curve shape not by T_m (Park *et al.*, 2009; Graham *et al*., 2005). Fluorescent-labelled primers were also able to distinguish between homozygotes and heterozygotes during HRM analysis (Gundry *et al*., 2003).

HRM has been used successfully to identifying SNPs in various crops including potato (Yuan *et al*., 2008), almonds (Wu *et al*., 2008; 2009), apple (Chagne *et al*., 2008), barley (Lehmensiek *et al*., 2008), lupin (Croxford *et al*., 2008; Lopez *et al*., 2008), grapevine and olives (Mackay *et al*., 2008), tomato (van Deynze *et al*., 2007), capsicum/pepper (Park *et al.*, 2009) and for the detection and quantification of mitochondrial RNA editing in *Arabidopsis* (Chateigner-Boutin and Small, 2007; Takenaka and Brennicke, 2009).

A comparison study on nine different instruments and two dyes found the greatest variance with plate HRM instruments and both dyes, LCGreen and SYBR Green were effective for genotyping (Hermann *et al*., 2006). The Corbett Rotorgene is specifically designed for HRM analysis and shows the least variation (Park *et al*., 2009). A high throughput study with SNPs in almond analysed 500 samples within eight hours including data analysis instead of three days with gel-based markers, thereby proving HRM analysis to be an efficient and cost-effective approach for SNP detection and analysis (Wu *et al*., 2009). Gel electrophoresis for SSRs markers can be problematic in the interpretation of stutter bands often produced by SSRs as well as being time consuming. Studies using HRM analysis with SSRs in grapevine and olive cultivars showed clear differentiation melting profiles with high reproducibility leading to reduced time to results for varietal certification (Mackay *et al.*,

2008) as well as in *Origanum* (Mader *et al*., 2008) confirming HRM is faster, more cost-effective and sensitive than standard protocols for SSRs analysis.

Marker-assisted selection

Marker–assisted selection (MAS) has been a plant-breeding tool since it was proposed by Sax in 1923 (Arus and Moreno-Gonalez, 1993). Marker-assisted selection (MAS) is the use of molecular markers in the selection process in plant breeding programmes and has been shown to be cost effective (Abalo *et al.,* 2009). These markers need to be closely linked to the target gene/locus and should be polymorphic for the breeding material used in the programme for them to be effective and reliable. Markers can be used for the introgression of recessive traits and for the selection of lines resembling the recurrent parent genome.

Successful plant breeding requires selecting many traits with complex inheritance. Desirable quantitative traits usually have both genetic and environmental components (Dudley, 1993), and separation of these components to achieve maximum efficiency in breeding programmes is necessary (Gebhardt and Salamini, 1992). Breeders originally depended on markers that had a morphological effect on the plant because these were the only markers available. However, most morphological marker types do not fit the description of a “good” marker because they have either dominance effects, late expression, exist in epistatic relationships, or have deleterious effects on the plant (Tanksley, 1983). The most recent markers are the SNPs, which appear useful for mapping, manipulation, and study of diseases in humans (Landegren *et al*., 1998), and will likely have comparable application in plant genomics (Cregan *et al*., 1998).

The development of molecular markers has great potential for increasing breeding efficiency due to many of the marker systems having large numbers of polymorphisms. Alternate alleles rarely have deleterious effects at the molecular or whole plant level and they are often co-dominant, allowing all genotypes to be distinguished in each generation. They rarely segregate in epistatic ratios. Scoring of molecular markers does not depend on gene expression and is not affected by the environment. The use of markers also reduces time and space necessary to evaluate plant populations. Molecular markers allow more efficient selection and offer a mechanism to eliminate undesirable traits associated with hybridizing diverse genotypes. A linkage map with many markers, especially when the genome is saturated with markers, can be used to locate genes of interest (Stalker and Mozingo, 2001).

Quantitative and recessive traits appear to have the most potential uses for MAS, because environmental effects confound selection for them. Since the cost to evaluate these complex

traits with low to moderate heritability is high, MAS would be more likely to show the greatest gain. Due to complex inheritance, low heritability, and confounding environmental effects, markers associated with quantative trait loci (QTL) have been difficult to find and once found have exhibited limited usefulness across a range of genetic backgrounds or environments. Efforts to utilize MAS for the improvement of quantitative traits have been limited, but may improve as better quantitative data is generated and denser linkage maps become available from map co-integration across laboratories (Freyre *et al*., 1998).

A review of MAS breeding on maize in Africa by Stevens (2008) found many research studies identifying QTLs for drought resistance, resistance to biotic stresses (sugar-cane mosaic virus, maize streak virus, grey leaf spot, stem borers, *Striga*) and micronutrient enhancement breeding (Vitamin A, iron and zinc). However the failure to use these markers is due to the variable effectiveness of the markers in predicting the desired phenotype, the low accuracy of QTL studies, and a lack of transferability across diverse germplasm and insufficient validation of markers. There is therefore a need to develop molecular markers that can overcome these limitations and be used to their full potential in breeding programmes.

For a practical plant breeding programme, a marker should have the following properties: co-dominance, reliability, quick, easy to use and cost-effective compared to traditional screening methods for the trait of interest.

The use of markers in backcross breeding programmes

Marker-assisted backcrossing (MABC) is an established tool in plant breeding. The main aim in MABC is the introgression of a trait of interest into the genetic background of a recipient genotype by recurrent backcrossing. The other aim is to recover the recurrent parent genome as rapidly and completely as possible. The use of markers to monitor the parental origin of alleles throughout the genome in MABC with restriction fragment length polymorphism (RFLP) markers was originally proposed by Tanksley *et al.* (1989) and was later called background selection (Hospital and Charcosset, 1997).

There are two goals of background selection: to reduce the proportion of the donor genome on the carrier chromosome of the target allele and to reduce the donor genome on the non-carrier chromosomes. Linkage drag is reduced by the selection of individuals that carry the target allele and are homozygous for the recurrent parent alleles at tightly linked marker loci (Frisch *et al.,* 1999). The use of background selection for a single gene has been widely investigated (Hospital *et al.,* 1992; Abalo *et al.,* 2009) and has now been used in maize for

two genes simultaneously (Frisch and Melchinger, 2001) and QTLs (Hospital and Charcosset, 1997; Bouchez *et al.,* 2002). Marker-assays can be advantageous in backcross breeding programme for both background and foreground selection (Hospital and Charcosset, 1997). Background selection can reduce the number of generations required for gene introgression from six to three (Frisch *et al.,* 1999). In recurrent backcrossing for recessive genes, if markers are not used, there is a need for progeny tests in each backcross generation to determine heterozygosity. The use of markers eliminates the need for progeny tests at each generation and needs to be only performed at the end of the backcross programme (Frisch, 2005).

The main objective of a backcross breeding programme is the reduction of the donor parent genome by 50% at each generation of backcrossing while retaining the trait of interest from the donor parent. There is, however, variation present in each backcross generation around the mean. Markers can be used to select for the donor trait as well as recombinant individuals that have genome composition closer (75%) to the recurrent parent. MAS is used to reduce the time and number of backcross generations required to achieve this goal. A major constraint has been the number of polymorphic markers required to cover the entire genome to effectively determine the amount of recurrent parent genome present.

The advantages of DNA markers in backcross (BC) breeding include the indirect selection of desirable gene(s) from donor parents; selection for regions of recurrent parent genome unlinked to the introgressed region and the reduction of linkage drag of unwanted donor parent genome near the introgressed region(s). These advantages provide a means of reducing the number of generations required for the recovery of the converted recurrent parent, which is confirmed by simulation studies and empirical reports (Lee, 1995).

The utility of MAS for achieving and improving genetic gain through BC breeding depends on the current and potential role of the breeding method. MAS is expected to result in greater rates of genetic gain during early generations of selection, with gain at later generations by traditional selection expected to surpass or approach that of MAS. BC breeding has been widely used for the introduction of monogenic characters and to a lesser extent for polygenic traits. According to Lee (1995) the efficiency of MAS for polygenic traits can be enhanced and more efficient than traditional selection under the following conditions: (1) the trait(s) under selection has low heritability; (2) tight linkage between the QTL and markers (<5 cM); (3) in earlier generations of selection prior to fixation of alleles at or near marker loci and recombinational erosion of marker-QTL associations; and (4) larger sample sizes for mapping and selection of QTL are used to improve estimates of QTL effects and to avoid rapid fixation of alleles, respectively.

Studies on the backcross introgression of traits have shown that markers can be used efficiently where the aim is to introgress a small part of the donor genome and simultaneously recover as much of the recurrent parent genome as quickly as possible (Hospital *et al.*, 1992; Visscher, 1996). It is recommended to use two to eleven markers per chromosome (Visscher, 1996) to select against the donor genome and recover the recurrent parent genome by two generations less than random selection of individuals with the introgressed gene (Hospital *et al.*, 1992). Frisch *et al.* (1999) recommended a four-stage selection approach with the first generations selection on the introgressed trait while Hospital *et al.* (1992) showed a single generation background selection is most efficient in the last backcross generation.

Moreau *et al.* (2004) compared the efficiency of MAS and conventional phenotypic selection for index selection combining grain yield and grain moisture at harvest on maize. It was found that genetic gain was significant for both methods; however the difference between phenotypic selection and combined MAS was not significant especially considering the low heritability of the trait. It was found that the method of marker only selection was efficient in fixing favourable QTL alleles in the initial population.

A study carried out on the cost effectiveness of conventional and MAS methods for the identification of plants with a mutant recessive form of the *opaque2* gene in maize in CIMMYT (Dreher *et al.*, 2003) produced four important insights that need to be considered when screening methods are to be selected. These are: (1) for any breeding project, a detailed budget analysis is needed to determine the cost effectiveness of MAS relative to conventional selection, (2) direct comparisons of unit costs are required but factors other than costs also are important in the choice of screening methods, (3) the choice between the methods is complicated as they are not always direct substitutes, and (4) spreadsheet-based budgeting tools with empirical data from actual breeding programmes can be used to increase efficiency of existing protocols and make informative decisions about future technology choices. Morris *et al.* (2003) compared MAS to conventional selection in a backcross programme showed conventional breeding schemes are less expensive and MAS-based breeding schemes can be completed in less time.

The choice to apply MAS in a breeding programme is dependent on a variety of factors. A detailed analysis of the field and laboratory costs would be required. The cost effectiveness of DNA markers depends on four parameters that can vary significantly: (1) the relative cost of phenotypic versus genotypic screening, (2) the time saving achieved using MAS, (3) the size and temporal distribution of benefits associated with the accelerated release of improved germplasm, and (4) the availability of operating capital to the breeding programme (Hoisington and Melchinger, 2005).

For traits that have recessive genes and/or have difficult or expensive methods of assessment of the trait, MAS can be especially useful in a breeding programme. In the case of low phytic acid gene (*lpa1-1*), it has both; a single recessive gene and a destructive colorimetric assay using mature seed. This method is not only time consuming and low repeatability but is not useful in breeding programmes to screening segregating material as it destroys the seed after the assay. A molecular marker that is able to identify the low phytic acid trait (*lpa1-1*) in early vegetative stages would be highly useful as it would eliminate the use of the colorimetric assay as well as increasing the efficiency and decrease time required for screening of the *lpa1-1* gene. The marker can be tested on DNA isolated from leaf material making detection of the trait quick and easy.

Seed germination and vigour

Selections in maize breeding programmes to improve crop productivity and nutritional quality are usually based on yield and disease resistance selections. There is little selection on the seed quality characteristics which is critical in early performance and growth of these crops. Seed quality is usually associated with germination and purity but seed vigour also needs to be included. The ISTA (International Seed Testing Association) defines seed vigour as “an index of the extent of the physiological deterioration and/or mechanical integrity of a high germinating seed lot which governs its ability to perform in a wide range of environments”. Vigour is a concept that describes several characteristics of the seed such as rate and uniformity of germination and growth, tolerance to environmental stresses after sowing and retention of performance after storage (Hrstková *et al.*, 2006).

The use of LPA mutants in plant breeding has been limited due to non-germination of genotypes with homozygous *lpa1* alleles (Raboy, 2000), reduced seed weight of *lpa1-1* (Raboy *et al.,* 2000), and the lower vegetative growth rate and impaired seed development due to the *lpa241* mutation (Pilu *et al.,* 2005). These LPA mutants have inferior agronomic and seed viability than their wild-type (WT) parents, leading to yield reduction (Raboy *et al.,* 2000) due to reduced seed weights and low vegetative growth.

There are no previous germination and vigour studies on the *lpa1-1* mutant lines, however there are two studies on the *lpa241* mutant which is allelic to *lpa1-1* (Pilu *et al*., 2005) and shows 90% reduction in seed phytic acid (Pilu *et al*., 2003). In the study with LPA mutant (*lpa241*) a 30% decrease in germination rate was observed when compared to the wild type (Pilu *et al*., 2003). Another germination study of the same LPA mutant line (*lpa241*) was tested and shown to have 72±15% germination under standard conditions which decreased

to 45±14% germination under accelerated aging conditions (Doria *et al.,* 2009). A negative correlation between yield and phytate was found when comparing 50 different maize lines with a suggestion that selection of larger kernel size should have a diluted concentration of phytate since 90% of phytate is found within the germ (Lorenz *et al*., 2007). The *lpa241* mutant has negative pleiotropic effects which are related to embryo development and size, germination rate, seedling growth rate and ear size (Pilu *et al.,* 2005). A study on rice found all rice LPA mutants showed reduced seed viability (Zhao *et al.,* 2008).

The LPA mutant lines also show significantly lower field emergence than their WT parents in rice (Zhao *et al.,* 2008), wheat (Oltmans *et al.,* 2005) and soybean (Meis *et al.,* 2003). This could be as a result of seed maturation in tropical environments with high temperatures at which the LPA genotypes have low stress tolerance, which results in reduction of germination and emergence (Raboy, 2007). Low field emergence is an important issue for LPA once lines have been developed and need to be adopted for commercial production as field emergence and not germination is important for seedling establishment.

Germination tests

Seed tests can be used to evaluate physical quality, genetic purity, viability and vigour. The most common seed test is the germination test which measures seed viability under ideal conditions. The germination tests are used to determine the maximum germination potential of seed for comparison to other seed lots and also to estimate the field planting value. The results are reported as percentage of germination of normal seedlings. Depending on the crop, different substrates are used for germination (ISTA, 1999).

The emphasis on seed germination as part of seedling morphology has little relationship with rapidity of growth which is a prime criterion of the potential for successful stand establishment. The germination percentage is the sum of strong and weak seedlings and the disadvantage of inclusion of the weak seedlings is that they seldom perform adequately under environmental stresses associated with field emergence. Germination is considered to be scale-less as the seed is either germinal or it is not, with no distinction between weak and strong seedlings. The seedlings that are considered to be germinal can show a wide range of variation in field performance, from weak to robust.

The germination test is well standardised and uses favourable conditions to ensure uniformity in test results. It establishes the maximum plant-producing ability of the seed lot. Under optimum conditions, it is able to accurately predict field performance of the seed lot and tends to overestimate field emergence values in suboptimal conditions. The germination test does not detect many seed weaknesses, thereby increasing the need for vigour testing

to act as quality control tool and marketing aid. Seed vigour testing has been developed to overcome these discrepancies.

Vigour tests

Seed viability and vigour are two characteristics of seeds that determine their value and utility of seeds. There are many different factors that determine and influence seedling germination and vigour. An important factor of seed quality is seed genetics and this plays a critical role in germination and seedling vigour. To attain good stand of a crop, it is necessary to have a high emergence percentage followed by high seedling vigour (Fakorede and Ojo, 1981). Maize has problems in maintaining high germination standards for certified seeds (80% parental lines, 90% hybrids), especially for low vigour parental lines (Basu *et al.,* 2004).

The vigour test is a more sensitive index of seed quality than the germination test as it is closely correlated with seed performance in the field under some conditions than the germination test. Vigour testing has become common practice especially for maize and soybeans to routinely market high quality seed and ensure the seed will perform well across a wide range of soil types and environmental conditions. There are various essential characteristics of a vigour test that make it useful, such as being inexpensively priced and requiring minimum investment in labour, equipment and supplies. Also required are a rapid testing period to minimize analyst time and germinator space with simple testing procedures without special training or experienced personnel. The test is objective with a quantitative or numerical index of quality to avoid subjective interpretations by analysts with high correlations of test results with field performance and test results should be reproducible between laboratories (McDonald, 1980).

Different vigour tests measure different aspects of seed quality under different soil conditions; therefore a combination of several vigour tests is able to provide information on the quality of a seed lot as well as its potential field performance (Byrum and Copeland, 1995). Seed storage under high relative humidity and/or high temperature and damage by insects are primary causes of poor seed quality. The two most commonly used vigour tests are the cold test and the accelerated aging test. The accelerated aging test is a vigour test that involves artificial aging of the seed, which causes integral membrane lipid peroxidation in the seeds (Basavarajappa *et al.,* 1991). In maize, accelerated aging causes associations between the starch granules, protein matrix and cell walls, leading to decreased solubility and functionality of starch and protein in aged grain due to protein oxidation (McDonough *et al.,* 2004). The accelerated aging test was developed by Delouche and Baskin (1973) to

assess the quality and storability of seed lots. These tests are also effective in evaluating seed vigour and germinative responses to accelerated aging are highly correlated with plant growth and development including yield (Delouche and Baskin, 1973).

The accelerated aging test was developed to predict seed storability (Delouche and Baskin, 1973). The test has been used extensively in maize to compare different types of vigour tests for prediction of field performance (Lovato *et al.,* 2005; Noli *et al.,* 2008), to determine relationship between total phenolics content and germination ability (Barla-Szabo *et al.,* 1990; Sredojević *et al.,* 2004), to improve vigour by using aerosol-smoke (Sparg *et al.,* 2006), to assess seed quality (Santipracha *et al.,* 1997; Munamava *et al.,* 2004); seedling vigour (Fakorede and Ojo, 1981) and to assess seed storability (Basu *et al.,* 2004). The vigour and viability of maize inbred lines is reduced by aging due to non-inheritable degenerative changes (Revilla *et al.,* 2009). In warmer tropical climates, the accelerated aging test has been used as an indicator of seed vigour. After the aging process, high vigour seeds are expected to show high rates of germination while low vigour seeds would show significant decrease in germination rate.

Studies on diallel crosses in maize, canola and rice have found seed germination and vigour traits to be under both additive and non-additive gene action with predominant non-additive gene effects (Barla-Szabo *et al.,* 1990; Akram *et al.*, 2007; Chapi *et al.,* 2008). There has been predominantly non-additive gene action found for germination percentage, seedling dry weight, average root length, average shoot length and vigour index (Barla-Szabo *et al.,* 1990; Akram *et al.*, 2007; Chapi *et al.,* 2008) while other studies on soybean and sugar beet have found mainly additive gene action (Cho and Scott, 2000; Sadeghian and Khodaii, 1998) for these traits.

The general combining ability (GCA) effects of germination percentage, fresh weight and dry weight of seedlings were used to select lines as better females and males in the seed vigour study on maize (Barla-Szabo *et al.,* 1990). GCA and specific combining ability (SCA) effects were used to select the best parent and cross for improving seedling traits in canola (Chapi *et al.,* 2008). Combining ability analysis was used to select the best parents and crosses for improving seed vigour, variability and field emergence potential for sorghum (Kannababu *et al.,* 2005). Sadeghian and Khodaii. (1998) suggested that seed traits are as important as root traits for development of varieties. In soybean, due to significant GCA effects for seed vigour, it was concluded that levels of seed vigour can be improved through breeding (Cho and Scott, 2000).

Summary

Phytic acid is an anti-nutritional compound found in mature maize grain. It chelates important minerals as it passes through the digestive system of monogastric consumers. There have been maize lines produced with lower levels of phytic acid, however these lines exhibit lower seed germination and vigour and yield. There is considerable evidence that diets with lower phytic acid grain show improved absorption of the essential minerals. However there is limited information of the seed germination, seedling vigour and yield performance of LPA lines compared to other maize lines. There is therefore a need to improve the seed germination and vigour as well as the yield of these LPA lines by using appropriate breeding strategies.

QPM maize lines were included in this study as there are calls to breed the LPA trait into QPM maize to improve the nutrient content of maize in a single variety. Due to low germination, low seedling vigour, and reduced grain yield shown by LPA and QPM lines, there are concerns that stacking the QPM and LPA traits in a single variety would lead to serious agronomic problems. The knowledge of the gene action controlling these traits is an important part of the selection of an appropriate breeding strategy. Also the knowledge of the combining ability of LPA, QPM and normal endosperm maize lines will aid in the selection of suitable lines and possible good crosses for improvement of these traits.

The level of phytic acid in grain is usually detected by a destructive colorimetric assay. There is a need for an improved assay for detecting phytic acid that is quicker and more reliable than the colorimetric assay. Molecular markers are non-destructive and can be used on leaf material to detect traits of interest. They are quicker and more reliable than the conventional assay for phytic assay. In backcross breeding programmes, markers can be used to track traits of interest as well as to recover the recurrent parent genome present in BC lines.

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Chapter Two

Development of a SNP marker for detection of the low phytic acid (*lpa1-1*) gene for use during maize breeding

Abstract

Reduced phytic acid in maize grain can increase the bioavailability of micronutrients to monogastric consumers. Breeding for low phytic acid (LPA) is hampered by a tedious and destructive colorimetric assay for phytic acid content in the mature dry grain. The aim of this study was to develop a molecular marker able to identify the low phytic acid (*lpa1-1*) trait that can be used at the early vegetative stage of the plant for evaluation of the phytic acid level present during breeding. The *lpa1-1* allele is known to result from a single amino acid change Ala to Val which we deduced must result from a C:T transition at position 1432 of the wild type sequence. PCR primers were designed to amplify across this region and the nature of the SNP was confirmed as a transition base change of C to T nucleotide causing the amino acid change. The parental inbred lines (one normal/wild type and one LPA) and the F_1 generation (heterozygote) were used to test and optimise the SNP marker. They all produced a PCR product of 150 bp which was then subjected to high resolution melt (HRM) analysis which was able to differentiate between the parental lines due to their definitive melting profiles. The melting profiles of the parental lines differed by 0.5°C in the high resolution melt analysis. The *lpa1-1* SNP marker was able to successfully differentiate between homozygous dominant (normal/wild type), homozygous recessive (LPA) genotypes by their melting profiles and heterozygote genotypes by normalised difference plots. To test the SNP marker-assay for high-throughput suitability, the costs of HRM analysis were compared to DNA sequencing and DNA extraction method. Similar melt profiles of DNA extracted with a DNA extraction kit (high quality) and DNA extracted with a crude extraction method were obtained, which showed that the low cost crude extraction was as efficient as the high quality extraction in distinguishing between the parental lines. The costs of DNA sequencing was compared to the costs of SNP marker amplification and HRM analysis. The SNP marker with HRM analysis was 8% of the cost of PCR amplification and DNA sequencing. The development of the SNP marker will avoid the use of the destructive colorimetric method of assessment for phytic acid content in maize grain. This will make breeding for low phytic acid content in maize efficient and fast.

Keywords: low phytic acid, SNP marker, HRM analysis

Introduction

Phytic acid is the amount and form of the major phosphorus (P) containing compound found in seeds. It is an anti-nutritional component found in many cereals and legumes. It is also a naturally occurring component of plant fibre. In maize, 90% of total seed P is in the form of phytic acid (O'Dell *et al.,* 1972). Phytic acid chelates essential minerals such as iron, zinc, potassium, magnesium and calcium as it passes through the digestive system of humans and other monogastric animals. This leads to increasing iron and zinc deficiencies in communities that subsist on maize as their daily food requirements. Iron deficiency is considered to be the most common and widespread nutritional disorder in the world and the condition is exacerbated by infectious diseases in developing countries where it affects mainly women and children (FAO, 2009). Zinc deficiency is estimated to affect billions of people. According to WHO statistics, zinc deficiency is rated the fifth most important health risk factor in developing countries and eleventh worldwide, with 60% of the population in developing countries affected (WHO, 2009).

Maize inbred lines containing lower levels of phytic acid have been produced using ethylmethane sulphonate-induced (EMS) mutagenesis (Raboy, 2000). These maize LPA mutants are distinguished according to differences in inositol and inositol phosphate content. The maize *lpa1-1* mutants show a reduction in phytic acid accompanied by inorganic phosphate, *lpa2* mutants show significant increases in inositol phosphate intermediates and *lpa3* mutants accumulate inorganic phosphate and myo-inositol but not inositol phosphate intermediates. The genes from *lpa1-1*, *lpa2* and *lpa3* have all been cloned (Shi *et al.,* 2003, 2005, 2007). The *lpa1-1* gene encodes a multidrug resistance associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi *et al.,* 2007) while *lpa2* gene encodes an inositol phosphate kinase (IPK) belonging to the Ins (1, 3, 4) P3 5/6 kinase gene family (Shi *et al.,* 2003) and *lpa3* gene encodes a myo-inositol kinase (MIK) gene (Shi *et al.,* 2005).

There have been studies comparing the effects of a LPA diet on humans (Agte *et al.*, 1999; Mendoza *et al.*, 1998, 2001; Adams *et al.*, 2002; Hurrell *et al.,* 2002, 2003 Ekholm *et al.,* 2003; Bohn *et al.,* 2004; Davidsson *et al.*, 2004; Hambigde *et al.,* 2004, 2005). There is a considerable amount of evidence to support the fact the dietary phytate has a negative effect on the bioavailability of dietary minerals in humans as the substitution of LPA grain in a maize based diet is associated with a substantial increase in zinc (Adams *et al.,* 2002; Hambidge *et al.,* 2004), iron (Mendoza *et al.,* 1998, 2001; Hurrell *et al.,* 2003), calcium (Hambidge *et al.,* 2005) and magnesium (Bohn *et al.,* 2004) absorption.

Phytic acid has very little bioavailability for monogastric animals due to their lack of phytase activity. A possible solution to the high PA content in cereals is the isolation of mutants accumulating less phytic P and more free phosphate in the seed. A result of high phosphorus excretion by monogastric animals is the environmental pollution of water and soils. Studies have been conducted to determine the effect and availability of phytic acid to pigs (Spencer *et al.,* 2000a,b; Veum *et al.*, 2001), chicks (Douglas *et al.,* 2000; Li *et al.,* 2000; Yan *et al.,* 2000; Peter and Baker, 2002), turkeys (Yan *et al.,* 2003) and rabbits (Marounek *et al.*, 2003). In particular, there were studies that used maize containing the *lpa1-1* allele (Spencer *et al.,* 2000a, b; Li *et al.,* 2000). In animal feeding studies using low phytate maize, there was an increase of 2-5 times the amount of bioavailable phosphorus observed (Douglas *et al.,* 2000; Li *et al.,* 2000; Spencer *et al.,* 2000a, b; Yan *et al.,* 2000, 2003; Veum *et al.,* 2001; Peter and Baker, 2002). It was found in rabbits that although inorganic phosphates hinder phytic acid hydrolysis, generally they were able to digest phytic acid fairly efficiently. Also, the addition of exogenous phytase to rabbit feeds could increase phytic acid hydrolysis in the upper part of the digestive tract and eliminate the need for inorganic phosphate supplements (Marounek *et al.*, 2003).

The *lpa1-1* mutants have 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g Pi (66% of total P) compared to the normal maize of 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g Pi (7% of total P) (Raboy *et al.,* 2000). The *lpa1-1* mutation shows 66% reduction in phytic acid P (Raboy, 2000). The *lpa1-1* gene has been mapped onto the chromosome 1 distal region and reported to be due to a single recessive mutation (Raboy *et al*., 2000). The gene was completely sequenced (5149 bp) and the *lpa1-1* phenotype reported to be probably due to a change of amino acids alanine to valine (Shi *et al.,* 2007). There are numerous examples of transitions mutations and SNPs to identify them. Some examples in rice include the fragrance gene (*fgr*) (Jin *et al*., 2003), semi-dwarfing gene (*sd-1*) (Sasaki *et al*., 2002) and blast resistance (Bryan *et al.*, 2000). There have been other LPA mutant lines showing similar amino acid changes which result in lower phytic acid content (Kim *et al.,* 2008; Xu *et al*., 2009).

While molecular markers have been used to detect LPA mutants in rice (Zhao *et al.,* 2008), soybean (Saghai-Maroof *et al.,* 2009; Scaboo *et al.,* 2009) and barley (Larson *et al.,* 1998; Roslinsky *et al.,* 2007), there have been no SNP markers and or HRM analysis used for detection of LPA mutants in maize. In rice, the *lpa1-1* mutation is due to a single base pair substitution and a cleavage amplified polymorphic sequence marker (*LPA*1-CAPS) was developed using *Xsp* I as the restriction enzyme. The *lpa1-2* mutation of rice was detected using an insertion/deletion marker (*LPA*1-InDel) which detects a 1475 bp fragment deletion (Zhao *et al.*, 2008). In barley, there was a co-dominant sequence tagged site-polymerase

chain reaction (STS-PCR) marker (aMSU21) that co-segregated with *lpa1-1* with a 200 bp deletion (Larson *et al.,* 1998). However this marker was found to be non-polymorphic among 2-rowed breeding materials and a repulsion phase sequence-characterised amplified region (SCAR) marker was developed that was positioned ~16cM away from the *lpa1-1* gene (Roslinsky *et al.,* 2007). This study also found an inter-simple sequence repeat (ISSR) marker found approximately 14.7cM away from *lpa3-1* and requiring enzyme digestion with *Cla* I for detection of the gene (Roslinsky *et al.,* 2007). In soybean, a single nucleotide mutation from A to T, resulting in substitution of a stop codon for an Arg residue was detected in the MRP gene on the LPA mutant, which was detected by sequencing after PCR (Saghai-Maroof *et al.,* 2009). Micro-satellite or simple sequence repeat (SSR) markers have been found to be linked to QTLs for phytate concentration in soybean (Scaboo *et al.,* 2009). These markers have been detected after PCR by either agarose gel-electrophoresis or DNA sequencing.

Since phytic acid is mainly found in the germ of the mature seed (O'Dell *et al.,* 1972), the method of detection has been a colorimetric assay of the crushed seed which effectively destroys the seed (Chen *et al.,* 1956). This method is detrimental in breeding programmes especially in cases of segregating material when screening for the LPA trait as once the seed has been crushed for the assay; it cannot be used to generate a seedling. The colorimetric method is long, has low repeatability and requires 11-12 hours of human labour to analyse 100 samples of which the majority is preparation of samples into plates, as well as having an overnight incubation (Lorenz *et al*., 2007). There is therefore a need for a non-destructive method of detecting the *lpa1-1* trait, particularly one that is reliable, quick, less labour intensive and that can be amendable to high throughput methods. Molecular markers can be used for detection of traits that are difficult to assess/phenotype or for recessive genes. In the case of phytic acid, the trait is both difficult to phenotype as well as being a single recessive mutation; hence molecular markers can be applied to identify the *lpa1-1* trait with increased reliability and accuracy. Marker-assisted selection (MAS) methods particularly those based on PCR are advantageous due to their objectivity and small amounts of sample tissue and results in accurate analysis of large numbers of individual plants early in the breeding programme.

SNPs are third generation markers defined as single base pair positions changes in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater (Brookes, 1999). There are two types of SNPs; substitution of one nucleotide for another (transitions / transversions) or an insertion/deletion (indel) of a nucleotide. A transition is a change of C/T or G/A, while transversions can be C/G, A/T/ C/A or T/G (Batley *et al.,* 2003). There is a

relative increase in the proportion of transitions over transversions (Batley *et al.,* 2003). A ten-fold increase in frequency of C to T mutation than average transitions was found after methylation (Coulondre *et al*., 1978). These direct markers are very useful as their sequence information shows exactly the allelic nature of the individual due to a high level of intraspecific nucleotide diversity.

SNP markers can occur naturally or through induced mutation techniques and are stable with high frequency in the genome and a higher inheritance than most other markers. In maize, SNP polymorphisms has been conservatively estimated at 20 million polymorphisms (Rafalski, 2002), thereby showing the value of using SNPs in MAS and plant breeding. The maize SNP studies covered 41.3% of the genome and found SNP occurrence rate of 8.9 SNPs/kb (Barker and Edwards, 2009), 1 SNP per 104 bp on chromosome 1 (Tenaillon *et al.*, 2001) and 1 SNP per 124 bp (coding regions), 1 SNP per 31 bp (non-coding regions) (Ching *et al*., 2002) and 1 SNP per 70 bp and 1 indel per 160 bp (Gupta *et al.,* 2001). A study on 36 maize inbred lines focusing on 18 genes found single nucleotide changes occur on average every 60.8 bp and most of these changes that occur in the protein coding regions are silent, with only five of eighteen changes resulting in an amino acid substitution (Ching *et al.,* 2002). These markers can have either gel and non-gel based assays for detection.

A limitation of SNP marker application to high-throughput is the method of detection of the sequence variation which usually involves sequencing the product. The sequencing of PCR products is costly and time consuming as well as the DNA sequencer being an expensive piece of equipment not widely available to most plant breeding programmes. High resolution melt (HRM) analysis is a post-PCR technology that under correct conditions, distinguish PCR amplicons on the basis of their melt profiles in the presence of an appropriate dye or probe. Instruments are available that perform stand-alone HRM or combine PCR followed by HRM in a single closed-tube analysis. This offers a more suitable solution with the added benefit of the equipment being easily obtainable and space efficient. The HRM analysis technology has the advantages of low cost, high volume, repeatability and accuracy of markers.

During HRM, fluorescence emittance of the dsDNA dyes is measured during an increase in temperature at specific intervals and product denaturation is observed as a rapid loss of fluorescence near the denaturation temperature. The fluorescence data is converted into melting curves by plotting the derivative of fluorescence with respect to temperature (dF/dt) over the temperature range in degrees Celsius. This technology is able to differentiate between alleles of homozygote recessive, homozygote dominant and heterozygotes. A major advantage of HRM over gel electrophoresis is the differentiation of PCR products of the same length but having different sequence having different melting curves. It eliminates

the post-PCR analyses such as gel-based or sequencing analyses, thereby increasing efficiency of marker detection assay as well as reducing costs, time and labour. The factors determining the melting curve profiles include GC content, length and sequence of the product (Ririe *et al.,* 1997). The Rotor-Gene 6000 real time rotary analyser (Corbett Research, Australia) is able to perform the HRM analysis immediately after PCR amplification.

HRM technology has been widely used in human genetics and is gaining more uses in animal and plant genetic studies especially in cases of SNP studies. It has been used successfully in identifying SNPs in various crops including potato (Yuan *et al*., 2008), in almonds (Wu *et al*., 2008, Wu *et al*., 2009), apple (Chagne *et al*., 2008), barley (Lehmensiek *et al*., 2008), lupin (Croxford *et al*., 2008, Lopez *et al*., 2008), grapevine and olives (Mackay *et al*., 2008), tomato (van Deynze *et al*., 2007), Capsicum/pepper (Park *et al.*, 2009) and detection and quantification of mitochondrial RNA editing in *Arabidopsis* (Chateigner-Boutin and Small, 2007, Takenaka and Brennicke, 2009).

The objectives of this study were to develop a SNP molecular marker/s for use at the early vegetative stages of the plant for evaluation of the *lpa1-1* gene present during breeding; to compare effect of high quality DNA extraction and crude extraction methods on the melting curves of the parental lines; and compare the cost of the HRM analysis to the conventional method of DNA sequencing for detection of the SNP marker. The sequence of the amplified PCR products was determined to validate the *lpa1-1* nucleotide change.

Materials and Methods

Germplasm

Two inbred maize lines were used in this study. The normal (wild type) tropical locally adapted line was P16 (CZL 00023) and the temperate *lpa1-1* source was CM32 (JUG 248 *LPA1-1*). The LPA source (CM32 – JUG 248 *LPA1-1*) was obtained from Dr V. Raboy (Iowa, U.S.A.) at the request of Prof P.T. Tongoona and P16 was obtained from CIMMYT maize germplasm collection. The heterozygote sample was the F_1 cross between P 16 and CM 32 inbred lines.

DNA extraction

Leaf material was sampled and ground in liquid nitrogen for high quality genomic DNA extraction using Wizard genomic DNA purification kit (Promega, Whitehead Scientific, Cape

Town, South Africa). Various crude extraction methods were tested in this study but a modification of the Edwards *et al.* (1991) method was selected. In the crude extraction method, leaf samples for PCR analysis were collected using the lid of a sterile 1.5 ml microcentrifuge tube to punch out a disc of leaf material into the tube. The tissue was macerated with extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS) and vortexed for a few seconds. The sample was heated for 10 mins at 65°C and centrifuged for 2 mins. The supernatant was removed, an equal volume of ice-cold isopropanol added and mixed before incubation at -20°C for 30 mins. The sample was centrifuged for 5 mins at 4°C and the pellet dried before re-suspending in 20 µl of TE (100 mM Tris-HCl, 1 mM EDTA, pH 8) buffer overnight. The DNA extracted using the genomic DNA extraction kit was visualised by screening on a 1% (w/v) agarose gel in TAE buffer

Primer design

The wild type *lpa1-1* gene was sequenced by Shi *et al.* (2007) who stated that the *lpa1-1* mutation is a result of an alanine to valine amino acids change at position 1432 and it was from this statement that it was inferred that there was a C to T transition occurrence. The wild type sequence of *lpa1-1* is available on National Centre for Biotechnology Information (NCBI) database (accession number: NM_001112590). Based on the nucleotide sequence, the amino acid sequence was used to identify the position of the *lpa1-1* mutation. The nine PCR primers were designed using the Primer3 programme (Rozen and Skaletsky, 2000) to flank the *lpa1-1* mutation position and had a predicted size range of 91 bp to 148 bp. The primers were purchased from Integrated DNA Technologies (Whitehead Scientific, Cape Town, South Africa). The primers were tested and optimized for annealing temperature.

PCR and HRM analysis

The Rotor-Gene 6000 real time rotary analyser (Corbett Research, Australia) was used for the PCR amplification and HRM analysis. PCR amplifications were performed in 20 µl reaction volumes consisting of 15 ng of genomic DNA template, Quantace SensiMixdT for the PCR reaction components (Celtic Diagnostics, Cape Town), 1 x SYBR Green I dye and 200 nM of forward and reverse primers. The PCR amplification was initiated with 10 min hold at 95°C as an initial denaturation step, followed by 40 cycles of 95°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec. A negative control was added in each set of PCR reactions with no DNA included to ensure non-contamination of PCR reagents. HRM analysis was performed automatically after the PCR and programmed to ramp temperature from 72°C to 95°C, raised by 0.1 degree/step after the final extension step. The Rotor-Gene 6000 real time

rotary analyser created the melting curves and the Rotor-Gene 6000 real time rotary analyser software version 1.7 was used to discriminate genotypes (Corbett Research, Australia). PCR products were screened initially for confirmation by electrophoresis in a 1.5 % (m/v) agarose gel in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized under UV light after staining with ethidium bromide (0.5 μg ml^{-1}). The optimization of the HRM method included the temperature range, checking for reliability between samples and between different runs and the ability to distinguish between homozygous dominant (WT), homozygous recessive (LPA) and the heterozygous genotypes.

Validation of *lpa1-1* SNP marker

The presence and identity of the *lpa1-1* SNP was confirmed and validated by forward and reverse sequencing the PCR products of the parental inbred lines (Inqaba Biotech, Pretoria, South Africa). Due to the small size of the product, the PCR product was first cloned into a vector and then sequenced to ensure the sequencing of the entire product. Sequence alignment was performed using the programme ClustalX (Ramu *et al.,* 2003).

Comparison of DNA extraction methods

Two DNA extraction methods were compared to determine the efficiency of the marker in detecting the different genotypes. The leaf material was ground in liquid nitrogen and the Wizard genomic DNA purification kit (Promega, Whitehead Scientific, Cape Town, South Africa) was used for the high quality DNA extraction. The crude extraction method was a modification of the Edwards *et al.* (1991) method using fresh leaf sample punch. The melt profiles of the parental lines were compared with high quality and crude extractions of genomic DNA to determine effectiveness of the *lpa1-1* SNP marker.

Comparison of costs of HRM analysis and sequencing

The costs of DNA sequencing were compared among different laboratories across South Africa to the cost of running the *lpa1-1* SNP marker on the Rotorgene 6000 with HRM analysis. The costing of the SNP marker with HRM analysis in a 20 µl PCR reaction included costs for DNA extraction, PCR reagents, SYBR green dye and PCR tube. The cost of a standard sequencing of one sample was used.

Results and Discussion

The parental inbreds were able to amplify a 150 bp PCR product at an annealing temperature of 55°C (Forward 5'- ATA ACT GGA GCG TGG GAC AG-3' and reverse 5'-CTG CGG ATG ATC TTT TGG AT-3'). The PCR optimisation used high quality genomic DNA of the maize inbred parental lines. There were nine PCR primer pairs tested in the initial optimisation for the *lpa1-1* SNP marker. The annealing temperature ranged from 50°C to 65°C, with the optimum annealing temperature at 55°C selected. The selection of the most suitable forward and reverse primers for the *lpa1-1* SNP marker was based on the most stable and reliable melt profiles as well as the largest melting temperature differences between the parental lines. It was hypothesized that shorter PCR products would show larger melting temperature differences between the parental inbred lines due to the ratio of similarity between the base sequences. However the shorter PCR products did not show significantly larger temperature shifts.

The raw data of the melt profiles is seen in Figure 2.1 showing the change of fluorescence over the increasing temperature range. The data was transformed (dF/dT) to give melting profiles of the parental lines and heterozygote PCR amplification products (Figure 2.2). Both parental lines were unmistakably distinguished from each other in the melt profiles by 0.5°C melt temperature difference (Figure 2.2). The wild type inbred line had a melting temperature of 83.05°C, *lpa1-1* inbred line 83.53°C and the heterozygote 82.80°C. The heterozygote profile was more clearly distinguishable in the difference plots and this was used to define the heterozygous genotypes.

In this study, the 150 bp product from the parental lines was clearly differentiated with the HRM analysis. It has been shown with HRM analysis, there is good (100%) sensitivity for PCR products of 300 bp or less in identifying heterozygotes and homozygote genotypes however this has decreased to 96.1% sensitivity and 99.4% specificity for 400 to 1000 bp products (Reed and Wittwer, 2004). This is due to the fact that as the product length increases, the difference between the melting curves decreases, thereby leading to errors in detection of the particular allele.

The differences between the parental inbred PCR products can be more clearly visualised and quantified by using the difference plots of normalised curves than the melting curve analysis. This is due to the difference between the melting temperatures of the parental line PCR products being very small or there being only one or a few bases that differ between the amplified products. A difference plot highlights the differences between individual curves relative to one of the sample melting curves plotted as a baseline. They can be used to differentiate between the homozygous and heterozygous genotypes more clearly than the

melting curves, especially in cases where the melting differences are very small. Figure 2.3 shows a difference plot in which CM 32 (red) and heterozygote (green) were normalised to the P16 (black) melt curve. The heterozygote curve weaves into the LPA curve, but this is seen more clearly in Figure 2.4 where samples are normalised to LPA and P16 is now positive with the heterozygote both positive and negative. The distinctive shape of the heterozygote is used to differentiate heterozygous from homozygous genotypes.

The PCR product of the heterozygote in this study was differentiated from the parental line PCR products due to its characteristic shape in the difference plots which was also found in other studies. The shape of the PCR amplicon for the heterozygotes is a characteristic "wave" shape in the difference plots, confirmed by the difference between genotypes visible in difference plots due to unique trace patterns (Wittwer *et al.,* 2003). Difference plots were able to successfully distinguish between homozygotes and heterozygotes in a F_2 population in pepper using Pp201 SNP marker (Park *et al.,* 2009) and allowed more distinct separation of grapevine varieties for DNA fingerprinting (Mackay *et al.,* 2008) than melting curve analysis alone. DNA melting analysis can also be used to detect heterozygotes as in the case of the PCR amplicon from the exon of the human N-methyl-D-aspartate receptor gene (NR1) that had an alanine to valine amino acid change due to C to T base change (Lipsky *et al.,*2001).

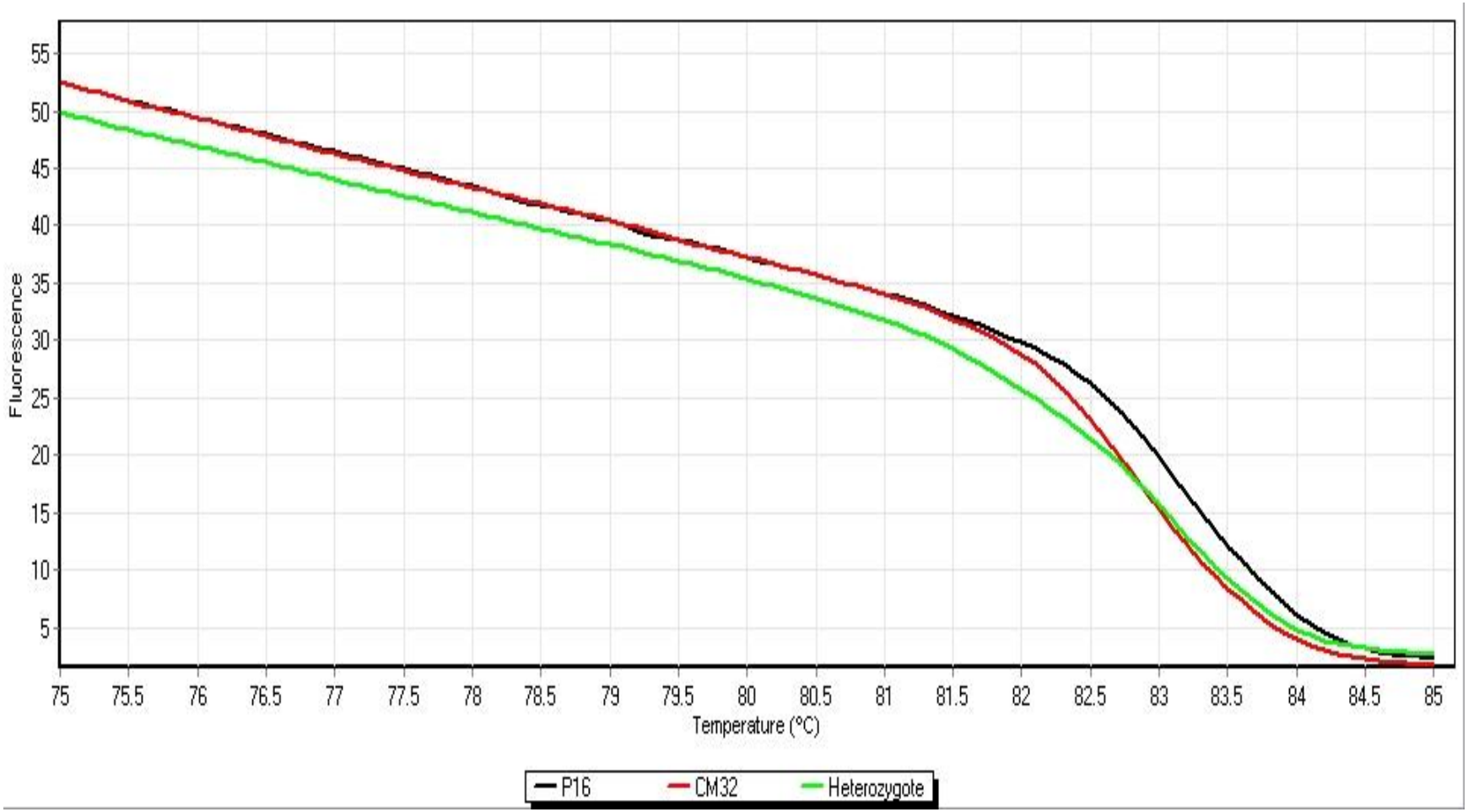

Figure 2.1: Change in fluorescence over increasing temperature range of P16, CM32 and heterozygote PCR amplification products

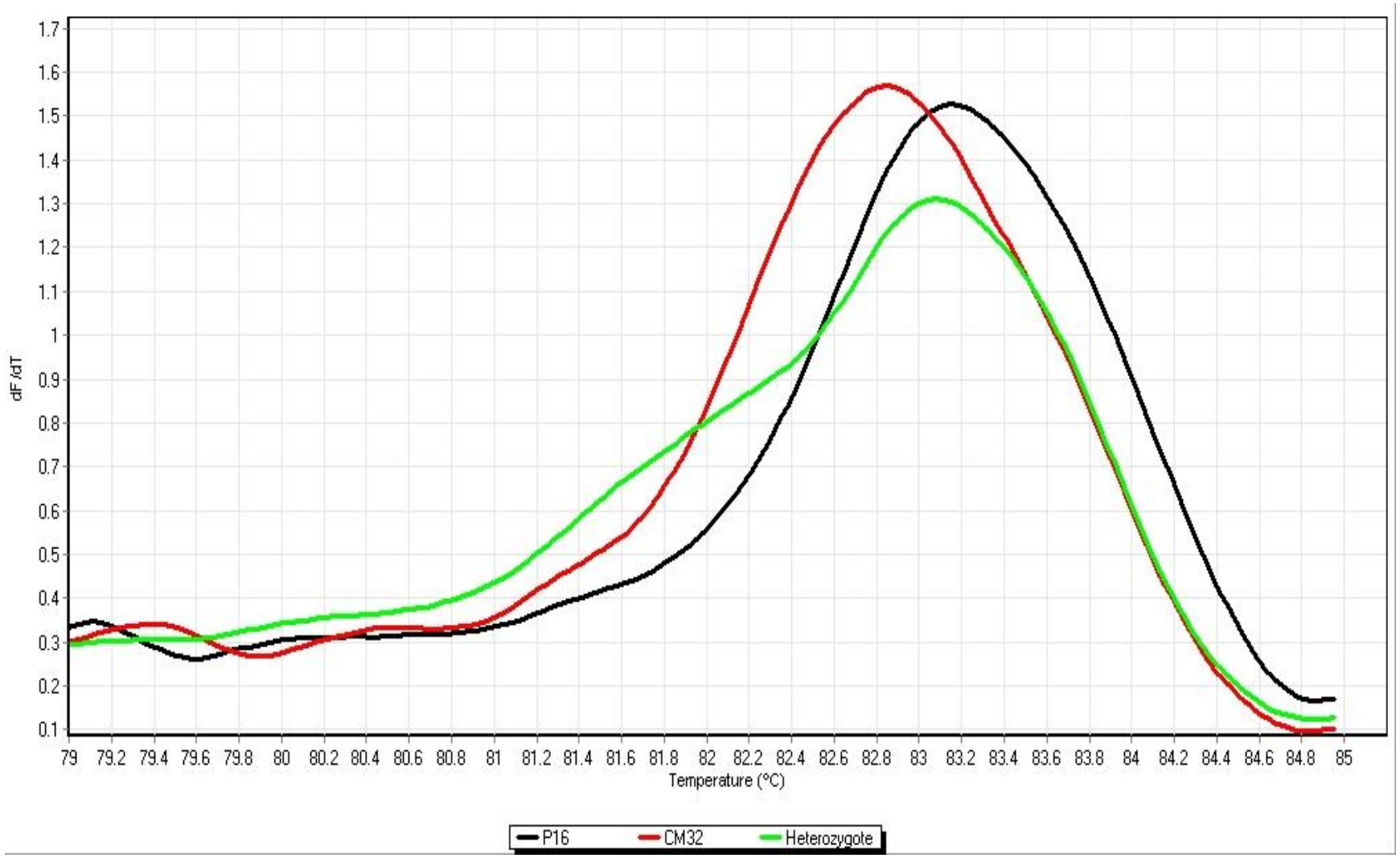

Figure 2.2: Melt profile of P16, CM32 and heterozygote PCR amplification products from HRM analysis

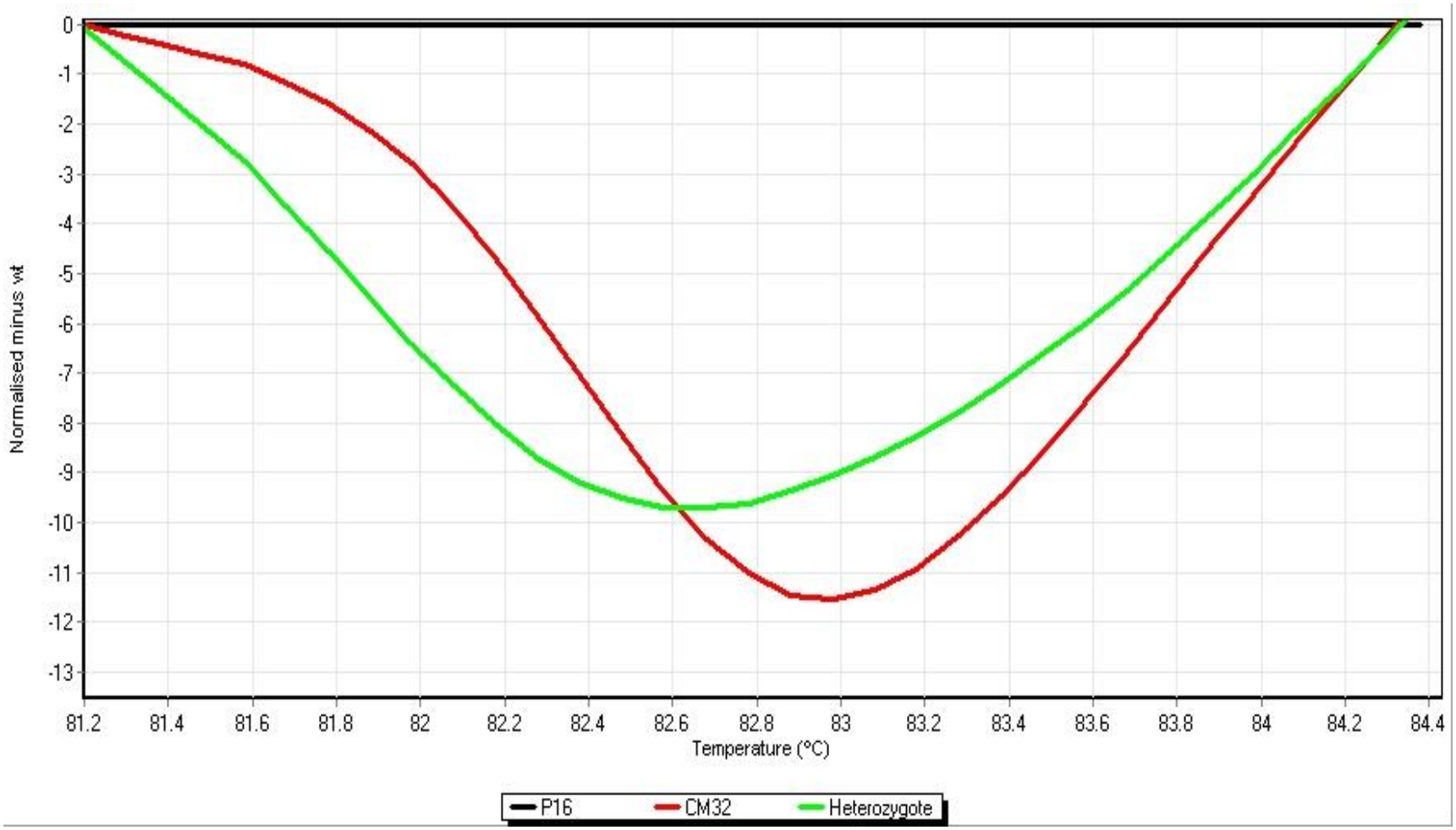

Figure 2.3: Difference plot of CM32 and heterozygote PCR amplification products with P16 normalized

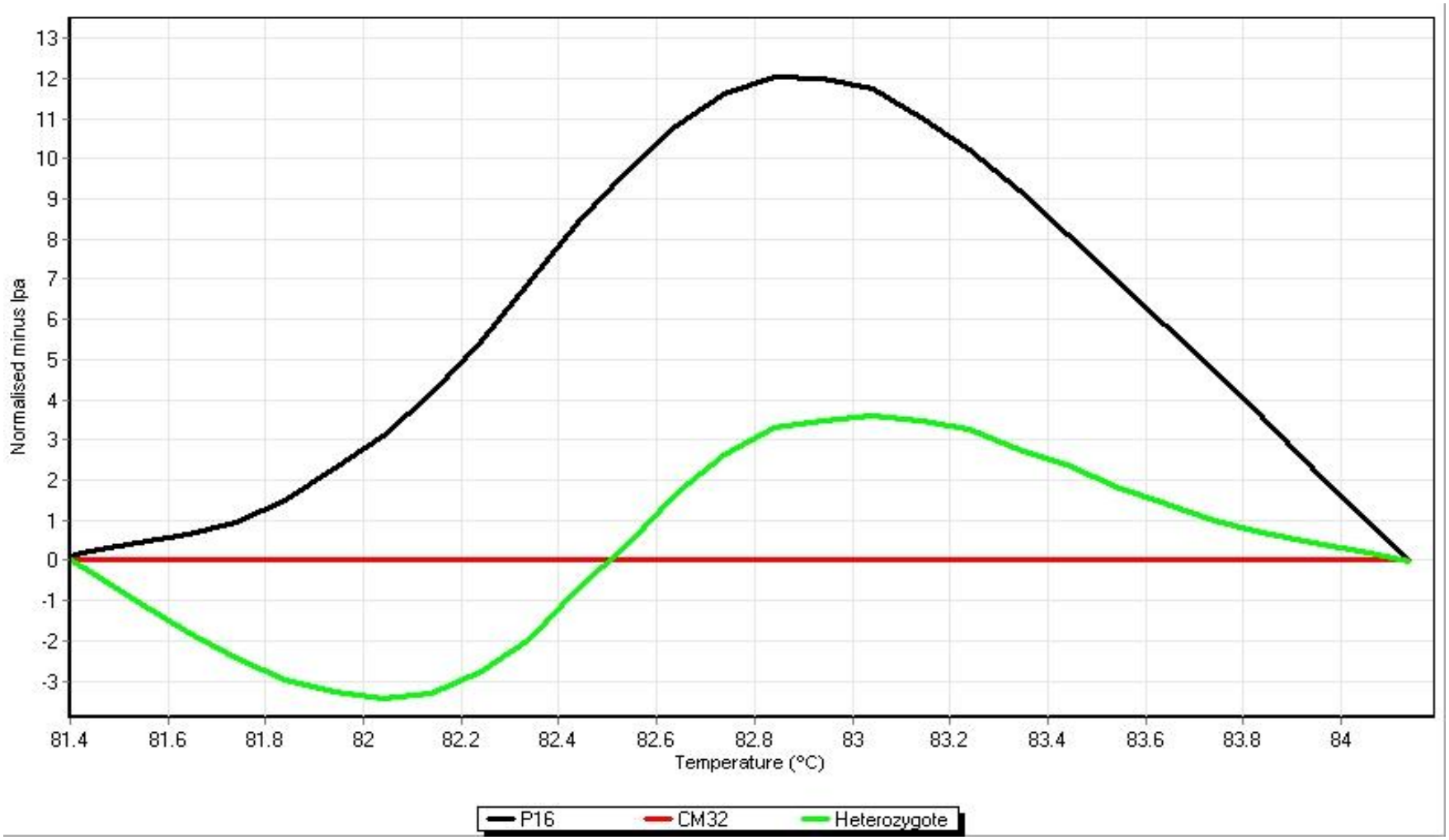

Figure 2.4: Difference plot of P16 and heterozygote PCR amplification products with CM32 normalized

The *lpa1-1* SNP marker was validated by forward and reverse DNA sequencing. Due to the small size of the PCR amplicon, it was cloned before sequencing. The sequences were aligned using the programme ClustalX (Ramu *et al.,* 2003) and were identical with only the single base change from C (wild type) to T (LPA) (Figure 2.5).

```
P16             CAAAAATTTTGGTACTCGATGAGGCGACAGCATCTGTCGACACAGCAACAGACAATCTTA
Reference       CAAAAATTTTGGTACTCGATGAGGCGACAGCATCTGTCGACACAGCAACAGACAATCTTA
CM-32           CAAAAATTTTGGTACTCGATGAGGCGACAGTATCTGTCGACACAGCAACAGACAATCTTA
                ***************************** *****************************
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Figure 2.5: Partial sequence alignment of P16 (wild type), reference (wild type) and CM32 (*lpa1-1*) showing the C to T nucleotide base change. The reference sample was the wild type of the *lpa1-1* sequence published on NCBI (accession number of NM_001112590).

The effect of high quality and crude extraction of genomic DNA was compared by the melting profiles obtained with the *lpa1-1* SNP marker. The melt profiles of the parental lines obtained from amplification with high quality genomic DNA showed very little difference from the melt profiles amplified with genomic DNA isolated with the crude extraction (Figure 2.6). The melting curves were very similar and the melting temperatures of P16 differed by 0.07°C and CM32 by 0.04°C. This proved that the crude extraction of genomic DNA was just as effective as the high quality genomic DNA extraction. The crude extraction method was more cost-effective as well and especially in cases of large sample volumes this is an added benefit.

The cost of the SNP marker with HRM analysis was approximately R5.50/sample with no additional post-PCR analysis costs as the Rotor-Gene 6000 is essentially maintenance-free. The cost of DNA sequencing however includes DNA extraction and PCR amplification as for the SNP marker with the additional cost of sequencing. The prices per sample for DNA sequencing a PCR product varied from R130 (University of Cape Town), R125 (University of Witwatersrand), R75 (Inqaba Biotech, Pretoria) to R60 (Central Analytical Facility, University of Stellenbosch) (prices accessed on internet websites on 15 November 2009). Therefore the cost of using the *lpa1-1* SNP marker with HRM (R5.50) is only 8% of the cost of DNA sequencing (R5.50 + R60). This is significantly lower than the DNA sequencing cost per sample even at the most cost effective laboratory. Additional factors to be taken into account are the labour and time required for “cleaning” the PCR product before DNA sequencing can occur, with the HRM analysis requiring much shorter time and less labour than DNA sequencing. All these factors validates the use of HRM analysis for *lpa1-1* SNP detection due to its lower cost (8%), the shorter time required for the HRM and less labour intensive than DNA sequencing.

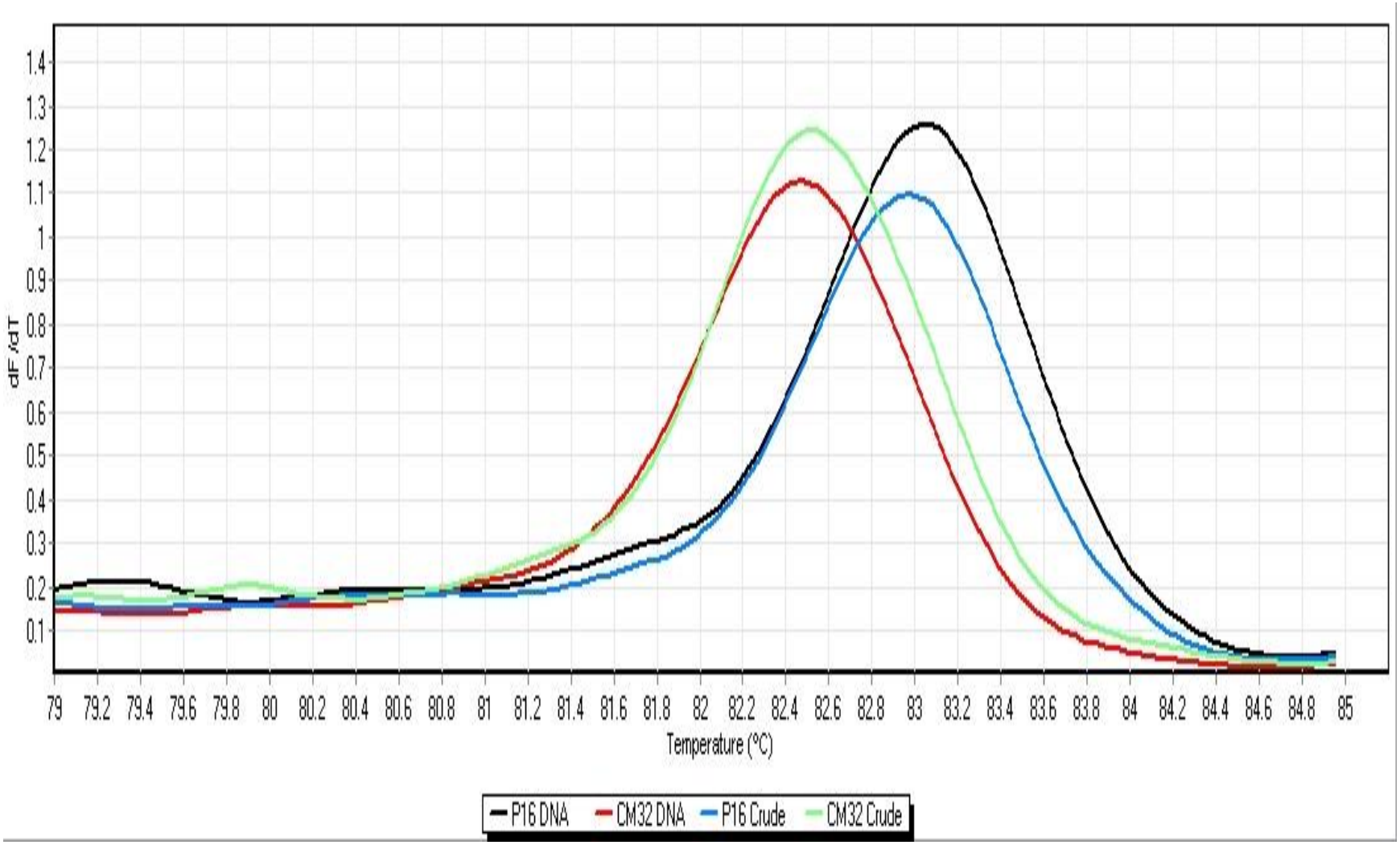

Figure 2.6: Melt profiles of high quality DNA extraction compared with crude DNA extraction of P16 and CM32

In plant breeding where there are large volumes of samples, screening methods that are quicker, less labour intensive and more cost effective than traditional screening methods are often sought. The characteristics that a molecular marker requires for use in plant breeding programmes include reliability, low technical skill and short turn-around time with most markers meeting one or some of these criteria. This often poses a challenge for molecular biotechnologists and limits the application of molecular markers in plant breeding programmes.

However in the case of SNP markers, this gap is slowly being bridged. These markers are widely distributed in the genomes and with the added technology of HRM analysis, SNPs can be used to successfully detect and distinguish between homozygous and heterozygous genotypes as well as being quick, reliable and very simple to use markers.

There have been other LPA mutant lines identified with SNP markers developed from similar amino acid changes which result in lower phytic acid content. These include LPA rice mutants isolated by Kim *et al.* (2008) and Xu *et al*. (2009). The rice mutant *Lpa* N15-186 is due to a single recessive gene with a single base pair change from the wild type (C to T transition) in the first exon of the gene resulting in a nonsense mutation. It is a mutant allele of the rice myo-inositol kinase (OsMIK) gene and shows 75% reduction in phytic acid P and was mapped to chromosome 3, showing a similar phenotype to maize *lpa3* mutant (Kim *et al.,* 2008). Another two rice LPA lines (homozygous lethal and homozygous non-lethal mutants) were identified that carried two allelic mutations of the rice orthologue (OsMRP5) of the maize *lpa1-1* gene (ZmMRP4) (Xu *et al.,* 2009). There was a C to T transition in the 6th exon of XS-*lpa2-1* which although caused a change of Proline to Serine amino acid but had a limited effect on the function of the protein as it does not change the tertiary structure of the protein. However, the other allelic mutation has a 5 bp deletion in the 1st exon of XS-*lpa2-2*, has a significant change as there is a frame shift mutation that introduces a premature stop codon after the deletion site in the first ABC-domain (Xu *et al.,* 2009).

The use of HRM analysis has been very limited in plants and in the case of the rice SNP (XS-*lpa2-1* and XS-*lpa2-2*) phytic acid levels were detected with the colorimetric assay and the gene sequence identified with PCR and RT-PCR (Xu *et al.,* 2009). The SNP marker linked to the fragrance gene in rice also shows a C to T transition resulting in the change of phenotype; however the SNP is detected by DNA sequencing of a 491 bp PCR product (Jin *et al.*, 2003). In this study, the maize *lpa1-1* SNP is also due to a C to T transition which results in the change of the phenotype to LPA, however the HRM analysis is able to successfully identify all the different forms of the alleles of *lpa1-1* gene. The potential to use HRM analysis for the rice SNP markers is thereby possible. Therefore, this study is novel and unique in the use of HRM analysis to successfully detect the *lpa1-1* SNP genotypes without the use of the colorimetric assay and DNA sequencing of PCR products.

Conclusions

The *lpa1-1* SNP marker was able to successfully differentiate the two inbred maize lines by the differences in melt profiles due to HRM analysis. The SNP marker was able to detect a single nucleotide base pair change (C to T) that causes the *lpa1-1* phenotype. The *lpa1-1* SNP marker is a co-dominant marker that is able to distinguish between homozygous dominant, homozygous recessive and heterozygous alleles of the *lpa1-1* trait. The inbred maize lines melting curves showed 0.5°C difference in melting temperature and the difference plots were used to characterise the different genotypes, especially the heterozygotes. The crude DNA extraction was as effective as high quality DNA extraction method for melt curve analysis. The SNP marker was validated by DNA sequencing with the C to T transition clearly shown. Breeding for *lpa1-1* has been hampered due to the fact that *lpa1-1* is single recessive gene, however with the *lpa1-1* SNP marker this can now be overcome and the gene can be easily tracked. The destructive colorimetric assay is no longer required for the assessment for phytic acid content in maize grain and can be replaced by the *lpa1-1* SNP marker that is stable, reliable and can be used on the early vegetative tissue of the plant instead of mature seed. The cost of the *lpa1-1* SNP marker with HRM analysis is 8% of the cost of DNA sequencing which is the conventional method of detecting SNP genotypes. A faster, reliable and cost effective SNP marker with HRM analysis for *lpa1-1* gene detection was developed for maize breeding programmes, leading to more efficient breeding for lower phytic acid in maize.

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Chapter Three

Marker-assisted selection for low phytic acid (*lpa1-1*) with SNP marker and AFLPs for background selection in a maize backcross breeding programme

Abstract

Phytic acid is difficult to assess in a maize breeding programme due to a tedious colorimetric assay. A co-dominant single nucleotide polymorphism (SNP) marker with high resolution melt (HRM) analysis was used to detect the low phytic acid (*lpa1-1*) gene in a BC_2F_1 population. Background selection for the recovery of the recurrent parent was by using six AFLP *Eco*RI/*Mse*I primer combinations. A BC_1F_1 population was developed from a locally adapted tropical normal inbred line (P 16) and CM 32 was the *lpa1-1* donor. The initial screening of 250 BC_2F_1 lines with the *lpa1-1* SNP marker with HRM analysis was able to select 11 homozygous recessive and 17 heterozygote genotypes for the *lpa1-1* mutation. The R^2 values for the SNP analysis for the heterozygotes showed a higher range of 90.95% to 99.59% than the *lpa1-1* homozygous recessives which ranged from 82.81% to 99.58%. These selected BC_2F_1 lines were fingerprinted with six AFLP *Eco*RI/*Mse*I primer combinations to determine the amount of recurrent parent genome present. There were 277 AFLP markers scored on the two parental lines and 28 BC_2F_1 lines with a mean of 46.2 markers/primer combination with 234 (84%) polymorphic and 43 (16%) non-polymorphic markers. All the BC_2F_1 selected lines were clearly differentiated from each other and the parental controls in the dendrogram based on the DICE coefficient of similarity with a similarity range from 62.12% to 92.15%. There were 13 BC_2F_1 lines (six heterozygotes and seven homozygous recessive) that showed the highest percentage of similarity to the recurrent parent (92.15% - 83.33%). The use of marker-assisted selection for foreground and background selection greatly increased the efficiency of detection of the homozygous recessive (99.58%) and heterozygous (99.59%) genotypes as well as improving the recovery of the recurrent parent (92.15%) in BC_2F_1 generation of the maize backcross breeding programme.

Keywords: Low phytic acid, SNP marker, AFLPs, marker-assisted backcrossing

Introduction

Phytic acid is an antinutritional compound found in seeds of cereals and legumes. It chelates essential minerals such as iron, zinc, magnesium, calcium and potassium as it passes through the digestive system of monogastric consumers such as humans and pigs. It is also a store of phosphorus therefore should not be eliminated completely. There are temperate maize lines that contain lower levels of phytic acid but these are not adapted to tropical and subtropical conditions. There are four low phytic acid (LPA) mutants (*lpa1-1*, *lpa2*, *lpa3*, *lpa241*) developed with different amounts of phytic acid reductions (Raboy, 2000; Pilu *et al.,* 2003). The *lpa1-1* mutation shows 66% reduction in phytic acid phosphorus and was generated by ethylmethane sulphonate-induced (EMS) pollen grain mutation (Raboy, 2000). The *lpa1-1* gene has been mapped onto chromosome 1 distal region and reported to be due to a single recessive mutation (Raboy *et al*., 2000). Shi *et al.* (2007) sequenced the *lpa1-1* gene (5149 bp) and reported the *lpa1-1* phenotype to be probably due to a alanine to valine change of amino acids. This *lpa1-1* gene was chosen to introgress the LPA trait into tropical adapted maize line (P16) due to its high percentage of reduction of phytic acid (66%), available gene sequence and the exact type and position of the mutation identified. However, due to its recessive nature, foreground marker-assisted selection with a single nucleotide polymorphism (SNP) marker was used to detect the *lpa1-1* gene in segregating backcross breeding material with high resolution melt (HRM) analysis to detect the various genotypes. Background marker-assisted selection was done to fingerprint the homozygous recessive and heterozygous genotypes with AFLPs to determine the amount of recurrent parent genome present.

Marker-assisted selection (MAS) entails the use of molecular markers in the selection process in plant breeding programmes and has been shown to be cost effective (Abalo *et al.,* 2009). These markers need to be closely linked to the target gene/locus and should be polymorphic for the breeding material used in the programme for them to be effective and reliable. Markers can be used for the introgression of recessive traits and for the selection of lines resembling the recurrent parent genome. Marker-assisted backcrossing (MABC) is an established tool in plant breeding. The main aim in MABC is the introgression of a trait of interest into the genetic background of a recipient genotype by recurrent backcrossing. The other aim is to recover the recurrent parent genome as rapidly and completely as possible. The use of markers to monitor the parental origin of alleles throughout the genome in MABC with restriction fragment length polymorphism (RFLP) markers was originally proposed by Tanksley *et al.* (1989) and was later called background selection (Hospital and Charcosset, 1997). There are two goals of background selection: to reduce the proportion of the donor genome on the carrier chromosome of the target allele and to reduce the donor genome on the non-carrier chromosomes. Linkage drag is reduced by the

selection of individuals that carry the target allele and are homozygous for the recurrent parent alleles at tightly linked marker loci (Frisch *et al.,* 1999). The use of background selection for a single gene has been widely investigated (Hospital et al., 1992; Abalo *et al.,* 2009) and has now been used in maize for two genes simultaneously (Frisch and Melchinger, 2001) and QTLs (Hospital and Charcosset, 1997; Bouchez et al., 2002). Marker-assays can be advantageous in backcross breeding programme for both background and foreground selection (Hospital and Charcosset, 1997).

Molecular markers that have a wide coverage of the genome and are highly polymorphic are preferred. Markers are based on detecting sequence variation in deoxyribonucleic acid (DNA) and amplified fragment length polymorphism (AFLP) markers in particular target the repetitive regions of the genome. The choice of marker system to use often depends on the species, objective of the work, genetic resolution and financial and technological resources available. They differ in information content, number of scorable polymorphisms per reaction, and degree of automation. Molecular markers are well established tools in plant breeding and genetics that improve the efficiency and sophistication of breeding. They have played a key role in improving the understanding of the genome organization, structure and behaviour for many major crops.

Since the late 1990s, SNPs have been used more as a marker system in plants (Gupta *et al.,* 2001; Rafalski, 2002b; Barker and Edwards, 2009). This marker is characterised by a single base substitution at a particular position, a type of polymorphism that is also recognised by the restriction fragment length polymorphism (RFLP) technique if the SNP occurs in a restriction enzyme recognition site (Brookes, 1999). The SNPs are regarded as the prototype of the third generation molecular markers (Gupta *et al.,* 2001). They are biallelic, abundant in genomes, have relatively low mutation rates, have even distribution in the genome, and are relatively easy to detect. The SNP density is generally higher in intergenic and intronic regions compared to that of exons (Brookes, 1999, Rafalski, 2002a). SNP markers are a consequence of either transition or transversion events (Batley *et al.,* 2003). There are eight possibilities for transversions but only four for transitions. If the mutation affects the first or second codon position within a protein coding region, the encoded amino acid is often substituted by a different one and such mutations are called nonsynonymous. These are called diagnostic SNPs and may be associated with certain diseases in humans and certain agronomic traits in plants (such as the *lpa1-1* SNP). However, the mutation is termed synonymous or silent if the amino acid sequence of the protein remains unchanged (Batley *et al*., 2003).

The choice of detection methods for SNP genotyping depend on many factors, including cost, throughput, equipment needed, difficulty of assay development and potential for multiplexing (Rafalski, 2002b). SNPs are the fastest and cheapest marker system available for map-based

cloning (Scheible *et al.,* 2005). SNPs are not commonly used in the plant sciences due to the high cost of developing SNPs detection assays, especially the re-sequencing of DNA samples and the requirements for expensive instruments and complex procedures to detect SNPs (Batley *et al.,* 2003; Kim and Misra, 2007). However with the advancement in technology leading to specific low density SNP chips and other affordable technologies, such as high resolution melt analysis (HRM), SNPs will be used more in DNA fingerprinting, genomic mapping and linkage analysis in the plant sciences (Gupta *et al.,* 2001, Rafalski, 2002b, Barker and Edwards, 2009). HRM is the most inexpensive, simple and rapid of these technologies to detect SNPs (Gundry *et al.,* 2003; Wittwer *et al.,* 2003).

In HRM analysis, the polymerase chain reaction (PCR) amplification and melt analysis can be in one closed tube reaction (Montgomery *et al.,* 2007) depending on the type of equipment used with the aid of intercalating fluorescence dyes (Worm *et al.,* 2001; Liew *et al.,* 2004; Zhou *et al.,* 2004, 2005; Montgomery *et al.,* 2007; Park *et al.,* 2009) or fluorescence-labelled primers (Gundry *et al.,* 2003). The PCR products are differentiated from each other by melting curve profiles (Ririe *et al.,* 1997) with heterozygous and homozygous genotypes identified (Gundry *et al.,* 2003, Wittwer *et al.,* 2003; Reed and Wittwer, 2004; Montgomery *et al.,* 2007). The melting curve is generated by plotting fluorescence as a function of time as the thermal cycler heats through the dissociation temperature of the product (Ririe *et al.,* 1997). The shape and position of the melting curve are due to the GC/AT ratio, length and sequence and can be used to differentiate PCR products separated by less than 2°C in melting temperature (Ririe *et al.,* 1997). There is no post-PCR analyses (Ririe *et al.,* 1997; Montgomery *et al.,* 2007) required therefore, increasing the efficiency of marker detection system. There is reduction of costs, time and labour in the HRM analysis (Ririe *et al.,* 1997; Zhou *et al.,* 2005).

The main objective of a backcross breeding programme is the reduction of the donor parent genome by 50% at each generation of backcrossing while retaining the trait of interest from the donor parent. There is, however, variation present in each backcross generation around the mean. Markers can be used to select for the donor trait as well as recombinant individuals that have genome composition closer (75%) to the recurrent parent. MAS is used to reduce the time and number of backcross generations required to achieve this goal. A major constraint has been the number of polymorphic markers required to cover the entire genome to effectively determine the amount of recurrent parent genome present.

AFLPs are a universal, multi-locus marker technique that can be applied to genomes of any source or complexity. It is a reproducible and reliable technique within and among mapping populations. No prior sequence information is required and highly informative fingerprints are produced due to the large number of bands produced per primer combination. AFLP is an

ingenious combination of RFLPs and PCR designed by Vos *et al*. (1995). It has become very popular and is a powerful approach to identify DNA polymorphisms. AFLP marker bands are mainly dominant but in some cases co-dominance can be detected.

AFLPs have been used extensively to assess genetic diversity in maize (Ajmone-Marsan *et al.,* 1998; Pejic *et al.,* 1998; Lübberstedt *et al.,* 2000; Heckenberger *et al*., 2003; Adawy *et al.,* 2004; Beyene *et al.,* 2005; Beyene *et al.,* 2006; Legesse *et al.,* 2007; Hartings *et al.,* 2008) and other crops such as soybean (Maughan *et al.,* 1996; Powell *et al.,* 1996; VanToai *et al.,* 1996), potato (McGregor *et al.,* 2000; Spooner *et al.,* 2005), barley (Russell *et al.,* 1997; Varshey *et al.,* 2007), wheat (Bohn *et al.,* 1999; Maccaferri *et al.,* 2007), rice (Saini *et al.,* 2004; Jeung *et al.,* 2005), groundnuts (Herselman, 2003), sugarcane (Lima *et al.,* 2002), lentils (Sharma *et al.,* 1996) and black pepper (Joy *et al.,* 2007). The AFLP technique was successful in detecting genetic diversity in all studies with high levels of polymorphism, effective multiplex ratio, marker index and genotype index observed. The technique was useful in assigning inbreds into heterotic groups, revealing pedigree relationships among lines, showing relationships between morphological and or phenotypic traits and AFLP marker data. It was concluded that SNP markers are better suited for characterizing and conserving genebank materials with AFLP markers being more suitable for diversity analysis, fingerprinting (Varshey *et al.,* 2007), cultivar identification, phylogenetic studies (Joy *et al*., 2007) and generally AFLP markers seem to be the most appropriate for various aspects of germplasm analysis (Lübberstedt *et al.,* 2000).

There are many studies on DNA fingerprinting of inbred lines in maize and other crops; however there are few studies on the use of AFLPs in backcross plant breeding programmes to determine the percentage of recurrent parent genome. These studies were on crops such as cotton (Zhong *et al.,* 2002); rice (Chen *et al.,* 2000, 2001; Zhou *et al.*, 2003; Gopalkrishan *et al.*, 2008); soybean (VanToai *et al.,* 1996) and beans (Mũnoz *et al.,* 2004).

There are numerous advantages of AFLPs including versatility as PCR with random primers. A high stringency is applied during PCR to ensure robustness and high reproducibility and limited set of AFLP primers can yield a large set of primer combinations, each with its own unique set of amplified fragments, the multiplex ratio is high and can be increased by altering the length of the 3'-nucleotide extensions and/or choice of enzyme. These advantages make AFLPs a very resourceful tool for numerous molecular applications. The pattern complexity can be in the range of 20 and 60 bands per primer combination in the fragment range of 50 to 500 bp, depending on the species. There are some disadvantages that include dominance of markers, clustering of markers (depending on the restriction enzyme choice), limited levels of polymorphisms in some species, and the requirement for good quality and high amounts of DNA as compared to other

markers. This is species-dependant as some species are highly polymorphic while others are not depending on the size of the genetic bases in these species.

A review of MAS breeding on maize in Africa by Stevens (2008) found many research studies identifying QTLs for drought resistance, resistance to biotic stresses (sugar-cane mosaic virus, maize streak virus, grey leaf spot, stem borers, *Striga*) and micronutrient enhancement breeding (Vitamin A, iron and zinc). However there is failure to use these markers due to the variable effectiveness of the markers in predicting the desired phenotype, the low accuracy of QTL studies, and a lack of transferability across diverse germplasm and insufficient validation of markers. There is therefore a need to develop molecular markers that can overcome these limitations and be used to their full potential in breeding programmes.

For a practical plant breeding programme, a marker should have the following properties: co-dominance, reliability, rapidity, ease of use and cost effectiveness compared to traditional screening methods for the trait of interest. The *lpa1-1* SNP marker is able to meet most of these criteria as it is co-dominant, reproducible, low technical need and is much more cost effective than the colorimetric assay for phytic acid determination.

Due to the maize LPA mutants which carry the *lpa1-1* gene, showing low vigour and slow growth (Pilu *et al.,* 2003) there is a need to introgress this trait into tropical inbred maize lines that have higher seed germination, vigour and local adapability. However the LPA lines show variation for vigour and therefore selection for vigour should be an important criterion in a backcross breeding programme. Due to the recessive nature of the *lpa1-1* gene and the tedious destructive colorimetric detection assay, the backcross breeding programme would be longer and more tedious. A backcross breeding programme was developed to introgress the *lpa1-1* gene into a wild type tropical and subtropical adapted maize inbred line using molecular markers for foreground and background selection.

The objectives of this study were to use the *lpa1-1* SNP marker to detect homozygous recessive and heterozygote individual genotypes from the BC_2F_1 population using HRM analysis; and use AFLP markers to determine the amount of recurrent parent genome (P16) present in each BC_2F_1 line.

Materials and Methods

Germplasm

Two inbred maize lines were used in the backcross breeding programme to introgress the *lpa1-1* gene into tropical and subtropical adapted germplasm. The normal (wild type) tropical locally adapted line P 16 (CZL 00023) and the temperate *lpa*1-1 source CM 32 (JUG 248 *LPA1-1*) were used in this study. The LPA inbred line was obtained from Dr V. Raboy (Iowa, U.S.A.) and normal inbred line from the CIMMYT germplasm. The normal line was crossed with the LPA line to produce the F_1 generation. The F_1 was backcrossed to the recurrent parent (P 16) to produce the BC_1F_1 generation. The BC_1F_1 generation was planted in pots in the greenhouse and backcrossed to the recurrent parent with no selection for the *lpa1-1* gene to generate BC_2F_1 generation.

Crude DNA extraction

Two hundred and fifty BC_2F_1 progeny were grown in pots in a greenhouse and leaves were sampled for crude DNA extraction. A crude DNA extraction method of Edwards *et al.* (1991) was used to extract DNA for the *lpa1-1* SNP marker screening. Leaf samples for PCR analysis were collected using the lid of a sterile 1.5 ml microcentrifuge tube to punch out a disc of leaf material into the tube. The tissue was macerated with extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS) and vortexed for a few seconds. The sample was heated for 10 mins at 65°C and centrifuged for 2 mins at 12 000 x *g*. The supernatant was removed, an equal volume of ice-cold isopropanol added and mixed before incubation at -20°C for 30 mins. The sample was centrifuged for 5 mins at 4°C at 12 000 x *g* and the pellet dried before re-suspending in 20 µl of TE (100mM Tris-HCl, 1mM EDTA, pH 8) buffer overnight.

PCR and HRM analysis

The Rotor-Gene 6000 real-time rotary analyser (Corbett Research, Australia) was used for the PCR amplification and HRM analysis. The PCR amplifications were performed in 20 µl reaction volumes consisting of approximately 15 ng of genomic DNA template, SensiMixdT for the PCR reaction components (Celtic Diagnostics, Cape Town), 1 x SYBR Green I dye and 200 nM of forward and reverse PCR primers. The PCR amplification was initiated with a 10 min hold at 95°C as an initial denaturation step, followed by 40 cycles of 95°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec. A negative control was added in each set of PCR reactions with no DNA included to ensure non-contamination of PCR reagents. The HRM analysis was performed automatically after

the PCR and programmed to ramp temperature from 72°C to 95°C, raised by 0.1 degree/step after the final extension step. The Rotor-Gene 6000 real time rotary analyser created the melting curves and Rotor-Gene 6000 software version 1.7 was used to discriminate genotypes by difference plots (Corbett Research, Australia).

Classification of BC_2 lines

The samples classified as heterozygous or homozygous recessive genotypes after analysis with the *lpa1-1* SNP marker by the R^2 values from the difference plots. The parental line, P 16 was used as the wild type standard genotype, CM 32 as the LPA recessive standard genotype and the F_1 cross between the two parental lines as the heterozygote genotype. The R^2 values were set at a threshold of 80%. Lines were selected for fingerprinting with AFLPs to determine the amount of recurrent parent present if their R^2 values ≥ 80% for heterozygous and homozygous recessive genotypes. Their leaf material was sampled and ground in liquid nitrogen for high quality genomic DNA extraction using Wizard genomic DNA purification kit (Promega, Whitehead Scientific, Cape Town, South Africa) as higher quality DNA is required for AFLP analysis. The DNA was screened on a 1% (w/v) agarose gel in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized under UV light after staining with ethidium bromide (0.5 μg ml^{-1}).

AFLP process

The three main steps in the AFLP process included template preparation (restriction digestion of genomic DNA and ligation of oligonucleotide adaptors), fragment amplification (preselective and selective amplification) and identification of amplified products by capillary electrophoresis. The AFLP reactions were performed using the AFLP plant kit modules according to the manufacturer's recommendations (Applied Biosystems, South Africa).

Genomic DNA was double digested by two restriction enzymes, *Eco*RI (rare cutter) and *Mse*I (frequent cutter). Double-stranded oligonucleotide adapters, with overhangs compatible to either 5´- or 3´-end generated fragments produced during the restriction digestion, were ligated to the DNA fragments. The ligated DNA fragments were pre-amplified by PCR using primers complementary to the adapter and restriction site sequence with only one additional selective nucleotide at their 3´-end (*Eco*-A, *Mse*-C). Ten microlitres of the pre-amplification reaction was screened by electrophoresis on a 1.5 % agarose gel (w/v) in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized under UV light after staining with ethidium bromide

(0.5 μg ml^{-1}), to ensure quality and quantity of pre-amplification products. Selective amplification was performed with the use of selective primers with three nucleotides for *Eco*RI and *Mse*I specific primers (Table 3.1) as per cycling conditions of the manufacturer. The amplified fragments of the parental profiles were initially screened on the Rotor-Gene 6000 (Corbett Research). The 10 primer combinations showing greatest differences between the parents were used for fingerprinting the BC lines. The pre-amplification and amplification PCR reactions for the BC_2 lines and parents were performed in tubes and 96 well microtitre plates (Applied Biosystems, South Africa) on GeneAmp PCR system 2700 (Applied Biosystems, South Africa) according to manufacturer's instructions.

The amplified samples were sent to the Central Analytical Facility at the University of Stellenbosch (Stellenbosch, Cape Town, South Africa) for analysis of products on the 3100 DNA sequencer. A ROX 500 (Applied Biosystems, South Africa) size standard was included in each sample for sizing of fragments amplified. The F filter set was used with a 36 cm capillary for the screening of the amplified products on the 3100 DNA sequencer.

Data analysis

There were only eight primer combinations selected for scoring due to incomplete data and poor amplification of the other two primer combinations. The eight *Eco*/*Mse* primer combinations selected run files were viewed using Peak Scanner software v1.0 (Applied Biosystems, freeware, www.appliedbiosystems.com) and raw data tables of the product sizes exported to Microsoft Excel for manual scoring. The parental amplification reactions were repeated in each sequencer run and only consistent bands were selected for scoring the individual BC_2 lines. Bands were conservatively manually scored in the size range of 100 to 500 bp.

Similarity matrices and dendrograms were generated for each primer combination separately and the combined data for the six primer combinations. For the similarity matrices construction, bands were scored as present (1) or absent (0). The fragment data was converted to binary data by generating a binary matrix (0,1) using the AFLP banding patterns of each individual. The NTSYS v2.1 software programme (Numerical Taxonomy and Multivariate Analysis for personal computers, Exeter Software, Setauket, NY, U.S.A.) was used to evaluate the genetic similarities between the maize BC_2F_1 lines and the parental lines (P 16 and CM 32). Pair-wise comparisons were made between the genotypes based on DICE (Dice, 1945) similarity coefficient. The resultant distance matrix data was used to construct a dendrogram using the agglomerative hierarchical un-weighted pair-group method with an arithmetic average (UPGMA) sub-programme of NYSTS (Rohlf, 1998).

Table 3.1: Sequences of adapters and primers used for the AFLP process and SNP analysis

Name	**Type**	**Sequence (5´-3´)**
*Eco*RI-AAG	Primer+3	GACTGCGTACCAATTCAAG
*Eco*RI-ACG	Primer+3	GACTGCGTACCAATTCACG
*Eco*RI-ACC	Primer+3	GACTGCGTACCAATTCACC
*Eco*RI-ACA	Primer+3	GACTGCGTACCAATTCACA
*Mse*I-CAA	Primer+3	GATGAGTCCTGAGTAACAA
*Mse*I-CAC	Primer+3	GATGAGTCCTGAGTAACAC
*Mse*I-CAG	Primer+3	GATGAGTCCTGAGTAACAG
*Mse*I-CAT	Primer+3	GATGAGTCCTGAGTAACAT
*Eco*RI-A	Pre-amplification	AGACTGCGTACCAATTCA
*Mse*I-C	Pre-amplification	GACGATGAGTCCTGAGTAAC
*Eco*RI	Adapter	5′-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5′
*Mse*I	Adapter	5′-GACGATGAGTCCTGAG TACTCAGGACTCAT-5′
lpa1-1 SNP *fwd*	Forward primer	5′-ATAACTGGAGCGTGGGACAG-3′
lpa1-1 SNP *rev*	Reverse primer	5′-CTGCGGATGATCTTTTGGAT-3′

Results and Discussion

There were 250 BC_2F_1 plants screened and 28 plants were selected that carried at least one copy of the *lpa1-1* gene and were therefore selected for fingerprint analysis with AFLPs. The Rotor-Gene 6000 software v1.7 was able to compute R^2 values based on the HRM analysis. The *lpa1-1* SNP marker is co-dominant and could therefore be used to detect homozygous dominant (wild type/normal), homozygous recessive (LPA) due to the melting profiles and heterozygous genotypes by difference plots using HRM analysis (Figure 3.1 and 3.2).

Figure 3.1 shows the melt curves of the parental inbred lines, heterozygote and three BC_2F_1 lines which are difficult to classify the BC_2F_1 lines into homozygous dominant, homozygous recessive and heterozygous genotypes due to the small melting temperature difference (0.5°C) between the parental lines. However in Figure 3.2 the difference plot graph with LPA normalized is more informative as the shape of the BC_2F_1 lines are more clearly defined with BC_2F_1 line 260 very closely resembling the CM 32 baseline, and BC_2F_1 lines 246 and 257 resembling the heterozygote curve shape. There were 11 homozygous recessive (LPA) and 17 heterozygote genotypes identified with the *lpa1-1* SNP marker according to their R^2 values and curve shape in difference plots. The heterozygotes showed a higher range of R^2 values of the *lpa1-1* SNP marker from 90.95% to 99.59% than the LPA recessives which ranged from 82.81% to 99.58% (Table 3.2). The HRM technology has been successfully used in other studies to detect homozygous and heterozygous genotypes based on melt curves and difference plots (Gundry *et al.,* 2003, Wittwer *et al.,* 2003; Reed and Wittwer, 2004; Montgomery *et al.,* 2007).

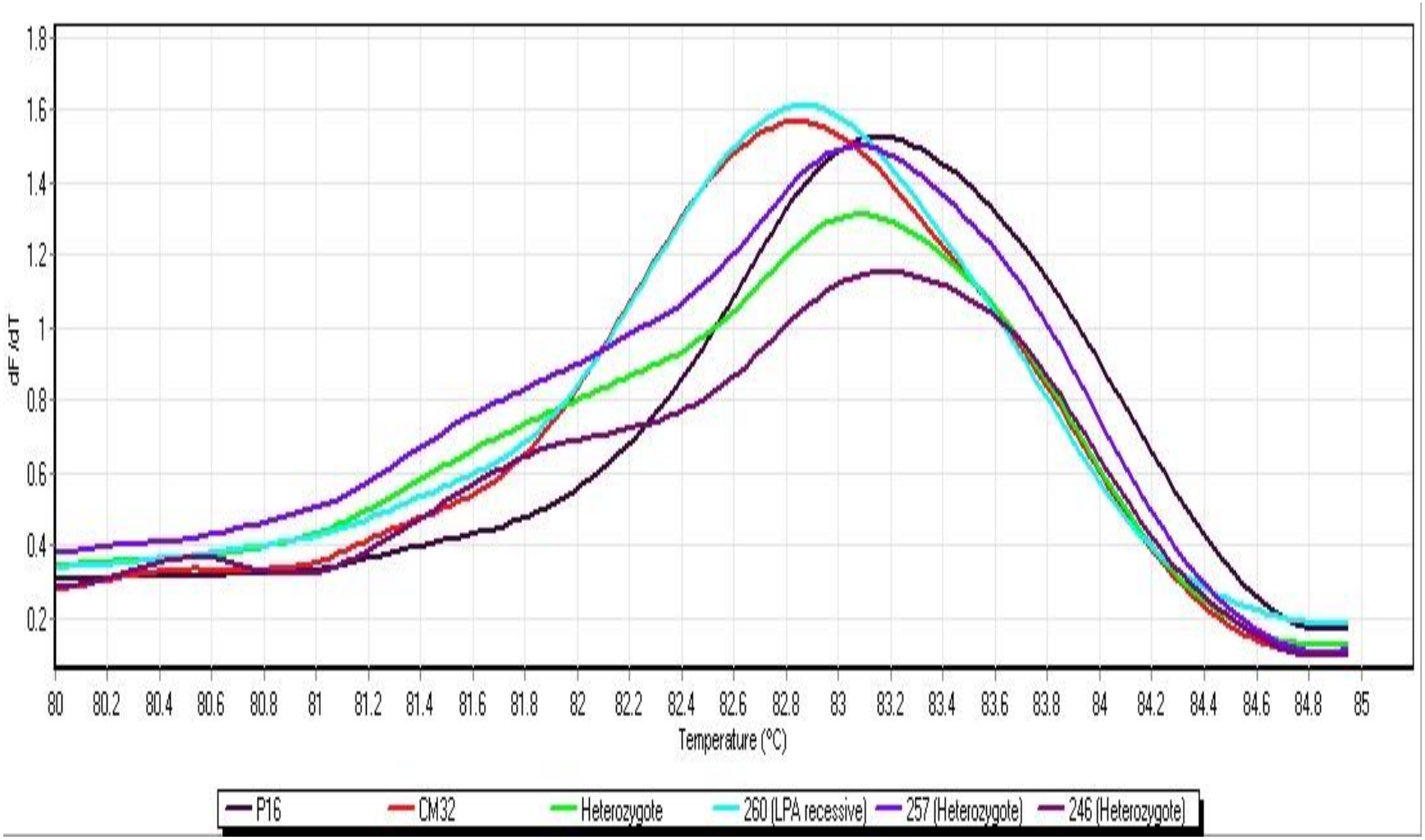

Figure 3.1: Melt profiles of parental lines, heterozygote and three of the selected BC_2F_1 lines (260, 257, 246)

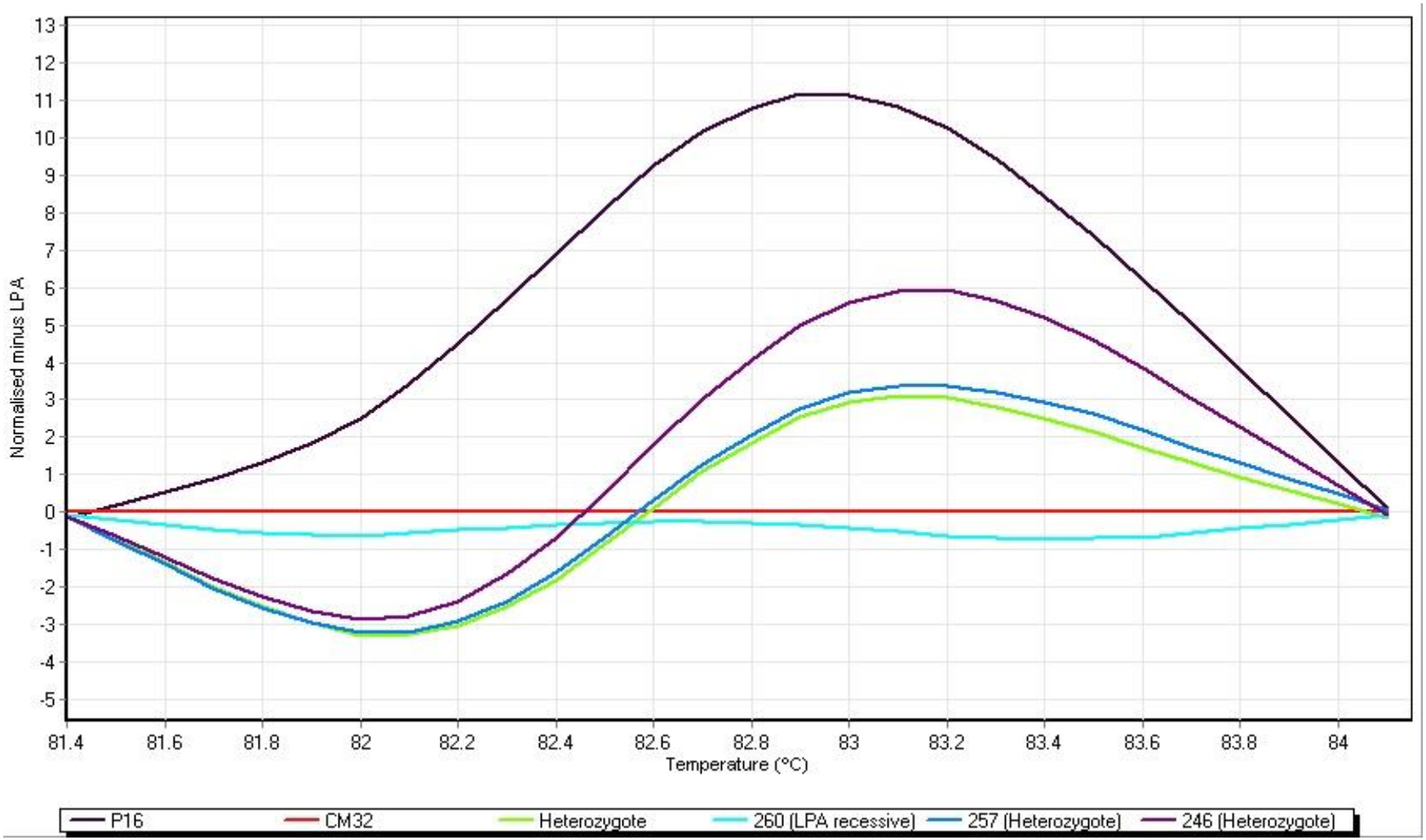

Figure 3.2: Difference plots of P 16, heterozygote and three of the selected BC_2F_1 lines with CM 32 (LPA) normalized

Table 3.2: R^2 values of SNP marker analysis and similarity percentage to recurrent parent for selected 28 BC_2F_1 individuals with six *Eco*RI/*Mse*I primer combinations of AFLP analysis

BC_2F_1 Line	**Genotype**	**R^2 (SNP)**	**Similarity % (recurrent parent P 16)**
119	Heterozygote	91.04	92.15
257	Heterozygote	99.55	90.65
235	LPA recessive	92.95	90.32
258	Heterozygote	96.55	89.80
246	Heterozygote	91.81	87.14
215	LPA recessive	90.15	86.54
264	LPA recessive	82.81	86.13
253	Heterozygote	96.04	85.79
117	LPA recessive	95.08	84.26
256	LPA recessive	97.20	83.94
136	LPA recessive	89.31	83.76
158	LPA recessive	93.12	83.37
271	Heterozygote	96.96	83.33
286	Heterozygote	93.77	82.07
176	Heterozygote	98.99	81.66
260	LPA recessive	99.58	81.05
138	LPA recessive	92.92	80.27
142	Heterozygote	91.89	80.09
252	LPA recessive	94.63	79.91
92	Heterozygote	91.31	79.82
242	Heterozygote	95.90	79.65
249	LPA recessive	92.88	79.19
78	Heterozygote	93.62	75.69
164	Heterozygote	99.59	73.73
145	Heterozygote	95.94	73.66
75	Heterozygote	98.60	72.90
265	Heterozygote	94.12	67.96
150	Heterozygote	90.95	62.12

In the initial AFLP screening of primer combinations on the two parental lines of the backcross breeding programme, there were ten *Eco*RI/*Mse*I primer combinations screened. Only eight *Eco*RI/*Mse*I primer combinations were selected for the 28 BC_2F_1 lines and the two parental lines of the backcross breeding programme. Due to incomplete data and poor amplification the other two primer combinations were not included in the combined data set. Each individual primer combination was analysed separately and a dendrogram based on the similarity values obtained. Two primer combinations were excluded from the combined data due to very high similarity percentage values between P 16 and CM 32 (*Eco*-AAG *Mse*-CAT and *Eco*-ACC *Mse*-CAC).These two combinations showed lower similarity vales for the BC lines than the donor parent. The data for six primer combinations were combined into one data set and analysed as the combined data set.

For the individual primer combinations, similarity values were obtained for each of the 28 BC_2F_1 lines and CM 32 (Table 3.3). The primer combination *Eco*-ACA *Mse*-CAT showed BC line 75 as zero percent similarity to the recurrent parent due to absence of similar bands between P 16 and 75. The amplification reaction of BC line 75 was repeated with the same results. It could be explained by the primer combination targeting an area that was not similar to the recurrent parent. Another possible explanation could be as a result of out-crossing that occured in this line. Also with this primer combination, there were two other BC lines that showed 100% similarity to the recurrent parent, having amplified exactly the same bands as the recurrent parent. Dendrograms were constructed for each of the primer combinations (Appendixes 1-8) with similarity matrices (Appendixes 9-16).

For the combined data, there was a total of 277 data points/bands scored with 43 (16%) non-polymorphic and 234 (84%) polymorphic bands scored between the parental lines of the backcross breeding programme (Table 3.4). The number of bands scored in each primer combination ranged from 34 (*Eco*-ACA *Mse*-CAT) to 59 (*Eco*-AGG *Mse*-CAT). The primer combinations, *Eco*-ACA *Mse*-CAA and *Eco*-ACA *Mse*-CAC showed the highest percentage of polymorphic bands (94%) with *Eco*-ACG *Mse*-CAA (90%) and *Eco*-ACG *Mse*-CAA (90%) and *Eco*-ACA *Mse*-CAT (88%) also showing high polymorphic rates. The primer combinations had a range of 6-28% of non-polymorphic bands and range of 72-94% of polymorphic bands. Positive polymorphic bands are defined as only present in the recurrent parent (P 16) (coupling markers) and negative polymorphic bands are present in the donor parent (CM 32). There were a total of 224 (81%) positive polymorphic bands in the combined data with 10 (4%) negative polymorphic bands (Table 3.4). All the primer combinations showed high positive polymorphic band percentages (>91%) showing the recurrent parent was highly polymorphic and generally amplified more fragments/bands compared to the donor parent.

There are numerous genetic diversity studies with AFLP markers on maize using different enzyme primer combinations showing differing polymorphic rates, average number of markers per primer combination and the range of number of markers amplified in each primer combination. The AFLP polymorphism rate differs due to the number for primer combinations used, number of polymorphic bands produced and the genetic background and number of inbred maize lines used. Examples of results using different *Eco*RI/*Mse*I primer combinations and bands produced include 284 polymorphic bands with 10 primer pairs (Hartings *et al*., 2008), 621 (59.8%) polymorphic bands with 16 primer combinations (Wu, 2000), 209 polymorphic bands (41.8%) with six primer combinations (Ajmone-Marsan *et al.,* 1998), 261 polymorphic bands (83%) with four primer combinations (Lübberstedt *et al.,* 2000), 232 polymorphic bands with six primer combinations (Pejic *et al.,* 1998) and 408 (81.7%) polymorphic bands with seven primer combinations (Legesse *et al.,* 2007). This study found 84% polymorphic bands with only six primer combinations tested which is relatively high considering only two inbred lines were compared. The type of restriction enzyme influences the polymorphism rate as *Eco*RI restriction sites are randomly located over the chromosome while *Pst*I is clustered at the methylation-specific sites and therefore *Pst*I primer combinations show a lower polymorphism ratio (Lübberstedt *et al.,* 2000).

Both polymorphic and non-polymorphic bands were selected for data points to determine the amount of similarity between the parental lines. The *lpa*1-1 source CM 32 showed a baseline similarity of 26.33% to P16, the recurrent parent with the combined data (Appendix 17). This is to be expected as they both are maize inbred lines although from different pedigrees and adaptation (tropical and temperate).

All the BC_2F_1 selected lines were clearly differentiated from each other and the parental controls in the combined data dendrogram based on the DICE coefficient of similarity (Figure 3.3). The combined similarity matrix (Appendix 17) generated by NTSYS was able to successfully differentiate between all 28 BC_2F_1 lines and donor parental line based on the similarity values to the recurrent parent. The similarity percentage values for the heterozygotes ranged from 62.12% to 92.15%, with the LPA homozygous recessives from 79.19% to 90.32% (Table 3.2). The BC lines were very similar to the recurrent parent and no groups were identified in the dendrogram due to the close clustering seen between the lines and parental lines.

A graphical representation of the selected BC_2F_1 lines R^2 values for the SNP analysis (HRM) and similarity percentage values (DICE coefficient) are shown in Figure 3.4.

Table 3.3: Similarity percentage values to the recurrent parent for the individual *Eco*RI/*Mse*I selective nucleotide primer combinations used for AFLP analysis of the 28 BC_2F_1 maize lines and CM 32

Line	*Eco*RI-AGG *Mse*I-CAA	*Eco*RI-AGG *Mse*I-CAT	*Eco*RI-AAG *Mse*I-CAT	*Eco*RI-ACA *Mse*I-CAA	*Eco*RI-ACA *Mse*I-CAC	*Eco*RI-ACA *Mse*I-CAT	*Eco*RI-ACC *Mse*I-CAC	*Eco*RI-ACG *Mse*I-CAA
CM32	28.26	27.12	70.49	5.56	6.00	11.76	37.50	7.69
75	84.78	67.80	54.10	55.56	42.00	0.00	58.33	78.85
78	76.09	69.49	60.66	58.33	50.00	55.88	66.67	57.69
92	71.74	74.58	62.30	52.78	60.00	55.88	75.00	78.85
117	80.43	71.19	67.21	63.89	60.00	88.24	79.17	78.85
119	97.83	88.14	75.41	72.22	74.00	94.12	62.50	88.46
136	80.43	76.27	47.54	61.11	54.00	88.24	87.50	76.92
138	84.78	69.49	55.74	50.00	50.00	82.35	79.17	71.15
142	69.57	72.88	50.82	66.67	54.00	76.47	70.83	69.23
145	63.04	54.24	47.54	55.56	50.00	79.41	62.50	59.62
150	30.43	52.54	47.54	47.22	34.00	58.82	50.00	53.85
158	78.26	72.88	62.30	58.33	58.00	94.12	50.00	75.00
164	63.04	57.63	37.70	47.22	56.00	73.53	37.50	57.69
176	78.26	71.19	57.38	63.89	48.00	85.29	66.67	75.00
215	84.78	77.97	59.02	75.00	64.00	97.06	75.00	67.31
235	91.30	76.27	63.93	69.44	74.00	94.12	79.17	92.31
242	84.78	76.27	54.10	47.22	28.00	97.06	70.83	71.15
246	86.96	74.58	49.18	77.78	66.00	100.00	75.00	69.23
249	78.26	64.41	65.57	58.33	50.00	91.18	79.17	63.46
252	78.26	71.19	62.30	58.33	50.00	79.41	70.83	65.38
253	82.61	77.97	62.30	55.56	0.00	91.18	79.17	71.15
256	78.26	77.97	4.92	61.11	64.00	91.18	79.17	67.31
257	93.48	81.36	4.92	72.22	66.00	100.00	83.33	90.38
258	97.83	77.97	4.92	61.11	72.00	97.06	83.33	86.54
260	80.43	76.27	4.92	61.11	40.00	97.06	45.83	63.46
264	86.96	76.27	4.92	69.44	64.00	79.41	54.17	80.77
265	78.26	54.24	34.43	41.67	24.00	58.82	4.17	57.69
271	91.30	69.49	67.21	63.89	48.00	94.12	4.17	71.15
286	80.43	77.97	62.30	50.00	54.00	85.29	79.17	71.15

Table 3.4: Levels of polymorphism and non-polymorphism among six *Eco*RI/*Mse*I selective nucleotide primer combinations used for AFLP analysis of the maize BC_2F_1 lines

Primer combination	Scored amplicon No.	Non-polymorphic amplicon No. (%)	Polymorphic amplicon No. (%)	Positive polymorphic amplicon No. (%)	Negative polymorphic amplicon No. (%)
*Eco*RI-AGG *Mse*I-CAT	59	16 (27%)	43 (73%)	39 (91%)	4 (9%)
*Eco*RI-AGG *Mse*I-CAA	46	13 (28%)	33 (72%)	32 (97%)	1 (3%)
*Eco*RI-ACG *Mse*I-CAA	52	5 (10%)	47 (90%)	45 (96%)	2 (4%)
*Eco*RI-ACA *Mse*I-CAT	34	4 (12%)	30 (88%)	30 (100%)	0 (0%)
*Eco*RI-ACA *Mse*I-CAA	36	2 (6%)	34 (94%)	33 (97%)	1 (3%)
*Eco*RI-ACA *Mse*I-CAC	50	3 (6%)	47 (94%)	45 (96%)	2 (4%)
Overall Total	**277**	**43 (16%)**	**234 (84%)**	**224 (81%)**	**10 (4%)**
Overall Mean	**46.2**	**7.2**	**39.0**	**37.3**	**1.7**

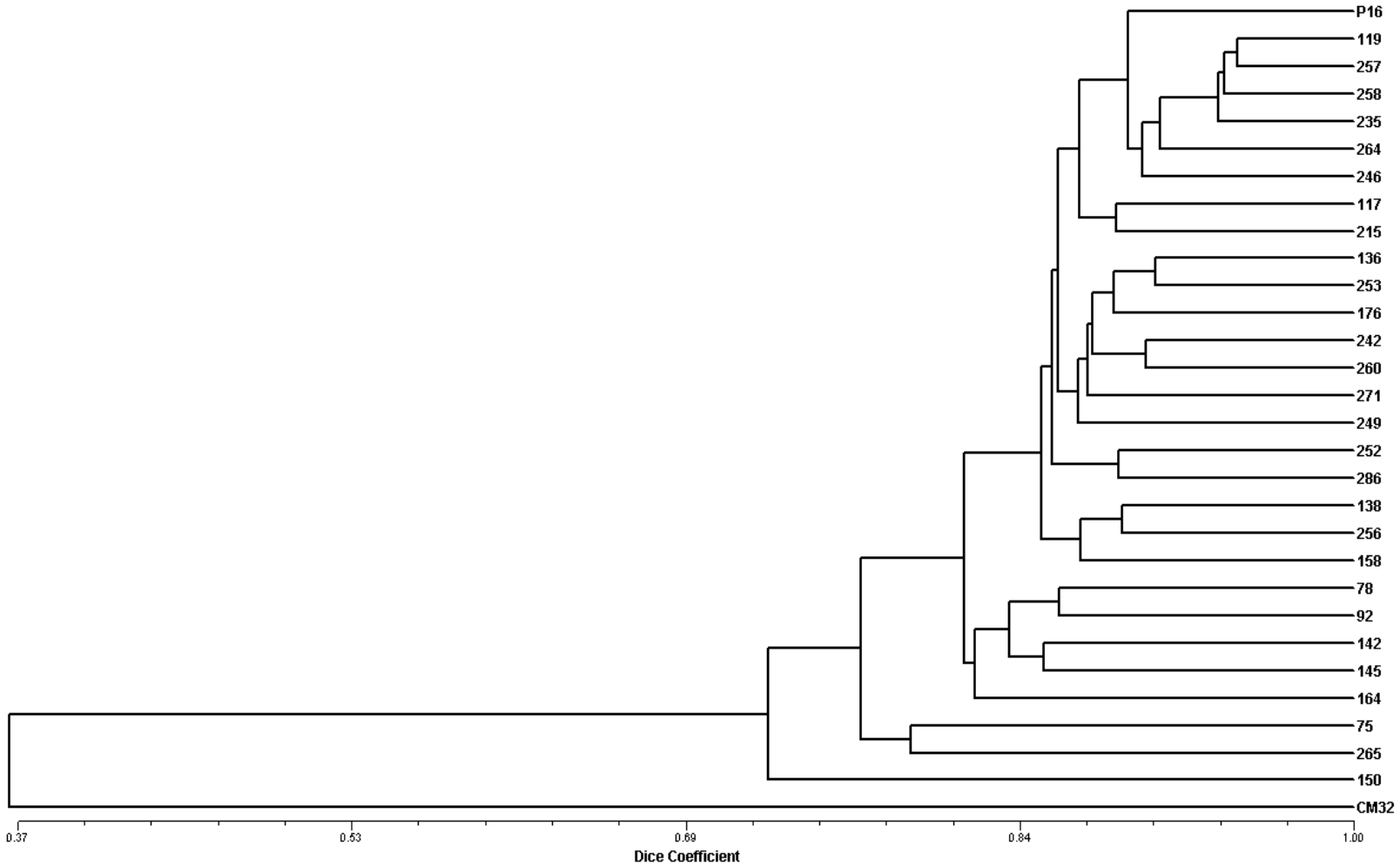

Figure 3.3: Dendrogram of fingerprinting 28 BC_2F_1 maize lines and the two parental lines (P 16 and CM 32) based on six primer combinations of *Eco*RI/*Mse*I AFLP data

For the combined data of all six primer combinations, there were 13 BC_2F_1 lines; seven LPA recessives [235 (90.32%), 215 (86.54%), 264 (86.13%), 117 (84.26%), 256 (83.94%), 136 (83.76%). 158 (83.37%)] and six heterozygous lines [119 (92.15%), 257 (90.65%), 258 (89.80), 246 (87.14%), 253 (85.79%), 271 (83.33%)] showing the highest similarity percentages therefore containing the highest percentage of the recurrent parent genome and can be selected for further improvement in the breeding programme.

According to the clustering on the dendrogram, CM 32 was closely related to three BC_2F_1 lines; 150 (50%), 265 (44%) and 75 (38%) (Appendix 17) therefore these lines have the lowest percentage of recurrent parent genome. Due to the high level of similiarity between the BC_2F_1 lines, it was not possible to group the lines in the combined data dendrogram (Figure 3.4). This is due to the BC_2F_1 generation which would have a high percentage of the recurrent parent genome (expected 87.5%) by this stage.

Studies on rice using AFLPs for background selection in the BC_1F_1 generation produced 364 polymorphic bands to obtain 81.4% of the recurrent parent genome (Jeung *et al.*, 2005), while a BC_3F_2 population had 98.8% recurrent parent recovery with 129 polymorphic bands (Chen *et al.*, 2001), and selection in the BC_3F_1 generation resulted in 84.2 to 100% recovery of the recurrent parent with 118 polymorphic bands (Zhou *et al.*, 2003). In this study for introgression of the *lpa1-1* gene into the recurrent parent, selection was in the BC_2F_1 generation with 234 polymorphic bands resulting in a maximum recovery of 92.15% of the recurrent parent genome. This was higher than the expected BC_2 recovery of 87.5% in the BC_2 generation.

Selection of suitable BC_2F_1 lines would be in the range of high R^2 SNP values with high similarity percentage values. The choice of heterozygous or homozygous recessive lines would also be part of the selection process, with homozygous recessive lines being preferred due to both alleles of the *lpa1-1* gene being present and fixed. All 28 BC_2F_1 lines will have to undergo further testing under field conditions to determine yield potential, adaptability to local environmental conditions and disease assessment. A recommendation for other MAS studies would be to perform foreground selection in the BC_1 generation and both foreground and background selection in the BC_2 generation. Due to the high percentage of recurrent parent recovered in the BC_2 generation, there is no need for a further backcross generation. A promising candidate for MAS for LPA mutant detection is the use of SNP markers accompanied by a high throughput analysis method such as HRM analysis. This will lead to an improved effective breeding programme enhanced with MAS to transfer genes of interest and accelerate the recovery of the elite parent genome in backcrossing breeding programmes.

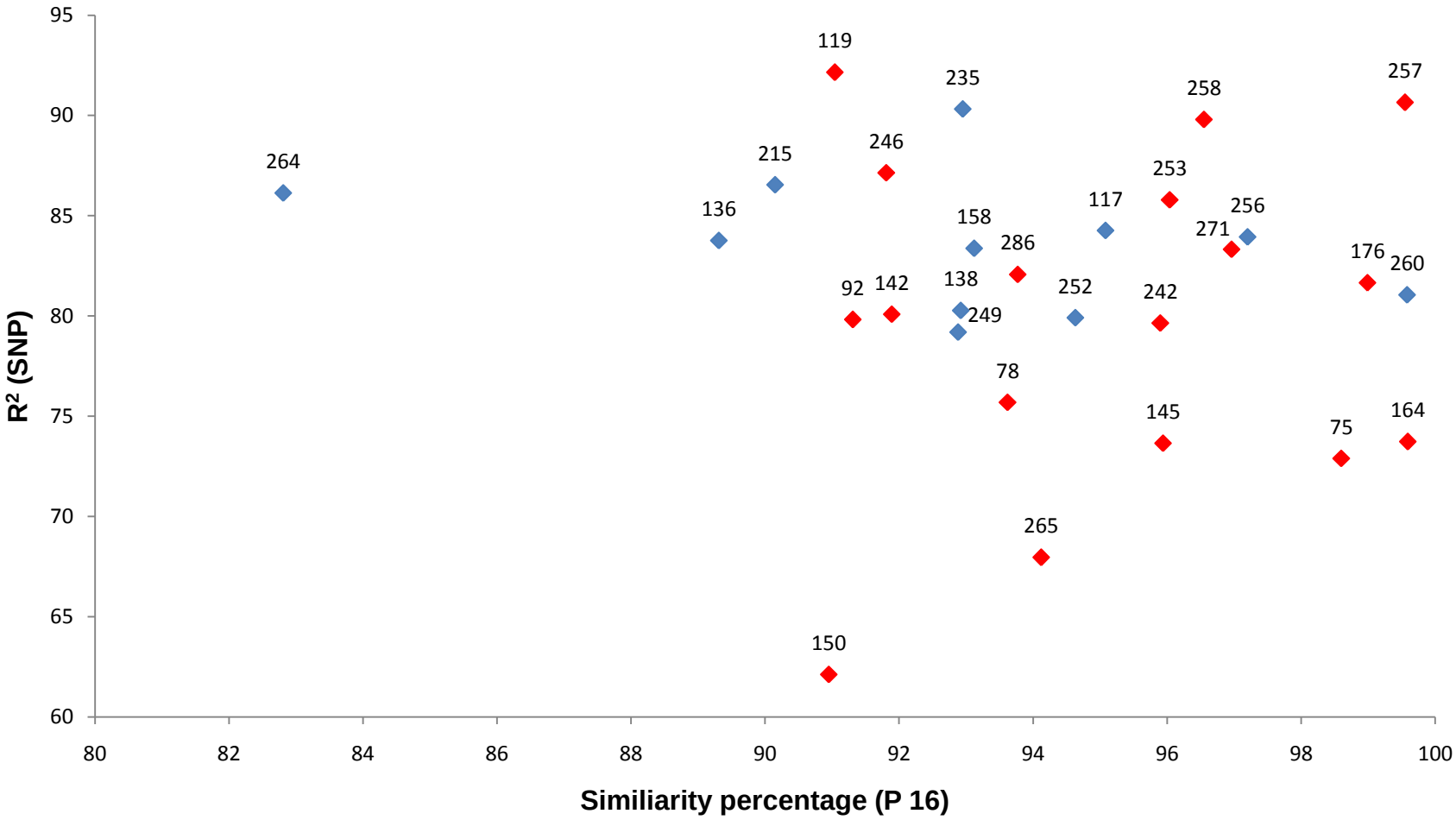

Figure 3.4: Graph of R^2 (SNP analysis) and similarity percentage to the recurrent parent (P 16) for 28 BC_2F_1 lines (Heterozygous genotypes are depicted with red diamonds and LPA recessives genotypes as blue diamonds)

Conclusions

The backcross breeding programme to introgress the *lpa1-1* gene into tropical and subtropical adapted inbred line (P 16) was successful with both foreground and background selection phases with molecular markers. The foreground selection for the *lpa1-1* gene was efficient and rapid with the *lpa1-1* SNP marker with HRM analysis for detection of the different alleles of the gene. Due to the co-dominant nature of the *lpa1-1* SNP, it was able to correctly identify the homozygous dominant (wild type), homozygous recessive (LPA) and heterozygote lines of the BC_2F_1 population. The marker-assay with the SNP marker and HRM proved to be effective and would be less time-consuming than the traditional approach. There were 250 BC_2F_1 lines screened for the *lpa1-1* gene with 11 homozygous recessive (LPA) and 17 heterozygous lines being selected for background selection using AFLP markers. The AFLP technique used six *Eco*RI/*Mse*I primer combinations to produce 84% of polymorphic bands between the parental inbred lines. The percentage of recurrent parent genome recovered ranged from 62.12% to 92.15%. There were 13 lines showing >83% of which four lines show >87.5% of the recurrent parent genome and are recommended for further advancement and field assessment. A further improvement would be to select in the BC_1 generation with the *lpa1-1* SNP marker and then perform both foreground and background selection using AFLPs in the BC_2 generation.

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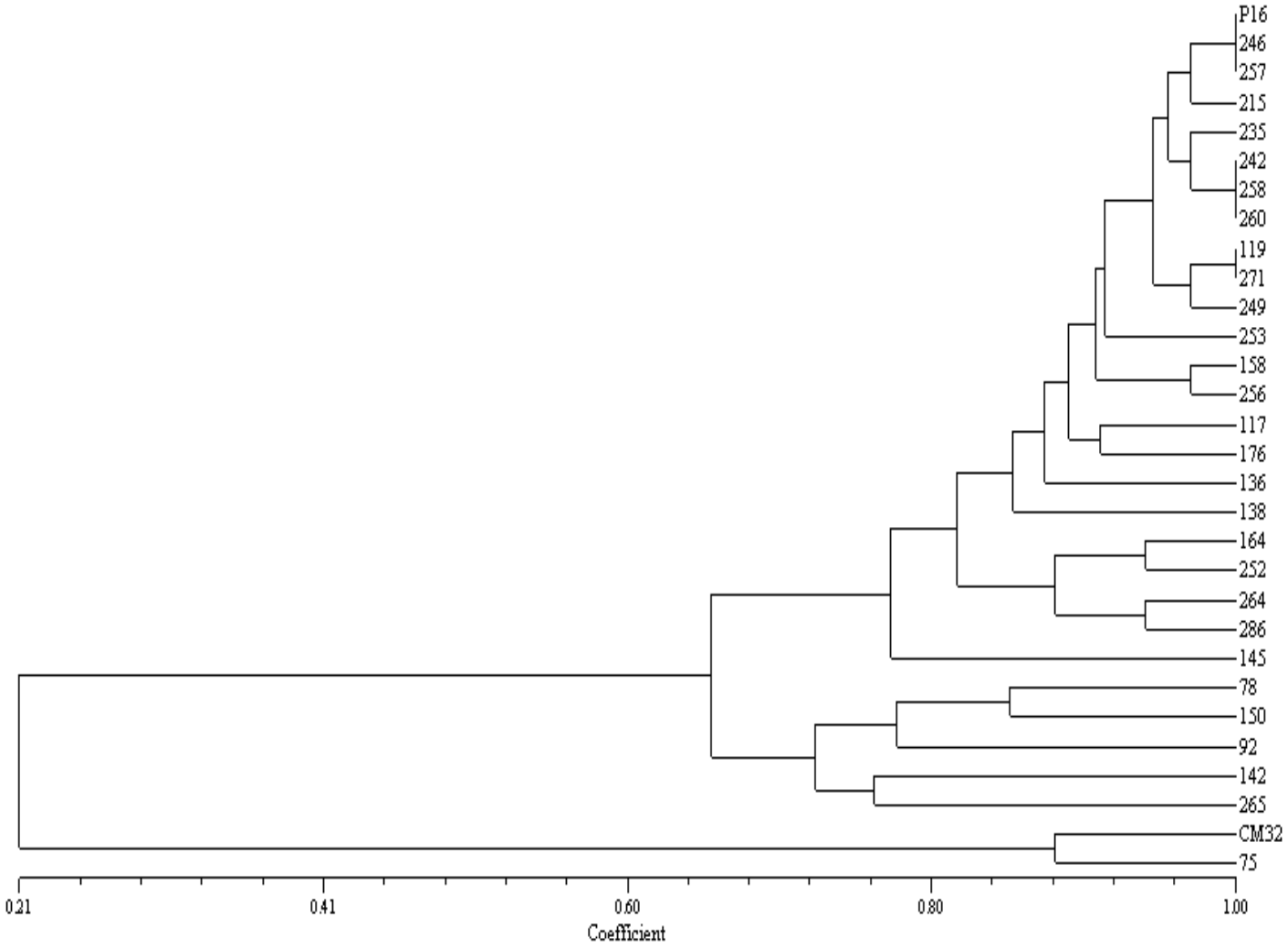
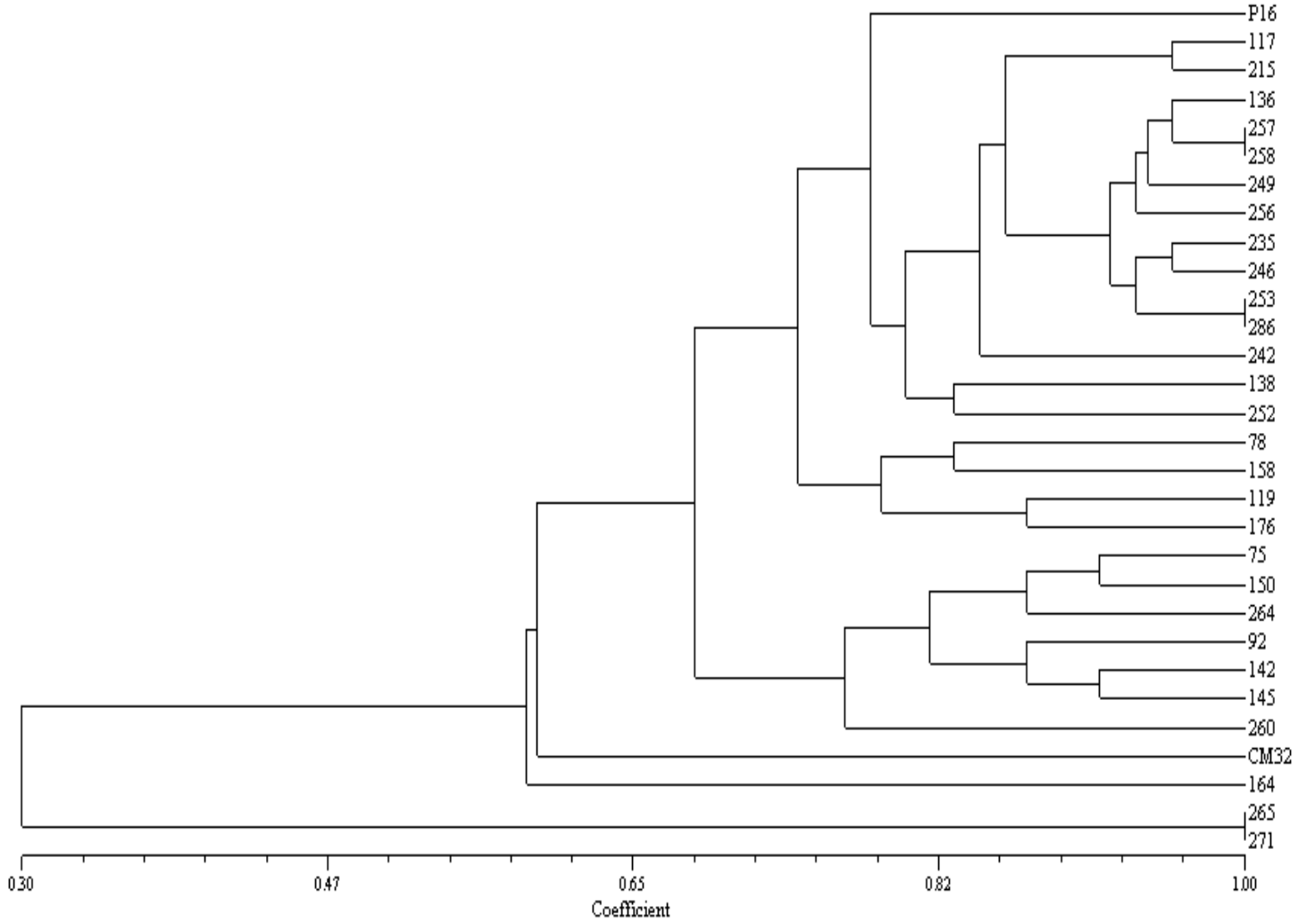
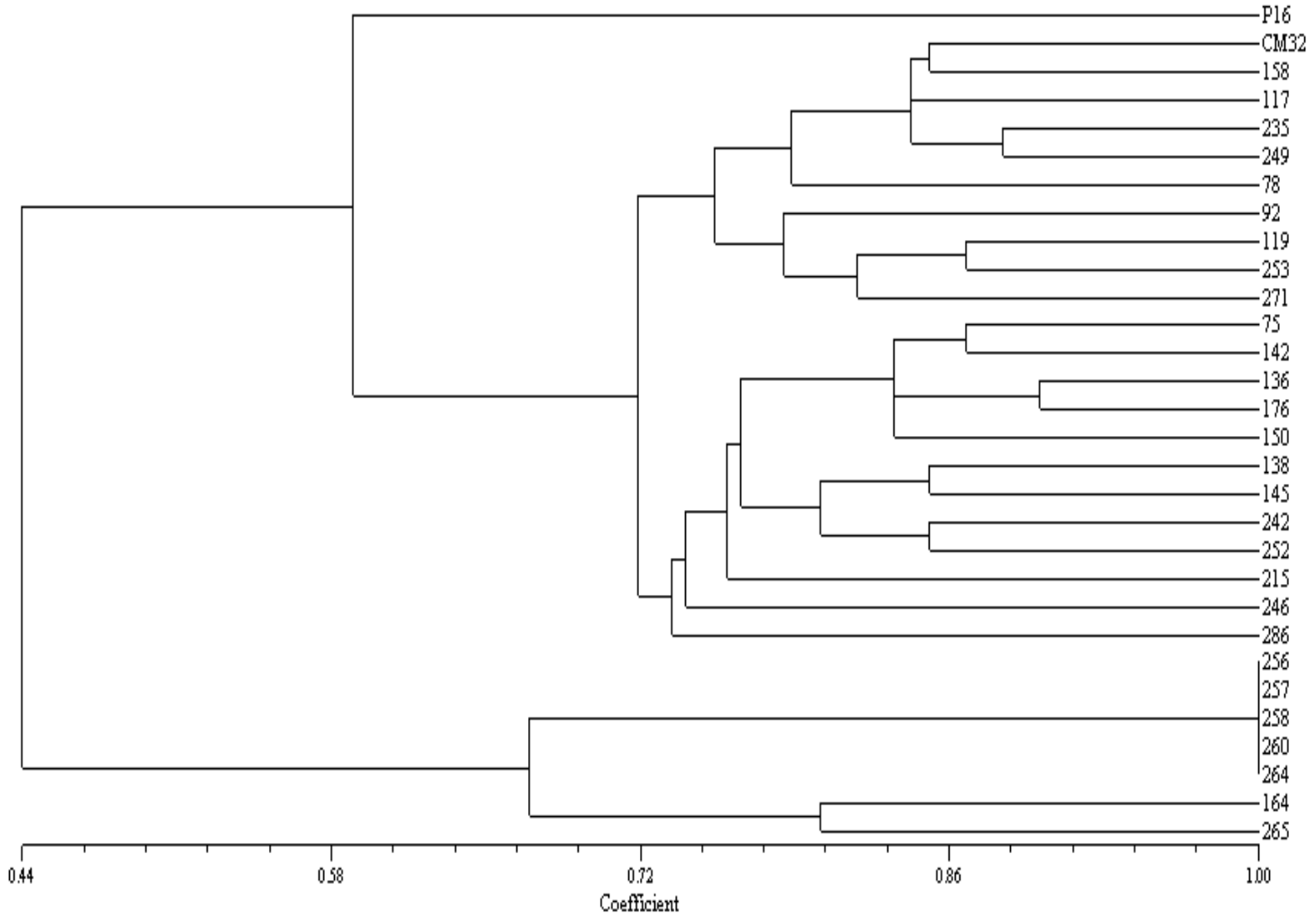
Appendix 1: Dendrogram of AFLP primer combination *Eco*RI-ACA *Mse*I-CAT



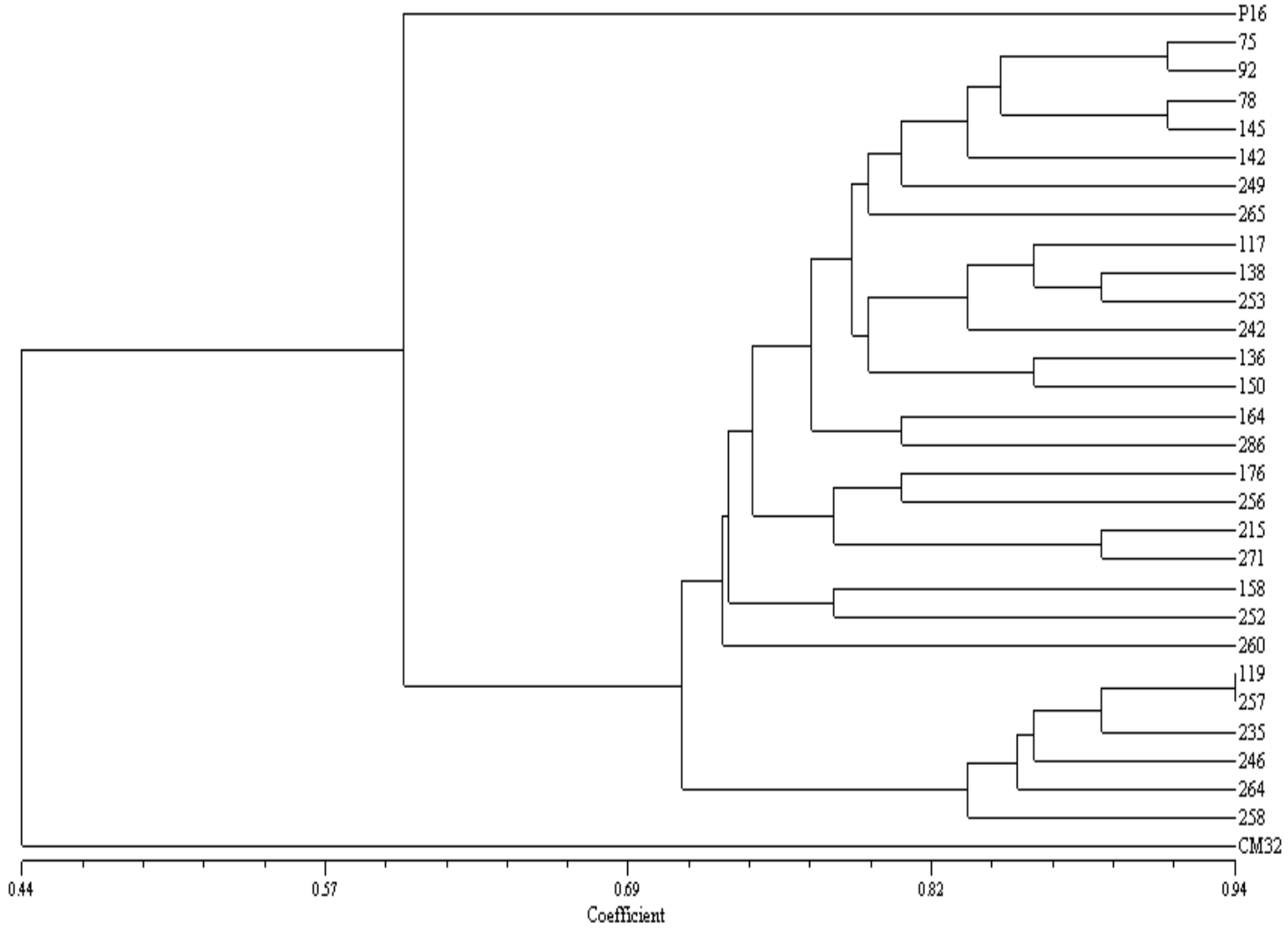
Appendix 2: Dendrogram of AFLP primer combination *Eco*RI-ACC *Mse*I-CAC



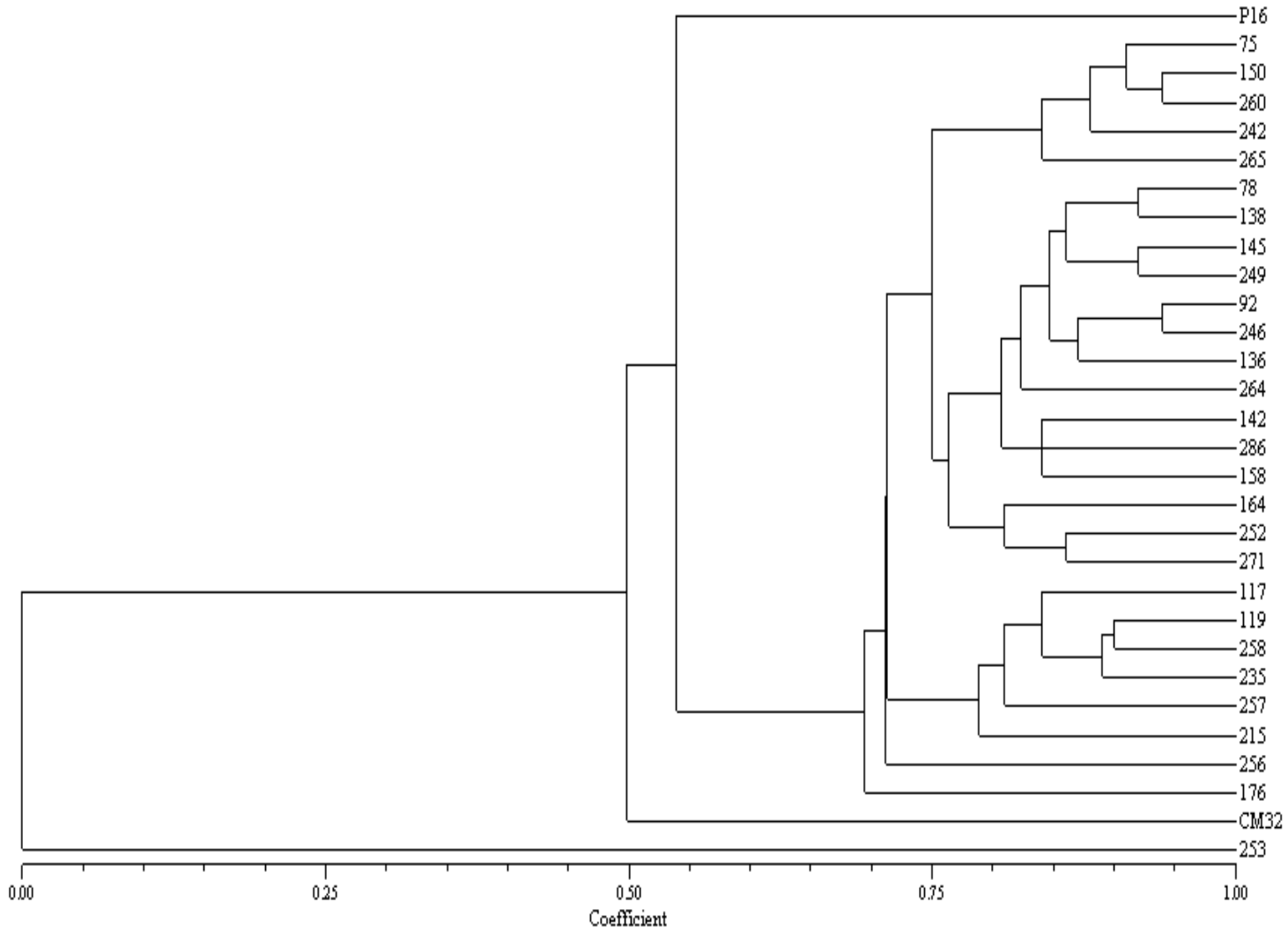
Appendix 3: Dendrogram of AFLP primer combination *Eco*RI-AAG *Mse*I-CAT



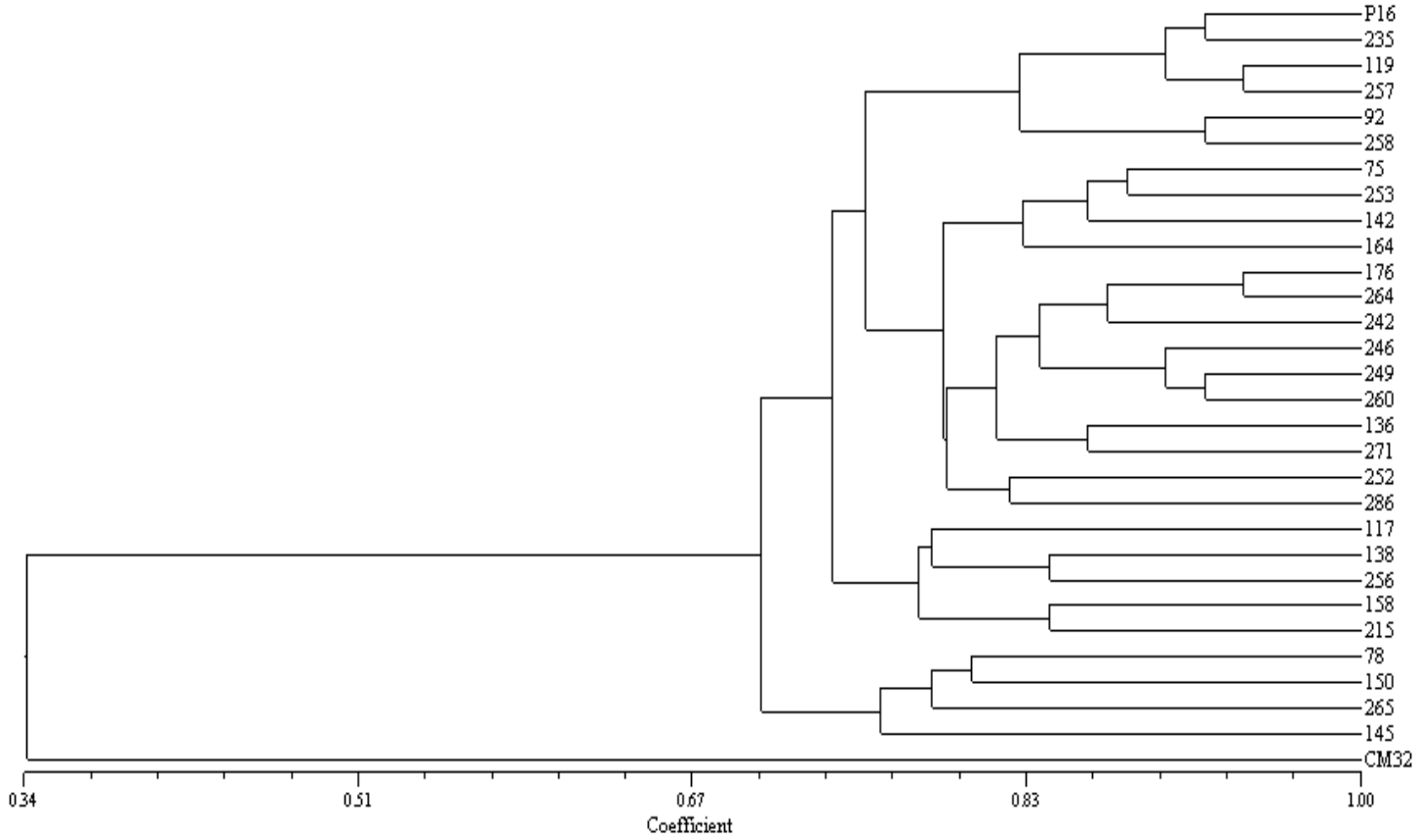
Appendix 4: Dendrogram of AFLP primer combination *Eco*RI-ACA *Mse*I-CAA



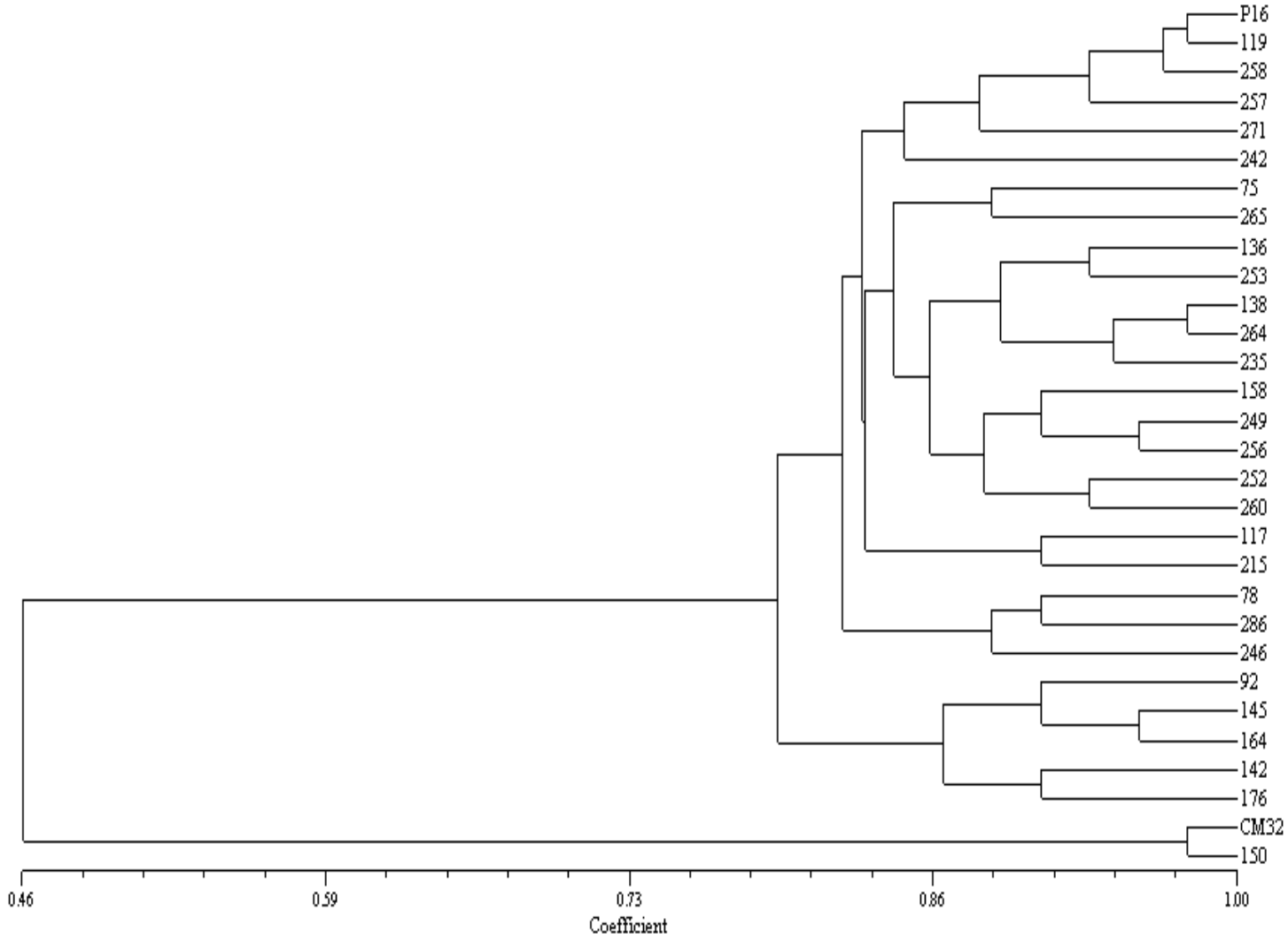
Appendix 5: Dendrogram of AFLP primer combination *Eco*RI-ACA *Mse*I-CAC



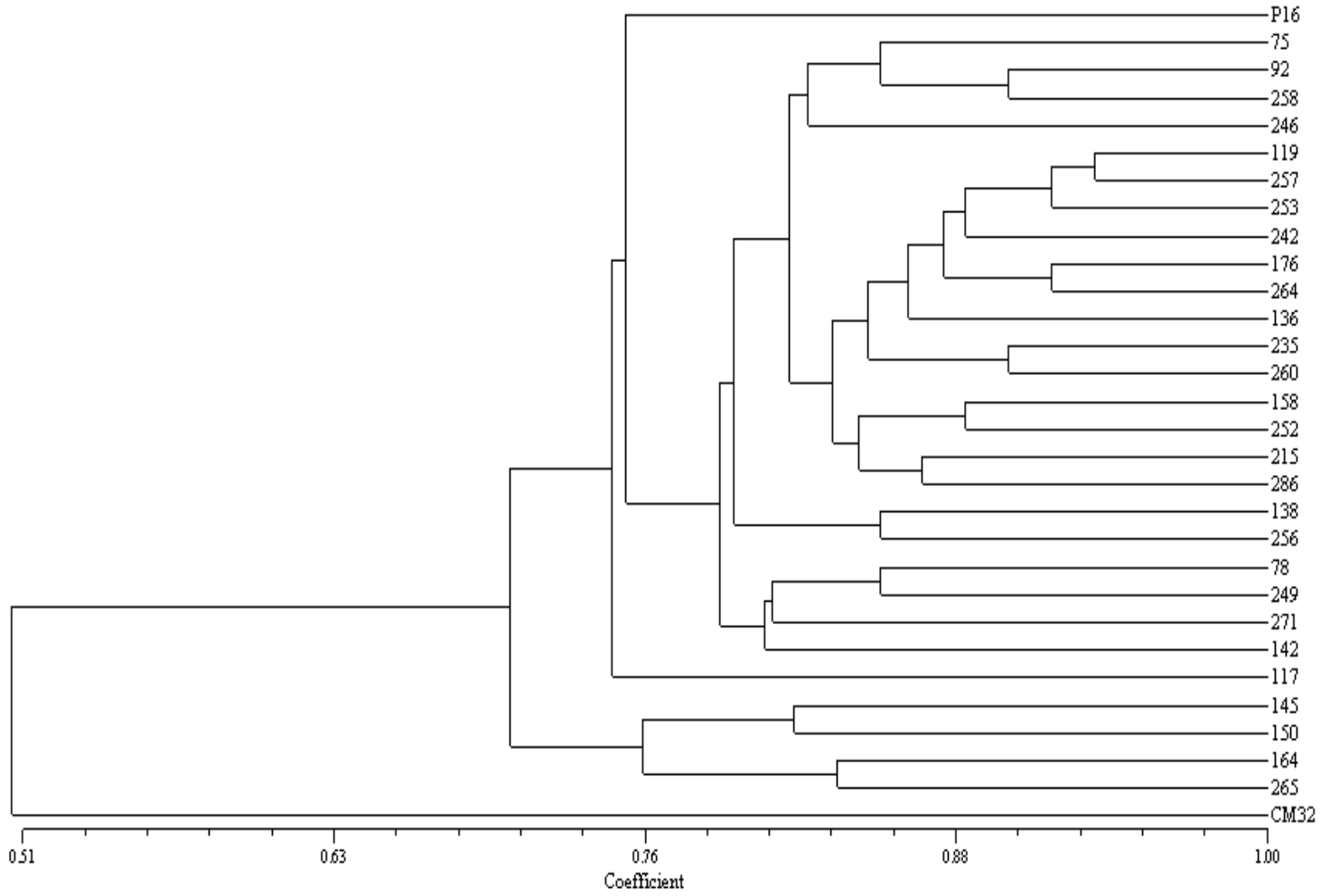
Appendix 6: Dendrogram of AFLP primer combination *Eco*RI-AGG *Mse*I-CAT



Appendix 7: Dendrogram of AFLP primer combination *Eco*RI-AGG *Mse*I-CAA



Appendix 8: Dendrogram of AFLP primer combination *Eco*RI-ACG *Mse*I-CAA



Appendix 9: Similarity matrix of AFLP primer combination *Eco*RI-AGG *Mse*I-CAA

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.28	1.00																												
75	0.85	0.43	1.00																											
78	0.76	0.52	0.83	1.00																										
92	0.72	0.57	0.83	0.87	1.00																									
117	0.80	0.43	0.83	0.78	0.87	1.00																								
119	0.98	0.30	0.83	0.78	0.74	0.78	1.00																							
136	0.80	0.43	0.83	0.74	0.83	0.87	0.78	1.00																						
138	0.85	0.43	0.87	0.83	0.87	0.91	0.83	0.91	1.00																					
142	0.70	0.59	0.76	0.80	0.85	0.85	0.67	0.80	0.80	1.00																				
145	0.63	0.65	0.78	0.87	0.91	0.78	0.65	0.78	0.78	0.89	1.00																			
150	0.30	0.98	0.41	0.50	0.54	0.46	0.33	0.46	0.46	0.61	0.63	1.00																		
158	0.78	0.50	0.80	0.76	0.85	0.85	0.80	0.85	0.89	0.78	0.85	0.52	1.00																	
164	0.63	0.65	0.78	0.83	0.91	0.78	0.65	0.78	0.78	0.89	0.96	0.63	0.85	1.00																
176	0.78	0.50	0.85	0.85	0.89	0.89	0.76	0.85	0.89	0.91	0.85	0.52	0.78	0.85	1.00															
215	0.85	0.39	0.78	0.78	0.83	0.91	0.83	0.78	0.83	0.76	0.74	0.37	0.76	0.74	0.80	1.00														
235	0.91	0.37	0.89	0.76	0.80	0.89	0.89	0.89	0.93	0.78	0.72	0.39	0.83	0.72	0.87	0.85	1.00													
242	0.85	0.43	0.83	0.78	0.74	0.83	0.83	0.78	0.87	0.76	0.70	0.46	0.76	0.70	0.85	0.78	0.85	1.00												
246	0.87	0.41	0.85	0.89	0.85	0.80	0.89	0.80	0.89	0.70	0.76	0.39	0.83	0.76	0.78	0.85	0.83	0.85	1.00											
249	0.78	0.50	0.85	0.85	0.93	0.89	0.80	0.85	0.93	0.83	0.85	0.52	0.91	0.85	0.87	0.80	0.87	0.80	0.87	1.00										
252	0.78	0.50	0.85	0.85	0.89	0.89	0.80	0.85	0.93	0.78	0.85	0.52	0.91	0.85	0.87	0.80	0.87	0.80	0.87	0.91	1.00									
253	0.83	0.46	0.85	0.76	0.85	0.80	0.80	0.93	0.89	0.83	0.80	0.48	0.83	0.80	0.87	0.76	0.91	0.76	0.78	0.87	0.83	1.00								
256	0.78	0.50	0.85	0.80	0.89	0.85	0.80	0.80	0.89	0.78	0.85	0.52	0.91	0.85	0.83	0.80	0.83	0.80	0.87	0.96	0.91	0.83	1.00							
257	0.93	0.35	0.87	0.78	0.78	0.87	0.91	0.87	0.91	0.76	0.70	0.37	0.80	0.70	0.85	0.87	0.93	0.87	0.89	0.85	0.85	0.85	0.85	1.00						
258	0.98	0.30	0.87	0.78	0.74	0.83	0.96	0.83	0.87	0.72	0.65	0.33	0.76	0.65	0.80	0.87	0.93	0.87	0.89	0.80	0.80	0.85	0.80	0.96	1.00					
260	0.80	0.48	0.83	0.83	0.83	0.87	0.78	0.83	0.91	0.80	0.83	0.50	0.85	0.83	0.89	0.83	0.85	0.87	0.85	0.85	0.93	0.80	0.89	0.87	0.83	1.00				
264	0.87	0.41	0.89	0.80	0.85	0.93	0.85	0.89	0.98	0.83	0.76	0.43	0.87	0.76	0.91	0.85	0.96	0.89	0.87	0.91	0.91	0.87	0.87	0.93	0.89	0.89	1.00			
265	0.78	0.50	0.89	0.76	0.80	0.85	0.76	0.80	0.85	0.83	0.80	0.52	0.83	0.80	0.87	0.76	0.87	0.80	0.74	0.87	0.83	0.83	0.87	0.85	0.80	0.85	0.87	1.00		
271	0.91	0.37	0.85	0.76	0.76	0.80	0.89	0.80	0.89	0.70	0.72	0.39	0.87	0.72	0.78	0.80	0.87	0.85	0.87	0.83	0.87	0.83	0.87	0.85	0.89	0.89	0.87	0.78	1.00	
286	0.80	0.48	0.87	0.91	0.91	0.87	0.83	0.78	0.87	0.80	0.83	0.46	0.80	0.83	0.89	0.87	0.85	0.78	0.89	0.89	0.89	0.80	0.85	0.87	0.83	0.83	0.89	0.80	0.76	1.00

Appendix 10: Similarity matrix of AFLP primer combination *Eco*RI-AGG *Mse*I-CAT

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.27	1.00																												
75	0.68	0.53	1.00																											
78	0.69	0.51	0.78	1.00																										
92	0.75	0.49	0.86	0.81	1.00																									
117	0.71	0.49	0.73	0.71	0.76	1.00																								
119	0.88	0.39	0.76	0.81	0.80	0.73	1.00																							
136	0.76	0.51	0.78	0.73	0.81	0.78	0.85	1.00																						
138	0.69	0.54	0.68	0.73	0.75	0.78	0.75	0.80	1.00																					
142	0.73	0.51	0.75	0.80	0.81	0.68	0.81	0.83	0.76	1.00																				
145	0.54	0.69	0.73	0.75	0.73	0.63	0.63	0.71	0.68	0.78	1.00																			
150	0.53	0.68	0.78	0.76	0.78	0.68	0.61	0.73	0.73	0.76	0.81	1.00																		
158	0.73	0.51	0.75	0.80	0.75	0.71	0.85	0.86	0.76	0.80	0.68	0.69	1.00																	
164	0.58	0.59	0.76	0.78	0.73	0.69	0.69	0.64	0.68	0.68	0.66	0.78	0.68	1.00																
176	0.71	0.46	0.80	0.78	0.83	0.73	0.83	0.85	0.81	0.81	0.63	0.75	0.81	0.76	1.00															
215	0.78	0.49	0.73	0.81	0.80	0.76	0.86	0.85	0.78	0.78	0.69	0.68	0.85	0.69	0.86	1.00														
235	0.76	0.47	0.81	0.80	0.81	0.75	0.85	0.80	0.73	0.69	0.64	0.73	0.80	0.75	0.81	0.81	1.00													
242	0.76	0.51	0.78	0.76	0.78	0.75	0.88	0.86	0.76	0.76	0.64	0.66	0.86	0.68	0.81	0.81	0.83	1.00												
246	0.75	0.49	0.80	0.75	0.83	0.76	0.80	0.85	0.71	0.75	0.69	0.71	0.78	0.73	0.83	0.83	0.81	0.81	1.00											
249	0.64	0.56	0.76	0.85	0.76	0.73	0.76	0.78	0.78	0.81	0.73	0.75	0.81	0.80	0.76	0.73	0.71	0.81	0.73	1.00										
252	0.71	0.56	0.73	0.75	0.73	0.80	0.83	0.85	0.78	0.75	0.66	0.71	0.88	0.69	0.76	0.83	0.81	0.88	0.76	0.76	1.00									
253	0.78	0.46	0.80	0.81	0.80	0.73	0.90	0.88	0.78	0.81	0.66	0.71	0.85	0.76	0.90	0.86	0.85	0.88	0.86	0.83	0.80	1.00								
256	0.78	0.49	0.73	0.85	0.76	0.80	0.86	0.81	0.85	0.75	0.66	0.68	0.78	0.73	0.80	0.83	0.85	0.85	0.76	0.83	0.80	0.86	1.00							
257	0.81	0.42	0.83	0.81	0.80	0.69	0.93	0.85	0.78	0.81	0.66	0.68	0.85	0.69	0.86	0.83	0.88	0.88	0.80	0.76	0.80	0.93	0.86	1.00						
258	0.78	0.49	0.83	0.81	0.90	0.76	0.90	0.88	0.81	0.81	0.73	0.71	0.81	0.73	0.83	0.83	0.85	0.88	0.83	0.83	0.80	0.90	0.86	0.90	1.00					
260	0.76	0.47	0.78	0.73	0.81	0.71	0.85	0.83	0.76	0.73	0.61	0.69	0.83	0.71	0.85	0.78	0.90	0.86	0.81	0.75	0.78	0.88	0.81	0.88	0.88	1.00				
264	0.76	0.44	0.81	0.83	0.81	0.78	0.88	0.86	0.80	0.80	0.64	0.69	0.86	0.78	0.92	0.85	0.80	0.90	0.85	0.85	0.81	0.92	0.85	0.88	0.88	0.83	1.00			
265	0.54	0.63	0.80	0.81	0.76	0.63	0.66	0.68	0.71	0.75	0.76	0.81	0.75	0.83	0.76	0.69	0.75	0.68	0.66	0.83	0.66	0.73	0.73	0.73	0.73	0.71	0.75	1.00		
271	0.69	0.51	0.75	0.83	0.71	0.68	0.81	0.80	0.73	0.80	0.64	0.73	0.80	0.75	0.85	0.81	0.80	0.80	0.75	0.78	0.75	0.88	0.85	0.85	0.78	0.80	0.83	0.71	1.00	
286	0.78	0.49	0.76	0.81	0.76	0.80	0.83	0.81	0.71	0.71	0.66	0.68	0.81	0.73	0.76	0.86	0.85	0.88	0.83	0.80	0.86	0.86	0.83	0.80	0.83	0.78	0.85	0.66	0.78	1.00

Appendix 11: Similarity matrix of AFLP primer combination *Eco*RI-AAG *Mse*I-CAT

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.70	1.00																												
75	0.54	0.64	1.00																											
78	0.61	0.77	0.67	1.00																										
92	0.62	0.72	0.79	0.75	1.00																									
117	0.67	0.84	0.70	0.84	0.72	1.00																								
119	0.75	0.79	0.72	0.75	0.80	0.75	1.00																							
136	0.48	0.67	0.80	0.67	0.72	0.67	0.66	1.00																						
138	0.56	0.75	0.72	0.82	0.74	0.79	0.67	0.75	1.00																					
142	0.51	0.67	0.87	0.70	0.75	0.67	0.69	0.84	0.82	1.00																				
145	0.48	0.74	0.70	0.74	0.69	0.70	0.59	0.80	0.85	0.77	1.00																			
150	0.48	0.67	0.80	0.67	0.69	0.67	0.62	0.84	0.82	0.87	0.84	1.00																		
158	0.62	0.85	0.69	0.79	0.74	0.85	0.70	0.72	0.74	0.69	0.72	0.72	1.00																	
164	0.38	0.54	0.77	0.54	0.66	0.54	0.56	0.74	0.62	0.80	0.70	0.77	0.59	1.00																
176	0.57	0.77	0.84	0.74	0.82	0.74	0.75	0.90	0.79	0.87	0.77	0.84	0.79	0.74	1.00															
215	0.59	0.75	0.72	0.72	0.74	0.72	0.70	0.75	0.77	0.75	0.75	0.75	0.77	0.62	0.82	1.00														
235	0.64	0.87	0.77	0.80	0.79	0.87	0.79	0.74	0.75	0.70	0.70	0.70	0.85	0.64	0.80	0.72	1.00													
242	0.54	0.80	0.70	0.77	0.69	0.77	0.62	0.80	0.82	0.74	0.84	0.74	0.82	0.64	0.80	0.75	0.77	1.00												
246	0.49	0.69	0.69	0.66	0.64	0.66	0.64	0.79	0.74	0.69	0.72	0.75	0.70	0.62	0.75	0.70	0.69	0.79	1.00											
249	0.66	0.82	0.79	0.75	0.84	0.82	0.77	0.79	0.77	0.75	0.72	0.75	0.84	0.66	0.85	0.80	0.89	0.75	0.70	1.00										
252	0.62	0.79	0.72	0.72	0.77	0.75	0.70	0.75	0.80	0.75	0.75	0.75	0.77	0.56	0.79	0.77	0.72	0.85	0.80	0.80	1.00									
253	0.62	0.72	0.72	0.75	0.77	0.75	0.87	0.75	0.67	0.72	0.62	0.72	0.70	0.66	0.82	0.74	0.79	0.62	0.67	0.77	0.67	1.00								
256	0.05	0.25	0.48	0.34	0.39	0.28	0.23	0.51	0.43	0.51	0.51	0.51	0.33	0.67	0.41	0.36	0.31	0.44	0.46	0.33	0.36	0.33	1.00							
257	0.05	0.25	0.48	0.34	0.39	0.28	0.23	0.51	0.43	0.51	0.51	0.51	0.33	0.67	0.41	0.36	0.31	0.44	0.46	0.33	0.36	0.33	1.00	1.00						
258	0.05	0.25	0.48	0.34	0.39	0.28	0.23	0.51	0.43	0.51	0.51	0.51	0.33	0.67	0.41	0.36	0.31	0.44	0.46	0.33	0.36	0.33	1.00	1.00	1.00					
260	0.05	0.25	0.48	0.34	0.39	0.28	0.23	0.51	0.43	0.51	0.51	0.51	0.33	0.67	0.41	0.36	0.31	0.44	0.46	0.33	0.36	0.33	1.00	1.00	1.00	1.00				
264	0.05	0.25	0.48	0.34	0.39	0.28	0.23	0.51	0.43	0.51	0.51	0.51	0.33	0.67	0.41	0.36	0.31	0.44	0.46	0.33	0.36	0.33	1.00	1.00	1.00	1.00	1.00			
265	0.34	0.51	0.74	0.57	0.56	0.61	0.52	0.74	0.72	0.77	0.74	0.80	0.59	0.80	0.67	0.59	0.57	0.67	0.72	0.59	0.62	0.59	0.67	0.67	0.67	0.67	0.67	1.00		
271	0.67	0.74	0.80	0.74	0.79	0.70	0.82	0.77	0.69	0.77	0.61	0.74	0.75	0.64	0.80	0.72	0.80	0.67	0.72	0.79	0.75	0.82	0.34	0.34	0.34	0.34	0.34	0.61	1.00	
286	0.62	0.72	0.72	0.69	0.70	0.66	0.64	0.69	0.77	0.75	0.75	0.82	0.74	0.59	0.75	0.70	0.72	0.69	0.70	0.70	0.74	0.64	0.36	0.36	0.36	0.36	0.36	0.62	0.72	1.00

Appendix 12: Similarity matrix of AFLP primer combination *Eco*RI-ACA *Mse*I-CAA

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.06	1.00																												
75	0.56	0.50	1.00																											
78	0.58	0.47	0.86	1.00																										
92	0.53	0.53	0.92	0.78	1.00																									
117	0.64	0.42	0.81	0.78	0.72	1.00																								
119	0.72	0.33	0.78	0.81	0.75	0.81	1.00																							
136	0.61	0.44	0.72	0.81	0.75	0.81	0.83	1.00																						
138	0.50	0.56	0.78	0.86	0.69	0.86	0.72	0.83	1.00																					
142	0.67	0.39	0.83	0.86	0.81	0.75	0.83	0.83	0.78	1.00																				
145	0.56	0.50	0.89	0.92	0.86	0.81	0.78	0.83	0.83	0.83	1.00																			
150	0.47	0.58	0.75	0.83	0.72	0.78	0.69	0.86	0.86	0.75	0.86	1.00																		
158	0.58	0.47	0.75	0.78	0.67	0.78	0.75	0.75	0.81	0.81	0.81	0.78	1.00																	
164	0.47	0.53	0.75	0.78	0.78	0.72	0.69	0.75	0.75	0.75	0.81	0.78	0.61	1.00																
176	0.64	0.42	0.69	0.72	0.67	0.72	0.69	0.81	0.69	0.75	0.75	0.78	0.67	0.72	1.00															
215	0.75	0.31	0.81	0.83	0.72	0.83	0.86	0.75	0.75	0.75	0.81	0.72	0.67	0.72	0.78	1.00														
235	0.69	0.36	0.75	0.72	0.72	0.78	0.86	0.69	0.64	0.75	0.69	0.56	0.67	0.61	0.61	0.78	1.00													
242	0.47	0.53	0.81	0.78	0.78	0.78	0.64	0.75	0.86	0.75	0.81	0.78	0.67	0.83	0.72	0.72	0.67	1.00												
246	0.78	0.28	0.72	0.75	0.75	0.69	0.89	0.78	0.61	0.83	0.78	0.64	0.69	0.69	0.69	0.81	0.81	0.64	1.00											
249	0.58	0.47	0.81	0.78	0.83	0.72	0.69	0.81	0.81	0.75	0.86	0.78	0.72	0.72	0.67	0.72	0.67	0.83	0.69	1.00										
252	0.58	0.47	0.75	0.72	0.78	0.78	0.75	0.75	0.69	0.75	0.81	0.72	0.78	0.72	0.72	0.72	0.78	0.72	0.75	0.78	1.00									
253	0.56	0.50	0.83	0.86	0.75	0.86	0.78	0.78	0.89	0.83	0.83	0.75	0.75	0.86	0.75	0.81	0.75	0.86	0.72	0.75	0.81	1.00								
256	0.61	0.44	0.67	0.75	0.64	0.75	0.67	0.72	0.78	0.67	0.72	0.75	0.69	0.75	0.81	0.81	0.64	0.81	0.67	0.75	0.81	0.83	1.00							
257	0.72	0.33	0.78	0.81	0.75	0.75	0.94	0.78	0.67	0.83	0.78	0.64	0.69	0.69	0.69	0.86	0.92	0.64	0.89	0.69	0.81	0.78	0.67	1.00						
258	0.61	0.44	0.78	0.81	0.81	0.64	0.83	0.72	0.67	0.83	0.72	0.64	0.64	0.69	0.64	0.75	0.86	0.69	0.78	0.69	0.69	0.72	0.61	0.89	1.00					
260	0.61	0.44	0.72	0.75	0.64	0.69	0.67	0.72	0.78	0.83	0.72	0.75	0.75	0.69	0.75	0.69	0.64	0.75	0.72	0.69	0.69	0.78	0.72	0.72	0.72	1.00				
264	0.69	0.36	0.75	0.72	0.72	0.72	0.81	0.69	0.64	0.81	0.69	0.56	0.72	0.61	0.61	0.72	0.89	0.67	0.86	0.67	0.78	0.75	0.64	0.86	0.81	0.75	1.00			
265	0.42	0.64	0.86	0.78	0.83	0.72	0.69	0.75	0.75	0.75	0.81	0.83	0.67	0.78	0.67	0.67	0.67	0.78	0.64	0.72	0.72	0.75	0.64	0.69	0.75	0.69	0.67	1.00		
271	0.64	0.42	0.81	0.83	0.72	0.78	0.75	0.75	0.81	0.81	0.81	0.78	0.67	0.72	0.78	0.89	0.72	0.78	0.69	0.72	0.72	0.81	0.75	0.81	0.81	0.81	0.67	0.78	1.00	
286	0.50	0.56	0.72	0.81	0.75	0.69	0.67	0.72	0.83	0.72	0.83	0.75	0.69	0.81	0.64	0.69	0.64	0.81	0.72	0.75	0.69	0.83	0.72	0.67	0.72	0.72	0.64	0.69	0.75	1.00

Appendix 13: Similarity matrix of AFLP primer combination *Eco*RI-ACA *Mse*I-CAC

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.06	1.00																												
75	0.42	0.60	1.00																											
78	0.50	0.56	0.84	1.00																										
92	0.60	0.46	0.78	0.90	1.00																									
117	0.60	0.46	0.66	0.78	0.80	1.00																								
119	0.74	0.32	0.64	0.68	0.78	0.86	1.00																							
136	0.54	0.52	0.76	0.84	0.86	0.70	0.72	1.00																						
138	0.50	0.56	0.80	0.92	0.82	0.70	0.68	0.84	1.00																					
142	0.54	0.52	0.80	0.84	0.78	0.66	0.68	0.84	0.80	1.00																				
145	0.50	0.56	0.88	0.88	0.86	0.74	0.72	0.84	0.80	0.88	1.00																			
150	0.34	0.68	0.92	0.80	0.70	0.66	0.60	0.76	0.76	0.76	0.84	1.00																		
158	0.58	0.48	0.76	0.84	0.78	0.74	0.72	0.76	0.80	0.84	0.84	0.72	1.00																	
164	0.56	0.50	0.74	0.74	0.80	0.76	0.74	0.78	0.66	0.74	0.74	0.74	0.58	1.00																
176	0.48	0.54	0.70	0.70	0.72	0.64	0.66	0.74	0.62	0.66	0.66	0.78	0.62	0.76	1.00															
215	0.64	0.42	0.58	0.66	0.72	0.84	0.82	0.70	0.62	0.70	0.66	0.58	0.70	0.76	0.60	1.00														
235	0.74	0.32	0.68	0.76	0.86	0.82	0.88	0.76	0.68	0.72	0.76	0.60	0.80	0.74	0.70	0.78	1.00													
242	0.28	0.74	0.86	0.74	0.64	0.60	0.54	0.74	0.70	0.74	0.78	0.90	0.66	0.72	0.72	0.56	0.54	1.00												
246	0.66	0.40	0.76	0.84	0.94	0.74	0.80	0.88	0.80	0.80	0.84	0.68	0.76	0.78	0.74	0.70	0.84	0.62	1.00											
249	0.50	0.56	0.84	0.92	0.86	0.78	0.72	0.88	0.84	0.84	0.92	0.80	0.76	0.82	0.70	0.70	0.76	0.78	0.84	1.00										
252	0.50	0.56	0.84	0.88	0.82	0.82	0.72	0.80	0.80	0.80	0.84	0.80	0.72	0.82	0.70	0.70	0.76	0.74	0.80	0.88	1.00									
253	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00								
256	0.64	0.42	0.66	0.82	0.76	0.72	0.70	0.74	0.82	0.70	0.70	0.62	0.82	0.60	0.60	0.68	0.74	0.56	0.78	0.74	0.74	0.00	1.00							
257	0.66	0.40	0.72	0.80	0.86	0.74	0.80	0.80	0.76	0.80	0.80	0.64	0.76	0.74	0.74	0.70	0.84	0.58	0.88	0.80	0.76	0.00	0.70	1.00						
258	0.72	0.34	0.66	0.78	0.84	0.84	0.90	0.82	0.74	0.74	0.74	0.62	0.74	0.76	0.72	0.80	0.90	0.56	0.86	0.78	0.78	0.00	0.72	0.86	1.00					
260	0.40	0.66	0.90	0.86	0.76	0.72	0.66	0.78	0.78	0.82	0.90	0.94	0.78	0.80	0.76	0.64	0.66	0.88	0.74	0.86	0.86	0.00	0.68	0.70	0.68	1.00				
264	0.64	0.42	0.70	0.82	0.80	0.72	0.78	0.86	0.78	0.82	0.82	0.70	0.78	0.72	0.72	0.72	0.82	0.64	0.86	0.82	0.78	0.00	0.76	0.78	0.88	0.76	1.00			
265	0.24	0.78	0.82	0.74	0.64	0.60	0.50	0.70	0.74	0.70	0.74	0.86	0.66	0.68	0.68	0.56	0.50	0.84	0.58	0.74	0.70	0.00	0.60	0.54	0.52	0.84	0.60	1.00		
271	0.48	0.58	0.78	0.82	0.76	0.84	0.70	0.70	0.74	0.74	0.74	0.74	0.74	0.80	0.72	0.72	0.74	0.72	0.70	0.82	0.86	0.00	0.68	0.70	0.72	0.80	0.68	0.72	1.00	
286	0.54	0.52	0.84	0.84	0.78	0.70	0.68	0.76	0.80	0.84	0.84	0.76	0.84	0.66	0.70	0.62	0.72	0.70	0.84	0.80	0.84	0.00	0.74	0.80	0.74	0.82	0.78	0.66	0.78	1.00

Appendix 14: Similarity matrix of AFLP primer combination *Eco*RI-ACA *Mse*I-CAT

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.12	1.00																												
75	0.00	0.88	1.00																											
78	0.56	0.56	0.44	1.00																										
92	0.56	0.56	0.44	0.82	1.00																									
117	0.88	0.24	0.12	0.68	0.56	1.00																								
119	0.94	0.18	0.06	0.62	0.56	0.94	1.00																							
136	0.88	0.24	0.12	0.68	0.62	0.88	0.88	1.00																						
138	0.82	0.29	0.18	0.68	0.56	0.88	0.88	0.82	1.00																					
142	0.76	0.35	0.24	0.74	0.68	0.76	0.76	0.71	0.65	1.00																				
145	0.79	0.32	0.21	0.65	0.71	0.79	0.79	0.74	0.74	0.74	1.00																			
150	0.59	0.53	0.41	0.85	0.74	0.71	0.65	0.65	0.76	0.71	0.68	1.00																		
158	0.94	0.18	0.06	0.62	0.62	0.88	0.94	0.88	0.88	0.76	0.79	0.65	1.00																	
164	0.74	0.38	0.26	0.82	0.76	0.79	0.79	0.68	0.74	0.85	0.76	0.79	0.79	1.00																
176	0.85	0.26	0.15	0.71	0.59	0.91	0.91	0.85	0.85	0.79	0.76	0.74	0.85	0.82	1.00															
215	0.97	0.15	0.03	0.59	0.59	0.91	0.97	0.91	0.85	0.79	0.82	0.62	0.97	0.76	0.88	1.00														
235	0.94	0.18	0.06	0.62	0.56	0.88	0.94	0.82	0.82	0.76	0.74	0.59	0.88	0.79	0.85	0.91	1.00													
242	0.97	0.15	0.03	0.59	0.53	0.91	0.97	0.85	0.85	0.74	0.76	0.62	0.91	0.76	0.88	0.94	0.97	1.00												
246	1.00	0.12	0.00	0.56	0.56	0.88	0.94	0.88	0.82	0.76	0.79	0.59	0.94	0.74	0.85	0.97	0.94	0.97	1.00											
249	0.91	0.21	0.09	0.65	0.59	0.91	0.97	0.91	0.85	0.74	0.76	0.68	0.91	0.76	0.88	0.94	0.91	0.94	0.91	1.00										
252	0.79	0.32	0.21	0.76	0.71	0.85	0.85	0.74	0.79	0.79	0.82	0.74	0.85	0.94	0.82	0.82	0.85	0.82	0.79	0.82	1.00									
253	0.91	0.21	0.09	0.65	0.53	0.91	0.91	0.85	0.85	0.68	0.76	0.68	0.85	0.76	0.88	0.88	0.91	0.94	0.91	0.88	0.82	1.00								
256	0.91	0.21	0.09	0.65	0.59	0.91	0.91	0.91	0.91	0.74	0.76	0.68	0.97	0.76	0.88	0.94	0.85	0.88	0.91	0.88	0.82	0.88	1.00							
257	1.00	0.12	0.00	0.56	0.56	0.88	0.94	0.88	0.82	0.76	0.79	0.59	0.94	0.74	0.85	0.97	0.94	0.97	1.00	0.91	0.79	0.91	0.91	1.00						
258	0.97	0.15	0.03	0.59	0.53	0.91	0.97	0.85	0.85	0.74	0.76	0.62	0.91	0.76	0.88	0.94	0.97	1.00	0.97	0.94	0.82	0.94	0.88	0.97	1.00					
260	0.97	0.15	0.03	0.59	0.53	0.91	0.97	0.85	0.85	0.74	0.76	0.62	0.91	0.76	0.88	0.94	0.97	1.00	0.97	0.94	0.82	0.94	0.88	0.97	1.00	1.00				
264	0.79	0.32	0.21	0.65	0.59	0.79	0.85	0.74	0.85	0.74	0.76	0.68	0.85	0.82	0.76	0.82	0.85	0.82	0.79	0.82	0.88	0.82	0.82	0.79	0.82	0.82	1.00			
265	0.59	0.53	0.41	0.85	0.68	0.71	0.65	0.65	0.59	0.76	0.68	0.71	0.59	0.79	0.74	0.62	0.65	0.62	0.59	0.62	0.74	0.68	0.62	0.59	0.62	0.62	0.62	1.00		
271	0.94	0.18	0.06	0.62	0.56	0.94	1.00	0.88	0.88	0.76	0.79	0.65	0.94	0.79	0.91	0.97	0.94	0.97	0.94	0.97	0.85	0.91	0.91	0.94	0.97	0.97	0.85	0.65	1.00	
286	0.85	0.26	0.15	0.71	0.65	0.85	0.91	0.79	0.85	0.74	0.76	0.68	0.91	0.88	0.82	0.88	0.91	0.88	0.85	0.88	0.94	0.88	0.88	0.85	0.88	0.88	0.94	0.68	0.91	1.00

Appendix 15: Similarity matrix of AFLP primer combination *Eco*RI-ACC *Mse*I-CAC

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.38	1.00																												
75	0.58	0.71	1.00																											
78	0.67	0.46	0.67	1.00																										
92	0.75	0.63	0.83	0.67	1.00																									
117	0.79	0.58	0.71	0.71	0.88	1.00																								
119	0.63	0.67	0.71	0.79	0.71	0.75	1.00																							
136	0.88	0.50	0.71	0.79	0.88	0.92	0.75	1.00																						
138	0.79	0.50	0.71	0.71	0.79	0.83	0.75	0.83	1.00																					
142	0.71	0.58	0.88	0.63	0.88	0.75	0.58	0.75	0.67	1.00																				
145	0.63	0.67	0.88	0.63	0.88	0.75	0.58	0.75	0.67	0.92	1.00																			
150	0.50	0.71	0.92	0.75	0.75	0.63	0.79	0.63	0.63	0.79	0.79	1.00																		
158	0.50	0.63	0.75	0.83	0.67	0.63	0.79	0.63	0.63	0.63	0.63	0.83	1.00																	
164	0.38	0.58	0.71	0.63	0.63	0.50	0.67	0.50	0.50	0.58	0.58	0.79	0.79	1.00																
176	0.67	0.54	0.67	0.83	0.67	0.71	0.88	0.79	0.79	0.54	0.54	0.75	0.75	0.63	1.00															
215	0.75	0.63	0.67	0.67	0.83	0.96	0.79	0.88	0.79	0.71	0.71	0.58	0.58	0.46	0.67	1.00														
235	0.79	0.58	0.71	0.88	0.79	0.83	0.83	0.92	0.83	0.67	0.67	0.71	0.71	0.58	0.88	0.79	1.00													
242	0.71	0.67	0.54	0.71	0.71	0.83	0.75	0.83	0.67	0.58	0.58	0.54	0.63	0.50	0.71	0.88	0.83	1.00												
246	0.75	0.63	0.75	0.83	0.83	0.88	0.88	0.88	0.88	0.71	0.71	0.75	0.75	0.63	0.83	0.83	0.96	0.79	1.00											
249	0.79	0.50	0.71	0.88	0.79	0.83	0.75	0.92	0.75	0.75	0.75	0.71	0.71	0.58	0.79	0.79	0.92	0.83	0.88	1.00										
252	0.71	0.67	0.79	0.71	0.88	0.83	0.83	0.83	0.83	0.75	0.75	0.71	0.63	0.58	0.71	0.88	0.83	0.75	0.88	0.75	1.00									
253	0.79	0.58	0.71	0.79	0.88	0.92	0.83	0.92	0.83	0.75	0.75	0.71	0.71	0.58	0.79	0.88	0.92	0.83	0.96	0.92	0.83	1.00								
256	0.79	0.58	0.63	0.79	0.79	0.92	0.75	0.92	0.75	0.67	0.67	0.63	0.63	0.50	0.79	0.88	0.92	0.92	0.88	0.92	0.75	0.92	1.00							
257	0.83	0.54	0.67	0.83	0.83	0.88	0.79	0.96	0.79	0.71	0.71	0.67	0.67	0.54	0.83	0.83	0.96	0.88	0.92	0.96	0.79	0.96	0.96	1.00						
258	0.83	0.54	0.67	0.83	0.83	0.88	0.79	0.96	0.79	0.71	0.71	0.67	0.67	0.54	0.83	0.83	0.96	0.88	0.92	0.96	0.79	0.96	0.96	1.00	1.00					
260	0.46	0.75	0.88	0.54	0.71	0.58	0.67	0.58	0.58	0.75	0.75	0.79	0.63	0.67	0.54	0.63	0.58	0.50	0.63	0.58	0.75	0.58	0.50	0.54	0.54	1.00				
264	0.54	0.67	0.88	0.79	0.79	0.67	0.67	0.67	0.67	0.83	0.83	0.88	0.79	0.67	0.63	0.63	0.75	0.58	0.79	0.75	0.75	0.75	0.67	0.71	0.71	0.75	1.00			
265	0.04	0.58	0.46	0.29	0.29	0.25	0.33	0.17	0.25	0.33	0.42	0.46	0.46	0.58	0.29	0.29	0.17	0.25	0.21	0.17	0.33	0.17	0.17	0.13	0.13	0.58	0.42	1.00		
271	0.04	0.58	0.46	0.29	0.29	0.25	0.33	0.17	0.25	0.33	0.42	0.46	0.46	0.58	0.29	0.29	0.17	0.25	0.21	0.17	0.33	0.17	0.17	0.13	0.13	0.58	0.42	1.00	1.00	
286	0.79	0.58	0.71	0.79	0.88	0.92	0.83	0.92	0.83	0.75	0.75	0.71	0.71	0.58	0.79	0.88	0.92	0.83	0.96	0.92	0.83	1.00	0.92	0.96	0.96	0.58	0.75	0.17	0.17	1.00

Appendix 16: Similarity matrix of AFLP primer combination *Eco*RI-ACC *Mse*I-CAC

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.08	1.00																												
75	0.79	0.29	1.00																											
78	0.58	0.50	0.79	1.00																										
92	0.79	0.29	0.77	0.75	1.00																									
117	0.79	0.29	0.73	0.67	0.73	1.00																								
119	0.88	0.19	0.87	0.69	0.79	0.83	1.00																							
136	0.77	0.31	0.75	0.73	0.83	0.75	0.81	1.00																						
138	0.71	0.37	0.69	0.75	0.73	0.81	0.75	0.75	1.00																					
142	0.69	0.38	0.87	0.81	0.79	0.71	0.77	0.85	0.71	1.00																				
145	0.60	0.44	0.73	0.75	0.65	0.62	0.67	0.67	0.77	0.79	1.00																			
150	0.54	0.54	0.71	0.81	0.67	0.67	0.65	0.69	0.75	0.77	0.75	1.00																		
158	0.75	0.33	0.77	0.75	0.73	0.81	0.83	0.83	0.81	0.79	0.69	0.79	1.00																	
164	0.58	0.50	0.79	0.81	0.71	0.63	0.69	0.77	0.60	0.85	0.71	0.81	0.67	1.00																
176	0.75	0.33	0.81	0.79	0.81	0.73	0.83	0.83	0.77	0.75	0.77	0.75	0.81	0.75	1.00															
215	0.67	0.37	0.69	0.67	0.65	0.77	0.75	0.79	0.69	0.67	0.65	0.71	0.85	0.71	0.73	1.00														
235	0.92	0.15	0.83	0.62	0.83	0.79	0.92	0.81	0.71	0.73	0.63	0.62	0.79	0.62	0.79	0.71	1.00													
242	0.71	0.37	0.77	0.75	0.77	0.77	0.79	0.87	0.73	0.79	0.73	0.71	0.81	0.79	0.85	0.81	0.75	1.00												
246	0.69	0.38	0.75	0.81	0.83	0.83	0.77	0.85	0.67	0.81	0.63	0.77	0.79	0.81	0.83	0.79	0.73	0.87	1.00											
249	0.63	0.40	0.73	0.79	0.73	0.81	0.75	0.83	0.73	0.79	0.73	0.75	0.81	0.75	0.85	0.81	0.67	0.85	0.90	1.00										
252	0.65	0.42	0.79	0.73	0.67	0.75	0.77	0.77	0.67	0.73	0.63	0.77	0.71	0.81	0.75	0.79	0.69	0.83	0.85	0.79	1.00									
253	0.71	0.37	0.88	0.83	0.73	0.73	0.83	0.83	0.65	0.87	0.73	0.75	0.77	0.87	0.85	0.73	0.75	0.85	0.87	0.85	0.83	1.00								
256	0.67	0.37	0.69	0.67	0.73	0.77	0.71	0.79	0.85	0.71	0.73	0.71	0.77	0.63	0.69	0.85	0.71	0.77	0.71	0.73	0.75	0.62	1.00							
257	0.90	0.17	0.81	0.63	0.77	0.81	0.94	0.79	0.77	0.71	0.65	0.60	0.77	0.63	0.81	0.69	0.90	0.77	0.71	0.69	0.71	0.77	0.69	1.00						
258	0.87	0.21	0.81	0.71	0.92	0.77	0.87	0.83	0.69	0.79	0.69	0.63	0.77	0.71	0.85	0.73	0.90	0.81	0.83	0.73	0.71	0.77	0.73	0.85	1.00					
260	0.63	0.40	0.73	0.79	0.77	0.73	0.75	0.79	0.65	0.75	0.69	0.79	0.77	0.83	0.85	0.81	0.67	0.85	0.90	0.92	0.83	0.85	0.69	0.69	0.77	1.00				
264	0.81	0.27	0.87	0.77	0.87	0.79	0.88	0.85	0.75	0.81	0.75	0.69	0.79	0.77	0.94	0.71	0.85	0.90	0.85	0.83	0.77	0.87	0.71	0.87	0.90	0.83	1.00			
265	0.58	0.50	0.75	0.81	0.75	0.67	0.65	0.65	0.67	0.81	0.79	0.77	0.67	0.77	0.67	0.56	0.62	0.71	0.73	0.67	0.65	0.75	0.60	0.60	0.67	0.67	0.73	1.00		
271	0.71	0.37	0.77	0.75	0.77	0.69	0.79	0.87	0.69	0.75	0.65	0.75	0.85	0.75	0.85	0.81	0.75	0.77	0.83	0.81	0.79	0.81	0.73	0.73	0.77	0.81	0.79	0.63	1.00	
286	0.71	0.33	0.85	0.79	0.65	0.77	0.83	0.75	0.73	0.75	0.69	0.75	0.77	0.75	0.81	0.77	0.75	0.77	0.79	0.85	0.83	0.85	0.73	0.77	0.69	0.85	0.79	0.63	0.77	1.00

Appendix 17: Similarity matrix for combination of six AFLP *Eco*RI/*Mse*I primer combinations

	P16	CM32	75	78	92	117	119	136	138	142	145	150	158	164	176	215	235	242	246	249	252	253	256	257	258	260	264	265	271	286
P16	1.00																													
CM32	0.26	1.00																												
75	0.73	0.38	1.00																											
78	0.76	0.40	0.81	1.00																										
92	0.80	0.38	0.82	0.86	1.00																									
117	0.84	0.35	0.75	0.80	0.82	1.00																								
119	0.92	0.31	0.78	0.82	0.83	0.88	1.00																							
136	0.84	0.36	0.76	0.82	0.85	0.86	0.88	1.00																						
138	0.80	0.37	0.75	0.84	0.81	0.87	0.84	0.87	1.00																					
142	0.80	0.36	0.78	0.85	0.84	0.80	0.83	0.87	0.81	1.00																				
145	0.74	0.41	0.76	0.83	0.83	0.78	0.79	0.82	0.81	0.85	1.00																			
150	0.62	0.50	0.70	0.77	0.73	0.71	0.68	0.74	0.75	0.76	0.78	1.00																		
158	0.83	0.35	0.75	0.82	0.81	0.85	0.88	0.88	0.87	0.85	0.82	0.74	1.00																	
164	0.74	0.41	0.75	0.83	0.83	0.79	0.80	0.80	0.76	0.83	0.80	0.77	0.77	1.00																
176	0.82	0.35	0.76	0.82	0.83	0.83	0.86	0.87	0.83	0.84	0.79	0.76	0.83	0.83	1.00															
215	0.87	0.35	0.73	0.81	0.81	0.89	0.90	0.86	0.83	0.82	0.79	0.69	0.86	0.80	0.85	1.00														
235	0.90	0.33	0.79	0.80	0.85	0.88	0.93	0.87	0.83	0.82	0.78	0.68	0.87	0.79	0.85	0.88	1.00													
242	0.80	0.38	0.77	0.80	0.79	0.83	0.85	0.87	0.84	0.82	0.78	0.73	0.84	0.80	0.86	0.84	0.84	1.00												
246	0.87	0.34	0.77	0.84	0.87	0.86	0.90	0.89	0.83	0.84	0.81	0.71	0.86	0.82	0.86	0.88	0.89	0.86	1.00											
249	0.79	0.36	0.76	0.85	0.84	0.86	0.85	0.88	0.86	0.84	0.84	0.74	0.87	0.83	0.84	0.84	0.84	0.87	0.88	1.00										
252	0.80	0.41	0.78	0.83	0.83	0.87	0.86	0.86	0.84	0.82	0.81	0.75	0.86	0.84	0.83	0.85	0.86	0.86	0.87	0.87	1.00									
253	0.86	0.37	0.80	0.85	0.82	0.86	0.90	0.91	0.86	0.87	0.81	0.74	0.87	0.86	0.90	0.87	0.90	0.90	0.89	0.89	0.87	1.00								
256	0.84	0.36	0.73	0.82	0.81	0.86	0.86	0.86	0.89	0.80	0.80	0.71	0.87	0.78	0.83	0.88	0.85	0.84	0.85	0.86	0.86	0.86	1.00							
257	0.91	0.31	0.79	0.82	0.84	0.86	0.95	0.89	0.86	0.85	0.80	0.68	0.87	0.79	0.87	0.88	0.94	0.85	0.90	0.85	0.86	0.91	0.86	1.00						
258	0.90	0.33	0.78	0.83	0.87	0.86	0.94	0.89	0.85	0.84	0.79	0.68	0.85	0.80	0.86	0.88	0.94	0.86	0.91	0.86	0.85	0.90	0.85	0.94	1.00					
260	0.81	0.38	0.76	0.82	0.81	0.84	0.85	0.86	0.84	0.83	0.80	0.76	0.87	0.82	0.88	0.85	0.86	0.90	0.88	0.88	0.87	0.90	0.84	0.87	0.87	1.00				
264	0.86	0.34	0.80	0.84	0.85	0.86	0.90	0.88	0.86	0.86	0.80	0.70	0.87	0.81	0.88	0.86	0.91	0.87	0.90	0.87	0.88	0.90	0.85	0.91	0.92	0.87	1.00			
265	0.68	0.44	0.79	0.82	0.78	0.75	0.74	0.76	0.77	0.80	0.79	0.75	0.76	0.80	0.78	0.73	0.76	0.78	0.74	0.79	0.76	0.81	0.74	0.75	0.76	0.78	0.77	1.00		
271	0.83	0.36	0.77	0.83	0.80	0.85	0.88	0.86	0.84	0.82	0.78	0.73	0.87	0.82	0.87	0.88	0.87	0.86	0.86	0.87	0.86	0.90	0.86	0.87	0.88	0.89	0.85	0.77	1.00	
286	0.82	0.38	0.79	0.86	0.82	0.85	0.86	0.84	0.85	0.82	0.82	0.73	0.86	0.82	0.84	0.85	0.86	0.86	0.88	0.87	0.89	0.90	0.85	0.86	0.85	0.87	0.87	0.74	0.85	1.00

Chapter Four

Seed germination and vigour analysis in diallel crosses among normal endosperm (Nm), low phytic acid (LPA) and quality protein maize (QPM) inbred lines under normal and accelerated aging conditions

Abstract

Seed germination and vigour are important traits especially for low phytic acid (LPA) and quality protein maize (QPM). A 10 x 10 half diallel was made between four temperate LPA, three tropical QPM and three tropical normal inbred lines. The seeds were subjected to the standard germination test and the accelerated aging test to assess germination and vigour. The seed traits were germination percentage, seedling dry weight, average root length, average shoot length and vigour index. The entry, GCA and SCA effects were significant ($P \leq 0.001$) for all traits tested under normal and accelerated aging conditions; therefore both additive and non-additive gene action was significant. In general, the SCA effects were superior to GCA effects for all traits under both test conditions, indicating that genes with non-additive effects were predominant. The LPA lines generally showed lower germination (70 - 27%) and vigour under accelerated aging conditions whilst the QPM lines performance was comparable to the tropical normal lines. The lines CM 31, QPM 7, P 12 and T 2 showed positive and mostly significant GCA effects and are recommended as sources for breeding for improved vigour and germination. There were nine crosses that showed positive SCA effects for all the traits across both environments, including one QPM x Nm, two LPA x QPM, two LPA x LPA and four LPA x Nm crosses. There were some lines identified as stress-tolerant due to increased/positive GCA effects and some crosses having increased/positive SCA effects under accelerated aging conditions. The LPA lines had the highest reduction under accelerated aging conditions for germination percentage (61%), dry weight (52%), average root length (23%), average shoot length (47%) and vigour index (34%). The QPM and normal inbred lines showed no differences for germination percentage and vigour index, increases in dry weight and average root length and slight decrease for average shoot length under accelerated aging conditions.

Keywords: LPA, QPM, germination, vigour, accelerated aging test

Introduction

Selections in maize breeding programmes to improve crop productivity and nutritional quality are usually based on yield and disease resistance. There is little selection on the seed quality characteristics which is critical in early performance and growth of these crops. Seed quality is usually associated with germination and purity but seed vigour also needs to be included. The ISTA (International Seed Testing Association) defines seed vigour as “an index of the extent of the physiological deterioration and/or mechanical integrity of a high germinating seed lot which governs its ability to perform in a wide range of environments”. Vigour is a concept that describes several characteristics of the seed such as rate and uniformity of germination and growth, tolerance to environmental stresses after sowing and retention of performance after storage (Hrstková *et al.*, 2006).

Phytic acid is an antinutritional factor found in seeds but has the potential to contribute to seed performance. It has been recently shown that phytic acid acts as an anti-oxidant by acting as protection against oxidative accelerated aging during the seed’s life span (Doria *et al.,* 2009). In maize there have been low phytic acid (LPA) mutants developed with reduced amounts of phytic acid (Raboy, 2000). These *lpa1-1* mutants have 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g P_i (66% of total P) compared to the normal maize of 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g Pi (7% of total P) (Raboy *et al.,* 2000). The yields of these lines have also been reduced to between 5 and 15% of the highest yielding commercial varieties (Raboy, 2000). There is no previous germination and vigour studies on the *lpa1-1* mutation lines, however there are two studies on the *lpa241* mutant line (90% reduction in seed phytic acid) (Pilu *et al*., 2003). In a study with a LPA mutant (*lpa241*), a 30% decrease in germination rate was observed when compared to the wild type (Pilu *et al*., 2003). Another germination study of the same LPA mutant line (*lpa241*) was tested and shown to have 72±15% germination under standard conditions which decreased to 45±14% germination under accelerated aging conditions (Doria *et al.,* 2009). A study on 50 different maize lines found a negative correlation between yield and phytate with a suggestion that selection for decreased whole-kernel phytate may result in lines with large kernel size that should have a diluted concentration of phytate (Lorenz *et al*., 2007).

Quality protein maize (QPM) has higher amounts of the amino acids, lysine and tryptophan than normal maize but show reduced grain yield, increased susceptibility to ear rot, soft floury endosperm and poor dry-milling properties. Normal maize compared to QPM maize has higher levels of phytate-phosphorus and inorganic phosphate with lower levels of myo-inositol leading to higher germination and vigour of normal maize (Modi and Asanzi, 2008). There were negative correlations found between grain yield and phytate (-0.47), lysine (-

0.25) and tryptophan (-0.25) suggesting that development of high yielding lines with low phytate was possible (Lorenz *et al*., 2007).

Seed tests can be used to evaluate physical quality, genetic purity, viability and vigour. The most common seed test is the germination test which measures seed viability under ideal conditions. Vigour testing has become common practice especially for maize and soybeans to routinely market high quality seed. The vigour test is a more sensitive index of seed quality than the germination test as it is closely correlated with seed performance in the field under some conditions than the germination test. There are various essential characteristics of a vigour test that make it useful, such as being inexpensively priced and requiring minimum investment in labour, equipment and supplies. Also required are a rapid testing period to minimize analyst time and germinator space with simple testing procedures without special training or experienced personnel. The test has numerous advantages including being objective with a quantitative or numerical index of quality, thus avoiding subjective interpretations by analysts, high correlations of test results with field performance and reproducible test results between laboratories (McDonald, 1980).

Different vigour tests measure different aspects of seed quality under different soil conditions; therefore a combination of several vigour tests is able to provide information on the quality of a seed lot as well as its potential field performance (Byrum and Copeland, 1995). The two most commonly used vigour tests are the cold test and the accelerated aging test. The accelerated aging test is a vigour test that involves artificial aging of the seed at high temperatures, with the cause due to integral membrane lipid peroxidation in the seeds (Basavarajappa *et al.,* 1991). In maize, accelerated aging causes associations between the starch granules, protein matrix and cell walls, leading to decreased solubility and functionality of starch and protein in aged grain due to protein oxidation (McDonough *et al.,* 2004). The accelerated aging test was developed by Delouche and Baskin (1973) to assess the quality and storability of seed lots. These tests are also effective in evaluating seed vigour and germinative responses to accelerated aging and are highly correlated with plant growth and development including yield (Delouche and Baskin, 1973).

The accelerated aging test has been used extensively in maize to compare different types of vigour tests for prediction of field performance (Lovato *et al.,* 2005; Noli *et al.,* 2008), to determine relationship between total phenolics content and germination ability (Barla-Szabo *et al.,* 1990; Sredojević *et al.,* 2004), to improve vigour by using aerosol-smoke (Sparg *et al.,* 2006), to assess seed quality (Santipracha *et al.,* 1997; Munamava *et al.,* 2004); seedling vigour (Fakorede and Ojo, 1981) and to assess seed storability (Basu *et al.,* 2004). In warmer tropical climates, the accelerated aging test has been used as an indicator of seed

vigour. After the aging process, high vigour seeds are expected to show high rates of germination while low vigour seeds would show significant decrease in germination rate.

The standard germination test is used to determine viability of the seed, whereas the accelerated aging test is used to determine the vigour by using different parameters such as root length, shoot length and dry weight of the seedling. Seed vigour is not only dependent on the size of kernel but the speed of germination is an important factor. The size of the kernel is an estimate of the amount of nutrients available to the seed during germination. However, even if the seed has large kernel size but has low speed of germination, it can be classified as low vigour. Seedling parameters such as the root length (indicator of potential to develop an adequate root system to access nutrients), shoot length (indicator of the competition ability to outgrow weeds in the field), and the dry weight of a seedling (effectiveness of the seed in producing biomass) are used to assess speed of germination and vigour.

Knowledge of the combining ability of maize inbreds is useful in devising an appropriate breeding strategy. A survey of the literature indicates that there is limited information regarding combining ability between LPA and QPM lines, especially for seed germination and vigour.

The objectives of this study were to:

- determine the gene action controlling inheritance of early vigour and germination and combining ability of tropical normal endosperm, temperate low phytate and tropical QPM germplasm for germination percentage, seedling dry weight, average shoot length, average root length and vigour index traits, and
- determine the germination capacity of LPA, QPM and normal lines.

Materials and Methods

Germplasm

There were four temperate *lpa1-1* lines, three tropical QPM lines and three tropical normal (Nm) inbred maize lines used in this study (Table 4.1). There were 45 F_1 crosses generated from a 10 x 10 half diallel mating design with no reciprocals and selfs. Two LPA x tropical normal crosses had insufficient seed set and were excluded from the seed testing. The F_1 hybrids were advanced to the F_2 generation in pots in the greenhouse. The F_2 seed was used for the seed tests as well as the parental inbred lines. Due to the recessive nature of the mutation, in the F_2 generation only 25% of the total number of seeds tested would show

the homozygous recessive genotype (1:2:1 ratio). The exact pedigrees of the LPA lines are not available however all the LPA lines used in this study were obtained from Dr V. Raboy (Iowa, U.S.A.) and contain the *lpa1-1* gene.

Table 4.1: Pedigrees of inbred lines used in diallel mating design

Line	Pedigree	Endosperm type	Adaptation	100-seed weight (g)
CM 31	TS3 LPA1-1	*lpa1-1*	temperate	20.26
CM 32	JUG 248 LPA1-1	*lpa1-1*	temperate	15.01
CM 33	Ex-UDSA[#]	*lpa1-1*	temperate	23.88
CM 34	CO63 LPA1-1	*lpa1-1*	temperate	27.29
QPM 3	CML 176	QPM	tropical	26.90
QPM 6	CZL 01006	QPM	tropical	34.65
QPM 7	OBATANPA-SRC1F3#-MALE	QPM	tropical	34.03
P 12	CZL 00008	Normal	tropical	42.26
T 2	PN7-2B	Normal	tropical	32.02
T 3	PN8-B	Normal	tropical	29.75

[#]: Actual pedigree information not available

The parental lines used in this study were different for adaptation type and the seed size/weight was also different (see 100-seed weight in Table 4.1). The parental inbred lines were divided into three groups: temperate LPA, tropical QPM and tropical normal endosperm. There were six groups of crosses generated between the three groups of parental lines: LPA x LPA, LPA x Nm, LPA x QPM, QPM x QPM, Nm x Nm and QPM x Nm. The means of each group was calculated and compared between the different groups. The groups were defined as: LPA group with 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g P_i (66% of total P), tropical normal and QPM group with 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g P_i (7% of total P) (Raboy *et al*., 2000).

Standard seed testing

The standard germination test used was the between-paper method. The seed sample was randomly sampled for two replicates of 50 seeds each. The germination bed used was rolled paper towel of size 54 cm x 30 cm (Anchor Paper, Agricol (Pty) Ltd, South Africa) (ISTA,

1999). Each sheet had 50 seeds (1 replication) placed equidistant on the upper half of the moistened paper towel with the radical end of the seed pointed toward the bottom end of the paper and the embryo end side up. Paper towels were folded in the middle to cover the seeds completely. The paper towels were rolled up and placed upright individually in polythene bags in a germination chamber. Approximately 2-3 cm distance was maintained between the seeds to reduce mutual influence and bacterial infection. The containers were labelled with the date, seed name and replicate number and were covered appropriately to prevent evaporation. The containers were placed into germination chambers (Labcon growth chamber). The between-paper method used a temperature 25±1°C and the seed germination measurements taken after seven days. During the period of seed germination, the chamber was checked daily to ensure suitable conditions for germination and the paper towel was maintained at the appropriate moisture and temperature for seven days before evaluation and counting. Seeds were visually assessed according to the International Seed Testing Association rules for percentage germination (ISTA, 1999).

Accelerated aging test

The 100 maize seeds were placed in a petri-dish with moistened filter paper. The seeds were incubated in closed petri-dishes in the germination chamber at 42°C (~100% RH) for 96 hours with regular checking to ensure sufficient moisture in the petri dish was maintained (ISTA, 1999). At the end of the aging period, the seeds were evaluated as per standard germination test, with two replications of 50 seeds each. The seeds were rated as normal and abnormal seedlings and dead seeds. A normal seedling has well developed root and shoot systems. An abnormal seedling has any abnormality in their root or shoots system. Seeds which were neither hard, fresh or have produced seedlings were classified as dead seeds (ISTA, 1999).

Data collection and analysis

All dead and defective seedlings were counted. The germinated seedlings were counted and weighed. The percentage germination was calculated as follows: (# of normal seedlings / # of seeds in sample) x 100. The percentage germinated, dead and abnormal was calculated for all the F_2 seeds tested. At the counting stage, 25 normal seedlings in each replicate were selected at random and the length of the root measured in millimetres and mean values calculated. The 25 normal seedlings that were selected for the root length measurement in each replicate was again used for shoot measurements (mm) and mean values were calculated. These values were used to calculate a vigour index. The vigour index of 1-week

old seedlings was calculated as VI = [(shoot length (mm) + root length (mm)] x percentage germination (Dhindwal *et al.,* 1991). The fresh weight of the germinated seedlings were taken at the counting stage and the seedling mass was subjected to 80°C for 24 hours to dry before the seedling dry weight was measured. General analysis of variance was performed for all data using the SAS programme version 9.1 (SAS Institute, 2002). Combining ability estimates were calculated for each seedling trait using the Diallel-SAS05 programme (Zhang *et al.*, 2005) in SAS.

Results

Gene action

Under standard germination conditions, the entry, GCA and SCA effects were significant (P≤0.001) for all the seed traits of germination percentage, seedling dry weight, average root length, average shoot length and vigour index (Table 4.2). A similar trend was observed under the accelerated aging conditions (Table 4.2).

The ratio of GCA/SCA sum of squares was in favour of SCA for all traits under both environments, showing predominance of non-additive gene action (Table 4.3). However, the GCA percentage was still high; therefore there is also additive gene action present for all the traits.

Combining ability

General combining ability

For all seed germination and vigour traits tested, positive GCA effects were desired. There were four lines (CM 31, QPM 7, P 12 and T 2) that showed positive GCA effects for germination percentage and seedling dry weight. Positive GCA effects were also shown by five lines (QPM 3, QPM 6, QPM 7, P 12, T 2) for the average root length, four lines (CM 31, QPM 6, QPM 7, P 12) for the average shoot length and four parental lines (QPM 6, QPM 7, P 12, T 2) for the vigour index (Table 4.3). The LPA lines showed varied GCA effects for all the traits with CM 31 generally showing positive GCA effects for all traits with significance for germination percentage and seedling dry weight (P≤0.001), and CM 32 and CM 33 having negative GCA effects for all traits with significance for CM33 for all traits (P≤0.001). However under accelerated aging conditions, CM 32 performed slightly better with positive GCA effects for average root length, average shoot length and vigour index. A similar trend was

displayed by CM 33 and CM 34 as again they showed negative GCA effects for all traits under accelerated aging conditions (Table 4.3).

QPM 7 was the best performing QPM line with significant and positive GCA effects for all traits under both environments (Table 4.3). The tropical normal lines also performed differently for all traits with P 12 and T 2 generally having positive GCA effects for all traits with significance at the P≤0.001 level for germination percentage and seedling dry weight for both conditions (Table 4.3). The tropical normal line T 3 generally had negative GCA effects for all traits under both conditions. From these trends, the inbred lines CM 31, QPM 7, P 12 and T 2 can be recommended as suitable parental lines to increase germination and vigour in the LPA lines.

Specific combining ability effects

Positive SCA effects were desired for all the seed germination and vigour traits. Generally the LPA x LPA crosses showed negative or reduced SCA effects under standard and accelerated aging conditions (CM 31 x CM 32, CM 32 x CM 33, CM 33 x CM 34). However there were a few LPA x LPA crosses that had positive or increased SCA effects under accelerated aging conditions (CM 31 x CM 33, CM 32 x CM 34). The QPM x QPM crosses showed different responses with QPM 3 x QPM 6 showing positive SCA effects for most of the traits under both standard and accelerated aging conditions; while QPM 3 x QPM 7 had negative SCA effects for most traits. Two of the three Nm x Nm crosses showed negative SCA values with only T 2 x T 3 showing positive SCA effects (Table 4.4).

There were five crosses that had positive SCA values for germination percentage, seedling dry weight and average root length under both standard and accelerated aging conditions (CM 34 x T 2, QPM 3 x QPM 6, QPM 3 x T 3, QPM 6 x T2 and QPM 7 x T3) (Table 4.4). For the average shoot length and vigour index there were five crosses with positive SCA effects (CM 31 x QPM 3, CM 31 x QPM 6, CM 31 x T2, CM 32 x QPM 3 and CM 32 x QPM 6) under both environments (Table 4.4). There were some crosses that had negative SCA effects under standard conditions but these crosses showed positive SCA effects under accelerated aging conditions (CM 31 x QPM 7, CM 31 x T 2, CM 34 x P 12, CM 34 x T 2, QPM 3 x QPM 6, QPM 3 x T 3, QPM 7 x T 3) thereby showing good tolerance to accelerated aging conditions. There were nine crosses that had positive SCA values for all the seed traits under both standard and accelerated aging conditions and were considered to be good combinations (CM 31 x CM 33, CM 32 x CM 34, CM 32 x T 3, CM 33 x QPM 7, CM 33 x P 12, CM 33 x T 2, CM 33 x T 3, CM 34 x QPM 3, QPM 6 x P 12).

Table 4.2: Mean square for seed traits of a 10 parent diallel evaluated under standard germination conditions (Std) and accelerated aging conditions (AA)

Source	df	Germination %		Seedling dry weight		Avg root length		Avg shoot length		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
Rep	1	613.6	1.1	8.5	0.8	5038.5	1605.3	5291.5	392.3	27865.4	6469.7
Entry	44	1372.4**	1651.9**	29.3**	33.8**	1650.4**	3436.2**	1302.6**	2043.9**	6187.6**	10099.8**
GCA	9	2216.0**	2693.9**	42.2**	50.4**	3222.9**	7491.9**	2465.72**	2859.5**	12128.6**	17445.7**
SCA	35	1155.5**	1383.9**	26.0**	29.5**	1246.0**	5.8**	1003.5**	1834.2**	4659.9**	8210.8**
Error	44	21.27	41.1	0.6	0.9	343.1	415.1	272.6	407.4	849.2	1208.0

Table 4.3: Percent contribution of GCA and SCA sum of squares to entry sum of squares and GCA effects for seed traits under standard germination (Std) and accelerated aging conditions (AA)

	Endosperm type	Germination %		Seedling dry weight (g)		Root Length (mm)		Shoot Length (mm)		Vigour index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
Percent contribution of GCA and SCA sum of squares to entry sum of squares											
GCA		33	33	29	31	40	45	39	29	40	35
SCA		67	67	71	69	60	55	61	71	60	65
GCA effects											
CM 31	LPA	6.65**	4.65	0.71*	0.29	0.45	-1.21	6.00	5.26	0.41	-4.00
CM 32	LPA	-2.65	0.71	-0.66	-0.28	-8.44	2.18	-9.24	2.83	-11.27	3.47
CM 33	LPA	-25.96**	-26.23**	-3.05**	-3.31**	-32.76**	-46.37**	-26.10**	-22.96**	-58.93**	-64.04**
CM 34	LPA	0.23	-1.73	-0.35	-0.45	0.75	-17.25	-3.90	-19.50**	1.33	-28.70
QPM 3	QPM	-3.03	-4.16	-1.19**	-1.10**	2.03	12.22	-2.84	3.51	-3.66	11.43
QPM 6	QPM	-2.28	-3.66	-0.58	-0.39	1.68	-8.30	13.22	5.37	10.98	-2.07
QPM 7	QPM	18.04**	21.21**	1.88**	2.41**	23.76**	20.69**	16.60**	12.00	48.47**	37.75**
P 12	Nm	8.04**	9.59**	1.57**	2.12**	8.28	21.17**	10.08	14.34	16.96	31.38*
T 2	Nm	8.10**	10.53**	2.29**	1.95**	5.19	24.62**	-0.90	11.30	12.69	40.60**
T 3	Nm	-7.15**	-10.91**	-0.62	-1.25**	-0.93	-7.75	-2.93	-12.14	-16.98	-25.81

** P≤0.001

Table 4.4: SCA effects of crosses under standard germination conditions and accelerated aging conditions for germination percentage, seedling dry weight, average root length, average shoot length and vigour index traits

Pedigree	Classification	Germination %		Seedling Dry Weight (g)		Root Length (mm)		Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
CM 31XCM 32	LPA x LPA	15.13**	4.16	3.02**	2.51**	4.98	-6.73	4.10	-4.81	19.90	-21.22
CM 31X CM 33	LPA x LPA	35.44**	40.60**	5.51**	6.48**	25.85	44.17	3.11	33.58	48.04	89.23**
CM 31XCM 34	LPA x LPA	12.26**	12.10	1.86*	2.17	6.14	-26.00	23.61	-20.43	48.45	-39.79
CM 31XQPM 3	LPA x QPM	-0.49	-0.47	-2.05**	-1.98	6.36	11.44	0.60	18.61	8.34	44.96
CM 31XQPM 6	LPA x QPM	6.76	13.03	1.89	2.86**	5.51	0.70	18.74	3.40	32.96	10.24
CM 31XQPM 7	LPA x QPM	-23.56**	-24.34**	-3.02**	-4.44**	-7.52	-9.09	-37.24	-4.63	-72.89**	-42.08
CM 31XP 12	LPA x Nm	-14.56**	-14.22	-1.14	-2.10	-6.04	-15.72	-12.17	-15.91	-18.90	-27.38
CM 31XT 2	LPA x Nm	-5.62	-1.15	-1.53	-0.98	-19.25	9.48	-9.24	12.62	-27.07	28.31
CM 31XT 3	LPA x Nm	-25.37**	-29.72**	-4.53**	-4.52**	-16.04	-8.25	8.49	-22.44	-38.83	-42.27
CM 32X CM 33	LPA x LPA	-48.24**	-44.47**	-6.17**	-5.49**	-69.71**	-78.57**	-71.26**	-87.79**	-127.68**	-149.69**
CM 32XCM 34	LPA x LPA	21.57**	26.03**	2.47**	3.44**	4.68	37.16	12.24	23.40	18.18	66.30
CM 32XQPM 3	LPA x QPM	14.82**	8.47	0.32	0.19	-1.90	2.15	6.73	25.19	2.96	9.27
CM 32XQPM 6	LPA x QPM	-16.43**	-10.03	-2.55**	-2.76**	9.85	22.47	12.97	19.54	6.62	12.97
CM 32XQPM 7	LPA x QPM	1.76	8.10	-0.65	0.29	-20.93	-6.08	5.99	4.35	-21.15	15.14
CM 32XP 12	LPA x Nm	-5.24	-10.28	0.23	-1.52	2.85	-19.11	3.07	-13.48	-7.22	-34.85
CM 32XT 2	LPA x Nm	-8.31	-6.22	-0.36	-0.01	30.08	23.14	11.74	6.50	34.25	24.12
CM 32XT 3	LPA x Nm	24.94**	24.22**	3.69**	3.35**	40.10	25.56	14.42	27.09	74.13	77.96
CM 33XCM 34	LPA x LPA	-38.12**	-40.03**	-5.28**	-5.08**	-25.60	-48.65**	-33.50	-30.46	-69.24**	-74.75
CM 33XQPM 3	LPA x QPM	-23.87**	-29.59**	-2.99**	-3.43**	-21.97	-53.11**	-22.35	-49.32**	-45.96	-94.88**
CM 33XQPM 6	LPA x QPM	-34.62**	-35.09**	-4.70**	-4.74**	-4.12	-31.94	18.08	-7.18	-0.59	-29.67
CM 33XQPM 7	LPA x QPM	21.07**	18.03**	2.75**	2.81**	4.50	32.92	16.65	36.39	23.11	56.01
CM 33XP 12	LPA x Nm	35.07**	30.66**	3.95**	3.25**	45.88**	54.24**	36.13	42.15	90.09**	93.58**
CM 33XT 2	LPA x Nm	36.01**	39.72**	5.79**	4.17**	25.51	48.44**	20.65	36.84	55.18	92.87**
CM 33XT 3	LPA x Nm	17.26**	20.16**	1.14	2.03	19.68	32.50	32.48	25.78	27.06	17.29
CM 34XQPM 3	LPA x QPM	11.94**	20.91**	3.01**	4.31	44.96**	45.57	28.20	11.87	66.08	55.39
CM 34XQPM 6	LPA x QPM	7.19	-7.59	-0.11	-2.15	22.36	2.34	2.48	8.86	18.43	-6.64

Pedigree	Classification	Germination %		Seedling Dry Weight (g)		Root Length (mm)		Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
CM 34XQPM 7	LPA x QPM	0.88	0.53	0.79	-0.70	3.98	9.80	10.15	-3.77	10.65	-7.62
CM 34XP 12	LPA x Nm	8.88	11.16	-0.05	0.44	-13.69	2.77	-10.27	22.59	-15.55	34.47
CM 34XT 2	LPA x Nm	10.82	14.22	2.38**	2.71**	3.55	16.67	-6.50	12.73	-3.09	33.97
CM 34XT 3	LPA x Nm	-35.43**	-37.34**	-5.07**	-5.14**	-46.39**	-39.66	-26.42	-24.78	-73.92**	-61.34
QPM 3XQPM 6	QPM x QPM	18.44**	24.84**	2.24**	2.65**	-9.46	28.68	-19.42	33.70	-15.58	64.38
QPM 3XQPM 7	QPM x QPM	-4.87	0.97	-0.47	-0.15	15.21	11.49	-3.75	-4.53	7.64	15.98
QPM 3XP 12	QPM x Nm	-3.87	-5.90	0.79	-0.86	-21.36	-1.20	15.92	4.79	-21.20	-13.02
QPM 3XT 2	QPM x Nm	-45.43**	-57.34**	-7.87**	-8.39	-31.38	-69.50**	-4.20	-53.13**	-55.72	-139.48**
QPM 3XT 3	QPM x Nm	33.32**	38.10**	7.03**	7.66**	19.54	24.47	-1.72	12.81	53.44	57.41
QPM 6XQPM 7	QPM x QPM	5.38	6.47	1.32	1.19	-13.14	-9.75	-13.62	-16.03	-16.03	-23.36
QPM 6XP 12	QPM x Nm	12.38**	18.10**	1.28	2.98**	-4.01	27.17	2.46	11.83	16.11	50.58
QPM 6XT 2	QPM x Nm	13.32**	19.16**	1.47	3.70**	6.12	8.52	-9.42	-9.78	4.97	8.58
QPM 6XT 3	QPM x Nm	-12.43**	-28.90**	-0.84	-3.74**	-13.11	-48.21**	-12.28	-44.35	-46.90	-87.08**
QPM 7XP 12	QPM x Nm	-5.93	-15.78*	-1.38	0.43	17.91	-28.27	1.98	-39.75	26.84	-55.62
QPM 7XT 2	QPM x Nm	-6.99	-10.72	-1.14	-1.30	-1.36	-15.22	21.80	4.99	17.83	-13.98
QPM 7XT 3	QPM x Nm	12.26**	16.72*	1.81*	1.86	1.36	14.20	-1.97	22.97	24.01	55.54
P 12XT 2	Nm x Nm	-2.99	-4.09	0.41	-0.51	-14.83	-20.40	-24.47	-12.95	-38.77	-32.32
P 12XT 3	Nm x Nm	-23.74**	-9.65	-4.08**	-2.11	-6.71	0.52	-12.64	0.74	-31.41	-15.43
T 2XT 3	Nm x Nm	9.19	6.41	0.85	0.61	1.57	-1.13	-0.37	2.17	12.42	-2.07

* P≤0.005, ** P≤0.001

There were two LPA x LPA and two LPA x QPM crosses that were included in this selection which is promising for LPA and QPM breeding programmes. These crosses showed good tolerance to accelerated aging by retaining or showing increased SCA effects. These crosses should be also included in the selection process of the breeding programme to improve germination and vigour in these different endosperm backgrounds.

Performance of parent inbred lines and classes

Tables 4.5 and 4.6 show the response of the 10 parental lines under standard and accelerated aging environments for the seed traits tested. The germination and vigour seed tests on the parental lines showed distinct differences between the different classes of endosperm classification between the LPA and the QPM and Normal groups. The germination and vigour of LPA lines were generally lower than the QPM and normal lines for all seed traits. The LPA lines had the highest reduction under accelerated aging conditions for germination percentage (61%), seedling dry weight (52%), average root length (23%), average shoot length (47%) and vigour index (34%). The QPM and normal inbred lines showed no differences for germination percentage and vigour index, increased for seedling dry weight and average root length and slight decrease for average shoot length under accelerated aging conditions.

The LPA group generally showed lower values for each trait than the overall mean. Under conditions of the accelerated aging test, all the LPA lines performed below the overall mean of all seed traits tested. The LPA lines showed the greatest percentage reduction under accelerated aging conditions for germination percentage and seedling dry weight traits. Lines P 12 and QPM 7 showed higher germination percentages, retained or increased dry weight, increased root length and increased vigour index under accelerated aging conditions compared to standard germination conditions. The lines QPM 6, QPM 7, P 12 and T 2 all showed increases in vigour index values under accelerated aging conditions than standard germination conditions which can be used as an indicator of stress tolerance.

Figures 4.1 to 4.5 show the mean values for each group under each seed trait tested. The LPA lines generally showed a decrease in values from the standard to accelerated aging conditions for all traits. All the LPA lines showed negative percentage reductions for germination percentage (44-80%), seedling dry weight (36-76%), average root length (1-52%), average shoot length (15-79%) and vigour index (4-67%) traits when placed under accelerated aging conditions. The number of abnormal and dead seedlings increased significantly for LPA lines when placed under accelerated aging conditions (Appendix 18). Only the LPA line, CM 32 showed very little reductions for average root length (1%) and vigour index (4%) traits under accelerated aging conditions compared to the other LPA lines.

In Figure 4.1, the average for the groups, LPA, QPM and Normal for germination percentage are shown with the LPA lines having the largest decrease in values from the standard to accelerated aging conditions (70% - 27%). The Normal and QPM group of lines showed almost unchanged germination percentage values under accelerated aging conditions. The graph for seedling dry weight (Figure 4.2) showed the Normal and QPM groups having increased values under accelerated aging conditions, while the LPA group showed decreased seedling dry weight (52% reduction). For average root length and vigour index traits, the QPM group remained the same while the Normal group showed a slight increase with the LPA group showing 23% reduction for average root length and 34% reduction for vigour index (Figure 4.3 and 4.5). However, for average shoot length the QPM group average decreased under accelerated aging conditions while the Normal group remained almost unchanged and the LPA group showed 47% reduction (Figure 4.4).

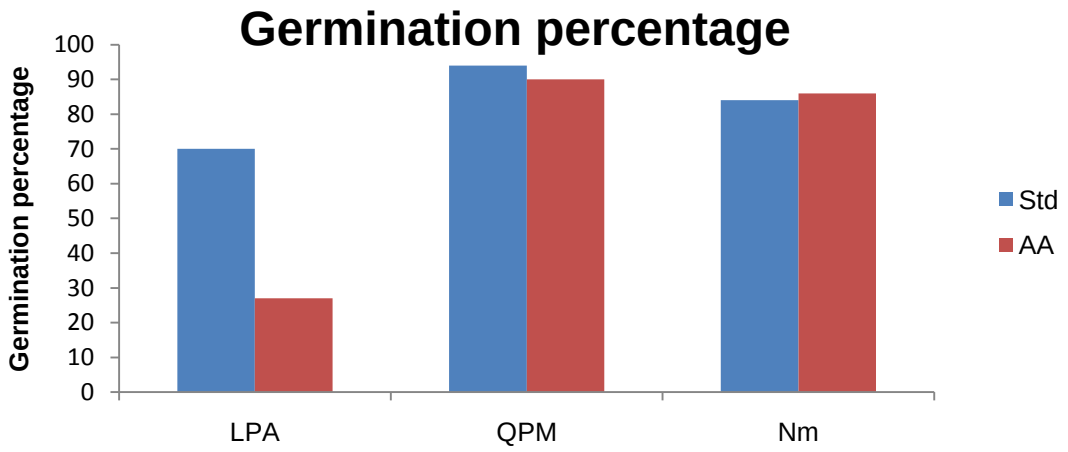


Figure 4.1: Averages of three groups of parental lines for germination percentage under standard germination (Std) and accelerated aging (AA) test conditions

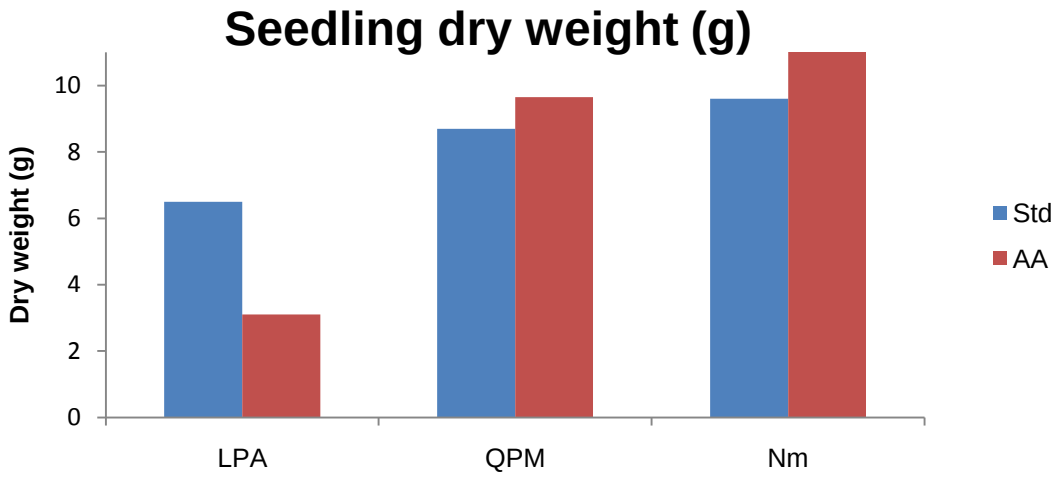


Figure 4.2: Averages of three groups of parental lines for seedling dry weight under standard germination (Std) and accelerated aging (AA) test conditions

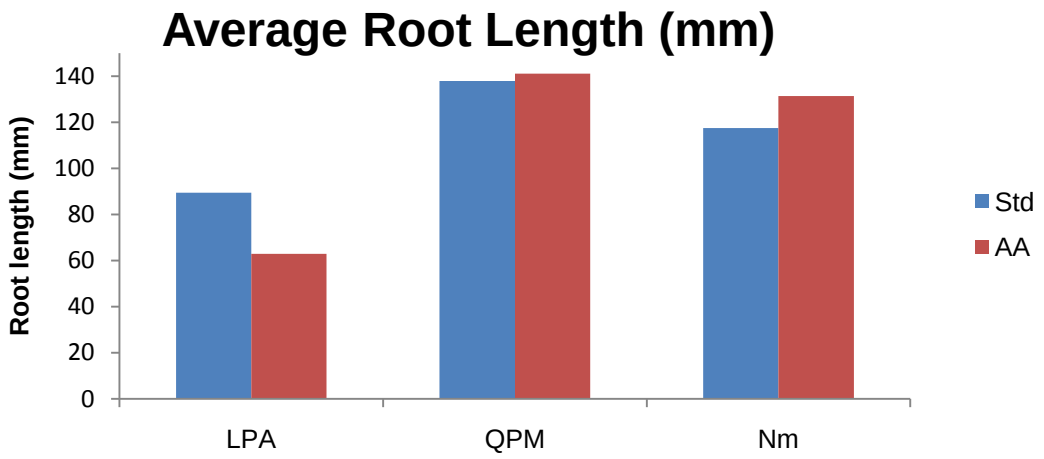


Figure 4.3: Averages of three groups of parental lines for average root length under standard germination (Std) and accelerated aging (AA) test conditions

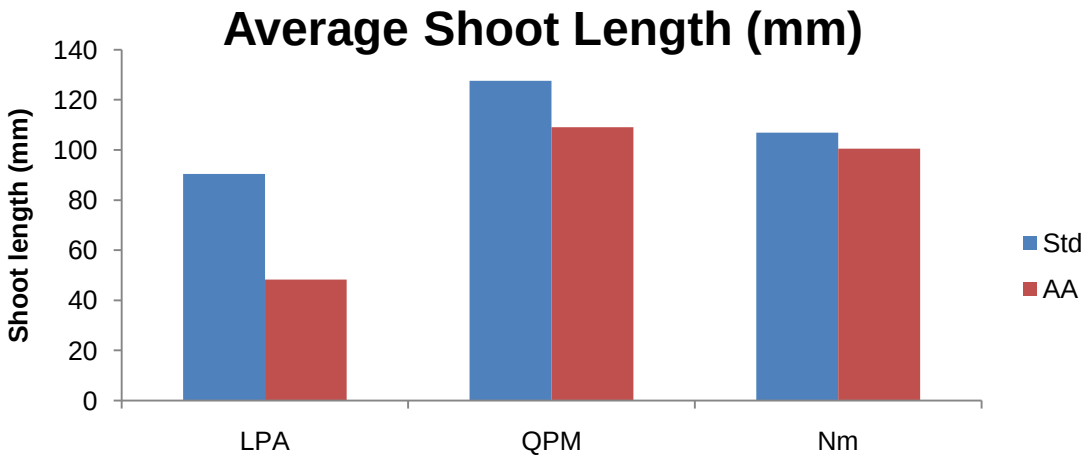


Figure 4.4: Averages of three groups of parental lines for average shoot length under standard germination (Std) and accelerated aging (AA) test conditions

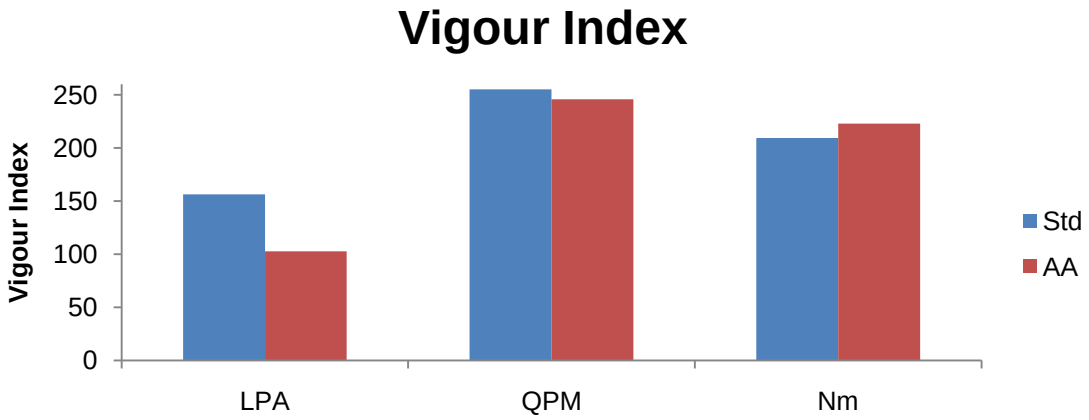


Figure 4.5: Averages of three groups of parental lines for the vigour index trait under standard germination (Std) and accelerated aging (AA) test conditions

Table 4.5: Mean values of 10 parental inbred lines under standard germination (Std) and accelerated aging (AA) for germination percentage, seedling dry weight (g) and average root length (mm) traits

Entry	Germination %			Seedling dry weight (g)			Average Root Length (mm)		
	Std	AA	% reduction[+]	Std	AA	% reduction[+]	Std	AA	% reduction[+]
LPA group mean	**69.75**	**27.00**	**61%**	**6.49**	**3.09**	**52%**	**89.53**	**62.95**	**23%**
CM 31	69.00	16.00	77%	5.35	1.30	76%	54.90	26.55	52%
CM 32	68.00	32.00	53%	8.60	5.50	36%	68.95	68.25	1%
CM 33	88.00	49.00	44%	7.95	4.55	43%	118.75	77.45	35%
CM 34	54.00	11.00	80%	4.05	1.00	75%	115.50	79.55	31%
QPM group mean	**93.67**	**89.67**	**4%**	**8.68**	**9.65**	**-11%**	**137.87**	**141.15**	**-2%**
QPM 3	93.00	85.00	9%	7.90	7.70	3%	167.30	130.00	22%
QPM 6	91.00	84.00	8%	8.55	9.80	-15%	117.30	145.10	-24%
QPM 7	97.00	100.00	-3%	9.60	11.45	-19%	129.00	148.35	-15%
Tropical normal group mean	**84.00**	**86.00**	**-2%**	**8.82**	**9.18**	**-4%**	**117.45**	**131.47**	**-12%**
P 12	89.00	98.00	-10%	10.80	10.80	0%	91.95	133.45	-45%
T 2	68.00	66.00	3%	6.65	7.05	-6%	138.55	146.25	-6%
T 3	95.00	94.00	1%	9.00	9.70	-8%	121.85	114.70	6%
P value	<0.0001	<0.0001		<0.0001	<0.0001		0.0033	0.016	
P value (ENV*Entry)									
Overall Mean	81.20	63.50		7.85	6.89		112.41	106.70	
CV (%)	5.45	9.55		6.69	9.24		15.29	18.38	
R^2 (%)	95.92	98.46		96.78	98.58		88.20	90.16	

[+] =100 - (standard germination – accelerated aging)

Table 4.6: Mean values of 10 parental inbred lines under standard germination (Std) and accelerated aging (AA) for average shoot length and vigour index traits

Entry	Average Shoot Length (mm)			Vigour Index		
	Std	AA	% reduction+	Std	AA	% reduction+
LPA group mean	**90.48**	**48.23**	**47%**	**156.32**	**102.79**	**34%**
CM 31	96.90	20.75	79%	129.02	43.09	67%
CM 32	68.05	58.05	15%	115.73	110.98	4%
CM 33	112.90	69.60	38%	222.89	135.44	39%
CM 34	84.05	44.50	47%	157.63	121.63	23%
QPM group mean	**127.60**	**109.02**	**15%**	**255.28**	**245.71**	**4%**
QPM 3	104.70	64.40	38%	266.59	180.99	32%
QPM 6	139.65	131.85	6%	234.64	276.96	-18%
QPM 7	138.45	130.80	6%	264.62	279.18	-6%
Tropical normal group mean	**106.83**	**100.50**	**6%**	**209.31**	**222.87**	**-6%**
P 12	114.25	109.10	5%	189.71	222.74	-17%
T 2	110.00	98.55	10%	228.66	237.28	-4%
T 3	96.25	93.85	2%	209.57	208.60	0%
P value	0.0003	<0.0001		0.0021	0.0005	
P value (ENV*Entry)						
Overall Mean	106.52	82.15		201.90	181.69	
CV (%)	7.85	14.60		12.90	17.30	
R^2 (%)	93.87	94.99		89.23	92.50	

+ =100-(standard germination – accelerated aging)

Performance of crosses and groups of crosses

The seeds of the cross CM 31 x CM 33 showed no germination (Table 4.7 and 4.8) due to high numbers of abnormal (97%) and dead (3%) seedlings under standard germination conditions which increased to 100% abnormal seedlings under accelerated aging conditions (data not shown). This LPA x LPA cross is clearly carrying detrimental genes for germination and vigour and is not suitable for further consideration in the breeding programme. There were 10 crosses that performed below the overall germination percentage mean across both environments. These included two LPA x LPA, two LPA x QPM, three LPA x Nm, two QPM x Nm and one Nm x Nm cross. The CV value for seedling dry weight under standard conditions was high (95.11%) (Table 4.7).

The crosses were grouped into six groups based on the type of the parental lines and their group averages calculated for each seed trait. All the groups except QPM x QPM showed decreases in germination percentages under accelerated aging conditions, with the QPM x QPM groups having the highest germination percentage overall. The LPA x LPA groups showed the lowest germination percentage (Figure 4.6).

For seedling dry weight trait, the QPM x QPM and LPA x LPA groups did not show much change under the different conditions, with QPM x Nm, LPA x Nm, LPA x QPM and Nm x Nm groups having slight decreases under accelerated aging conditions (Figure 4.7). The two groups of LPA x LPA and LPA x QPM showed similar values under standard and accelerated aging conditions for the average root length trait showing stability under accelerated aging conditions. In all the other groups (LPA x Nm, Nm x Nm, QPM x Nm, QPM x QPM) a higher average root length value was obtained under accelerated aging conditions than standard conditions, thereby showing a positive response and adaptation to stress conditions (Figure 4.8) compared to other above-ground parameters measured in this study.

For the average shoot length only the LPA x Nm group showed increased average values under accelerated aging conditions, while the QPM x Nm group decreased under accelerated aging conditions. The other four groups had similar values under both conditions showing stability under accelerated aging conditions (Figure 4.9). The LPA x LPA group had decreased vigour index values under accelerated aging conditions and LPA x QPM group showed no change under the different aging conditions. The LPA x Nm, QPM x Nm, Nm x Nm and QPM x QPM groups all showed slight increased vigour index values under accelerated aging conditions (Figure 4.10).

Table 4.7: Means for F_2 seeds generated under standard germination (Std) and accelerated aging (AA) conditions for germination percentage, seedling dry weight (g) and average root length (mm) traits

Pedigree	Classification	Germination %		Seedling dry weight (g)		Average Root Length (mm)	
		Std	AA	Std	AA	Std	AA
CM 31 x CM 32	LPA x LPA	96.00	79.5	12.95	11.60	107.90	117.00
CM 32 x CM 33	LPA x LPA	0.00	0.00	0.00	0.00	0.00	0.00
CM 32 x CM 34	LPA x LPA	96.00	95.00	11.35	11.80	107.90	144.85
CM 31 x CM 33	LPA x LPA	93.00	89.00	13.05	12.55	104.45	119.35
CM 31 x CM 34	LPA x LPA	96.00	85.00	12.10	11.10	118.25	78.30
CM 34 x CM 33	LPA x LPA	13.00	2.00	1.20	0.61	53.30	16.62
LPA x LPA		**65.67**	**58.42**	**8.44**	**7.94**	**81.97**	**79.35**
CM 32 x T 3	LPA x Nm	92.00	84.00	12.30	10.90	141.65	142.75
CM 33 x P 12	LPA x Nm	94.00	84.00	12.35	11.15	132.30	151.80
CM 33 x T 2	LPA x Nm	95.00	94.00	14.90	11.90	108.85	149.45
CM 34 x P 12	LPA x Nm	94.00	89.00	11.05	11.20	106.25	129.45
CM 34 x T 2	LPA x Nm	96.00	93.00	14.20	13.30	120.40	146.80
CM 34 x T 3	LPA x Nm	34.50	20.00	3.85	2.25	64.35	58.10
T 2 x CM 32	LPA x Nm	74.00	75.00	11.15	10.75	137.75	172.70
CM 31 x T 2	LPA x Nm	86.00	84.00	11.35	10.35	97.30	155.65
T 3 x CM 33	LPA x Nm	61.00	53.00	7.35	6.55	96.90	101.15
CM 31 x T 3	LPA x Nm	51.00	34.00	5.45	3.60	94.40	105.55
LPA x Nm		**77.75**	**71.00**	**10.40**	**9.20**	**110.02**	**131.34**
CM 32 x QPM 6	LPA x QPM	86.00	75.00	8.35	7.9	102.60	139.30
CM 32 x QPM 7	LPA x QPM	55.50	57.00	6.10	5.65	114.00	139.10
CM 33 x QPM 3	LPA x QPM	24.00	10.00	2.65	1.25	58.20	35.50
CM 33 x QPM 7	LPA x QPM	90.00	83.00	11.45	11.00	106.40	130.00
CM 34 x QPM 6	LPA x QPM	82.00	57.00	8.85	6.10	135.70	99.55
CM 34 x QPM 7	LPA x QPM	96.00	90.00	12.20	10.35	139.40	136.00
CM 31 x QPM 3	LPA x QPM	80.00	70.00	7.35	6.30	119.75	145.20
QPM 3 x CM 34	LPA x QPM	86.00	85.00	11.35	11.85	158.65	163.30
QPM 6 x CM 33	LPA x QPM	14.00	5.00	1.55	0.65	75.70	36.15
CM 31 x QPM 6	LPA x QPM	88.00	84.00	11.90	11.85	118.55	113.95

Pedigree	Classification	Germination %		Seedling dry weight (g)		Average Root Length (mm)	
		Std	AA	Std	AA	Std	AA
QPM 7 x CM 31	LPA x QPM	78.00	71.50	9.45	7.35	127.60	133.15
QPM 7 x CM 32	LPA x QPM	94.00	100.00	10.45	11.50	105.30	139.55
LPA x QPM		**72.79**	**65.63**	**8.47**	**7.65**	**113.49**	**117.56**
T 2 x P 12	Nm x Nm	90.00	86.00	14.15	12.65	109.55	148.15
T 3 x P 12	Nm x Nm	54.00	59.00	6.75	7.85	111.55	136.70
T 3 x T 2	Nm x Nm	87.00	76.00	12.40	10.40	116.75	138.50
Nm x Nm		**77.00**	**73.67**	**11.10**	**10.30**	**112.62**	**141.12**
QPM 3 x P 12	QPM x Nm	78.00	69.50	11.05	9.25	99.85	154.95
QPM 3 x T 2	QPM x Nm	36.50	19.00	3.10	1.55	86.75	90.10
QPM 6 x P 12	QPM x Nm	95.00	94.00	12.15	13.80	116.85	162.80
QPM 7 x P 12	QPM x Nm	97.00	85.00	11.95	14.05	160.85	136.35
QPM 7 x T 2	QPM x Nm	96.00	91.00	12.90	12.15	138.50	152.85
T 2 x QPM 6	QPM x Nm	96.00	96.00	13.06	14.35	124.90	147.60
T 3 x QPM 3	QPM x Nm	100.00	93.00	15.10	14.40	131.55	151.70
T 3 x QPM 6	QPM x Nm	55.00	26.50	7.85	3.70	98.55	58.50
T 3 x QPM 7	QPM x Nm	100.00	97.00	12.95	12.10	135.10	149.90
QPM x Nm		**83.72**	**74.56**	**11.12**	**10.59**	**121.43**	**133.86**
QPM 3 x QPM 6	QPM x QPM	90.00	87.00	10.35	10.25	105.15	155.35
QPM 3 x QPM 7	QPM x QPM	87.00	88.00	10.10	10.25	151.90	167.15
QPM 6 x QPM 7	QPM x QPM	98.00	94.00	12.50	12.30	123.20	125.40
QPM x QPM		**91.67**	**89.67**	**10.98**	**10.93**	**126.75**	**149.30**
Overall Mean		77.12	69.98	11.02	9.40	113.63	126.99
P value		<0.0001	<0.0001	0.1142	<0.0001	0.0002	<0.0001
P value (ENV*Entry)							
CV (%)		6.08	9.38	95.11	10.30	16.82	16.74
R^2 (%)		98.53	97.57	60.15	97.05	78.31	85.46

Table 4.8: Means for F_2 seeds generated under standard germination (Std) and accelerated aging (AA) conditions for average shoot length (mm) and vigour index traits

Pedigree	Classification	Average Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA
CM 31 x CM 32	LPA x LPA	107.45	111.20	206.93	188.51
CM 32 x CM 33	LPA x LPA	0.00	0.00	0.00	0.00
CM 32 x CM 34	LPA x LPA	105.70	114.65	206.12	251.33
CM 31 x CM 33	LPA x LPA	89.60	123.80	187.40	231.45
CM 31 x CM 34	LPA x LPA	132.30	73.25	248.07	137.77
CM 34 x CM 33	LPA x LPA	43.10	68.56	71.04	42.77
LPA x LPA		**79.69**	**81.91**	**153.26**	**141.97**
CM 32 x T 3	LPA x Nm	108.85	125.70	243.76	265.88
CM 33 x P 12	LPA x Nm	126.70	141.45	246.00	271.18
CM 33 x T 2	LPA x Nm	100.25	133.10	206.83	279.69
CM 34 x P 12	LPA x Nm	102.50	125.35	200.63	247.41
CM 34 x T 2	LPA x Nm	95.30	112.45	208.82	256.14
CM 34 x T 3	LPA x Nm	73.35	51.50	108.32	94.41
T 2 x CM 32	LPA x Nm	108.20	128.55	233.55	278.45
CM 31 x T 2	LPA x Nm	102.45	137.10	183.92	275.18
T 3 x CM 33	LPA x Nm	110.05	98.60	149.03	137.70
CM 31 x T 3	LPA x Nm	118.15	78.60	142.48	138.19
LPA x Nm		**104.58**	**113.24**	**192.33**	**224.42**
CM32 x QPM6	LPA x QPM	101.25	139.45	185.91	234.43
CM32 x QPM7	LPA x QPM	123.55	135.65	204.21	224.63
CM33 x QPM3	LPA x QPM	55.30	39.15	89.33	62.77
CM33 x QPM7	LPA x QPM	113.75	133.35	210.53	239.98
CM34 x QPM6	LPA x QPM	118.40	102.65	228.63	172.85
CM34 x QPM7	LPA x QPM	129.45	96.65	258.33	211.69
CM31 x QPM3	LPA x QPM	110.35	135.30	202.98	262.65
QPM3 x CM34	LPA x QPM	128.05	103.80	261.64	248.38
QPM6 x CM33	LPA x QPM	111.80	83.15	149.35	114.48
CM31 x QPM6	LPA x QPM	144.55	121.95	242.24	214.43

Pedigree	Classification	Average Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA
QPM 7 x CM 31	LPA x QPM	91.95	120.55	173.88	201.94
QPM 7 x CM 32	LPA x QPM	119.95	127.10	213.93	266.62
LPA x QPM		**112.36**	**111.56**	**201.75**	**204.57**
T 2 x P 12	Nm x Nm	91.30	120.60	188.77	249.93
T 3 x P 12	Nm x Nm	101.10	110.85	166.46	200.4
T 3 x T 2	Nm x Nm	102.40	109.25	206.01	222.99
Nm x Nm		**98.27**	**113.57**	**187.08**	**224.44**
QPM 3 x P 12	QPM x Nm	129.75	130.55	189.98	240.05
QPM 3 x T 2	QPM x Nm	98.65	69.60	151.20	122.82
QPM 6 x P 12	QPM x Nm	132.35	139.45	241.94	290.15
QPM 7 x P 12	QPM x Nm	135.25	94.50	290.16	223.78
QPM 7 x T 2	QPM x Nm	144.10	136.20	276.88	274.64
T 2 x QPM 6	QPM x Nm	160.25	114.80	120.08	257.37
T 3 x QPM 3	QPM x Nm	99.10	112.10	230.68	253.29
T 3 x QPM 6	QPM x Nm	104.60	56.80	144.99	95.30
T 3 x QPM 7	QPM x Nm	118.30	130.75	253.38	277.75
QPM x Nm		**124.71**	**109.42**	**211.03**	**226.13**
QPM 3 x QPM 6	QPM x QPM	97.55	150.50	189.63	283.99
QPM 3 x QPM 7	QPM x QPM	116.60	118.90	250.33	275.42
QPM 6 x QPM 7	QPM x QPM	122.80	109.25	241.31	222.59
QPM x QPM		**112.32**	**126.22**	**227.09**	**260.67**
Overall Mean		110.51	111.64	196.35	210.26
P value		0.0107	<0.0001	<0.0001	<0.0001
P value (Env*Entry)					
CV (%)		19.37	17.74	18.78	16.87
R^2 (%)		70.14	78.23	84.94	89.51

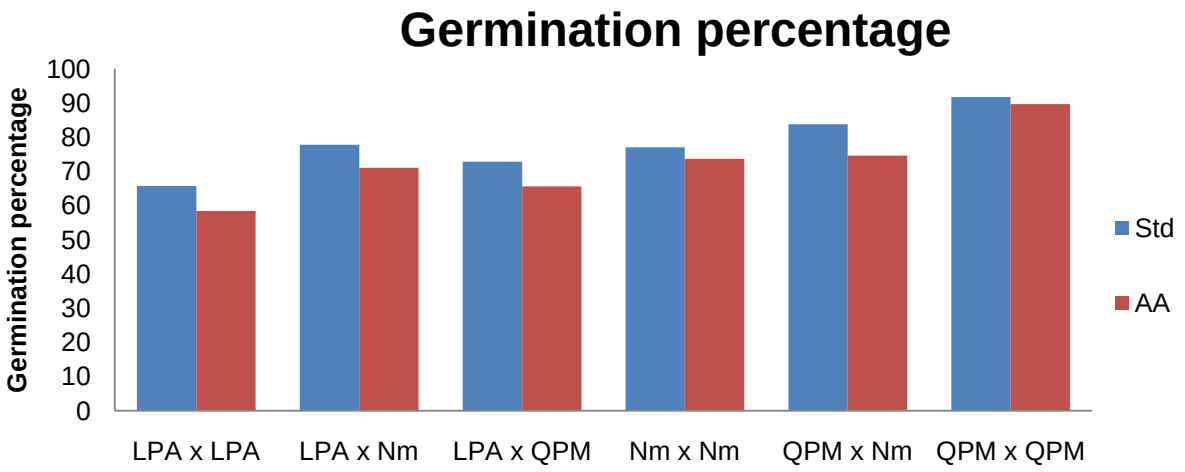


Figure 4.6: Averages of five groups of crosses for the germination percentage under normal germination (Std) and accelerated aging (AA) test conditions

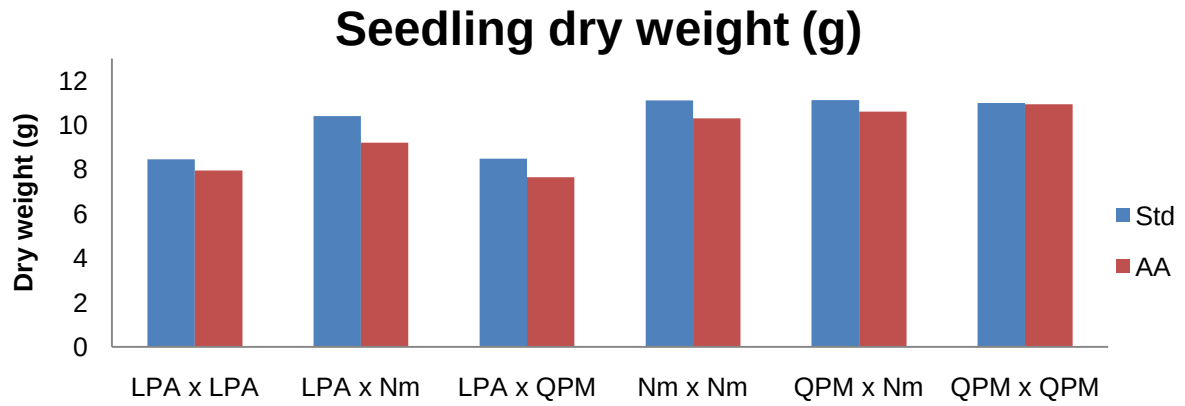


Figure 4.7: Averages of five groups of crosses for the seedling dry weight trait under normal germination (Std) and accelerated aging (AA) test conditions

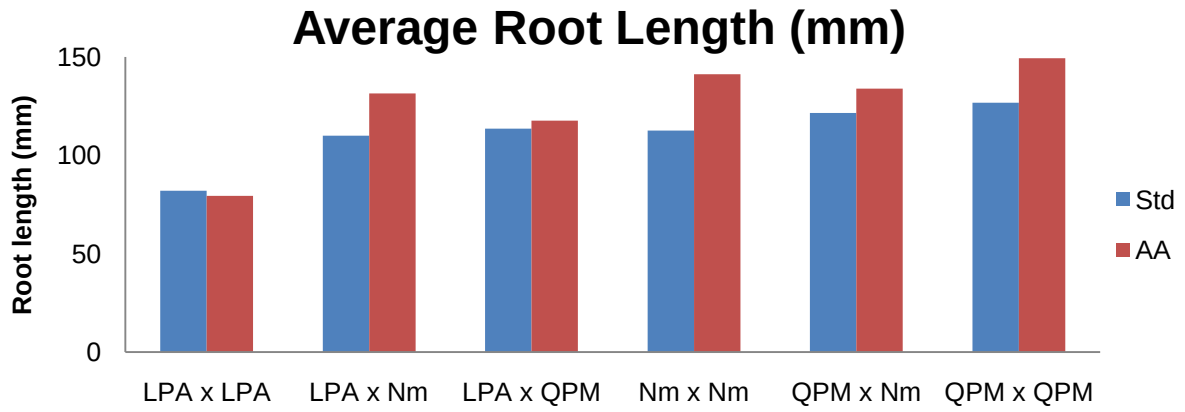


Figure 4.8: Averages of five groups of crosses for the average root length under normal germination (Std) and accelerated aging (AA) test conditions

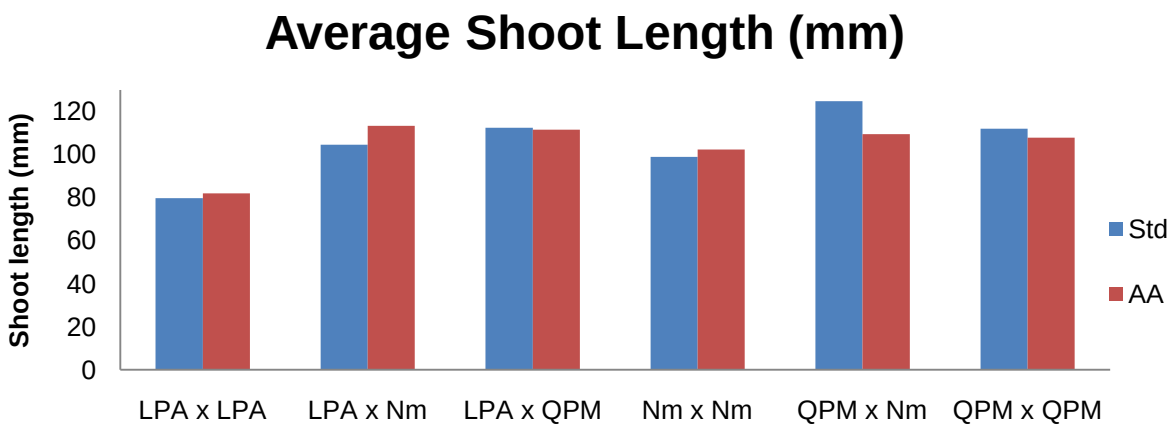


Figure 4.9: Averages of five groups of crosses for the average shoot length under normal germination (Std) and accelerated aging (AA) test conditions

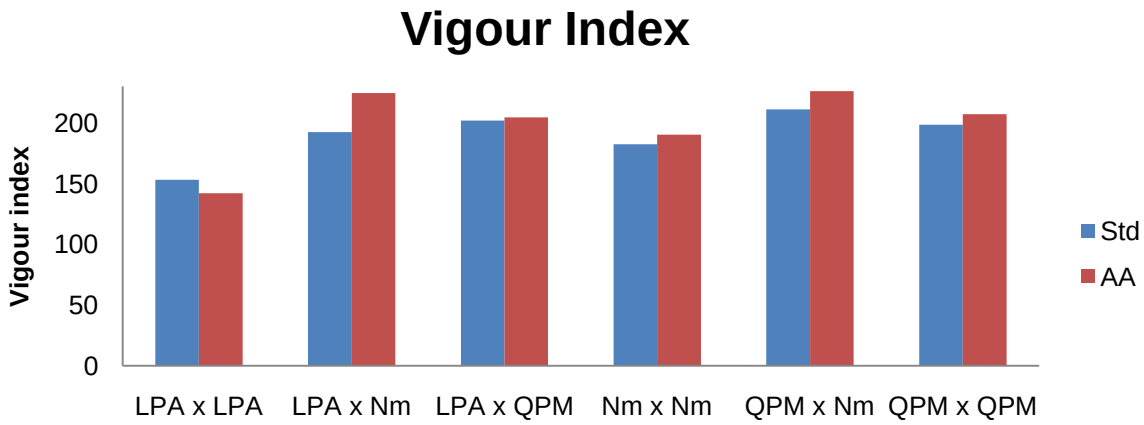


Figure 4.10: Averages of five groups of crosses for the vigour index under normal germination (Std) and accelerated aging (AA) test conditions

Discussion

Due to the recessive mutation of the *lpa1-1* gene and the segregation in the F_2 generation, it should be stated that only 25% of the F_2 seeds were expected to be homozygous recessive for the LPA gene. However, this is sufficient to show differences in the seed germination and vigour traits, as well as the added contribution of the heterozygous genotypes in the population. Under standard and accelerated aging conditions, the entry, GCA and SCA effects were significant for all the seed traits tested ($P \leq 0.001$). The lines that show significant GCA effects can be selected to be used in the breeding programme. The ratio of GCA/SCA sum of squares was in favour of SCA for all traits under both environments, showing

predominance of non-additive gene action present. However, the GCA percentage was still high; therefore there is also additive gene action present for all the traits. There are other studies that have also found that both additive and non-additive gene action was present with non-additive gene effects more important (Barla-Szabo *et al.,* 1990; Akram *et al.*, 2007; Chapi *et al.,* 2008) as in this study.

The LPA line, CM 31 showed positive GCA effects for germination percentage, dry weight and average shoot length under both standard and accelerated aging conditions, however CM 32 only showed positive GCA effects under accelerated aging conditions for average root length, average shoot length and vigour index thereby showing stress tolerance. Line QPM 7 had positive and significant GCA effects for all traits under both conditions. The normal tropical lines, P 12 and T 2 were the best performing normal lines with positive GCA effects for most of the traits. Therefore the following lines can be recommended for use in breeding programme to improve germination and vigour; CM 31, CM 32, QPM 7, P 12 and T 2.

The QPM and normal lines were most efficient in accumulating dry matter content by having retained or increased dry weight under accelerated aging conditions. Lines QPM 6 and QPM 7 were also able to increase the root length under accelerated aging showing stress tolerance. The shoot length was decreased for all lines during accelerated aging, however QPM 6, QPM 7, P 12 and T 3 showed the least reductions (<6%) while showing stress tolerance. Due to the significance of SCA effects, these can be exploited for hybrid production.

There were nine crosses with positive SCA effects for all traits under both standard and accelerated aging conditions and are considered to be stress tolerant (CM 31 x CM 33, CM 32 x CM 34, CM 32 x T 3, CM 33 x QPM 7, CM 33 x P 12, CM 33 x T 2, CM 33 x T 3, CM 34 x QPM 3, QPM 6 x P 12). There were two LPA x LPA and two LPA x QPM crosses that were included in this selection. These crosses showed stress tolerance to accelerated aging by retaining or showing increased positive SCA effects. There were some crosses that had negative SCA effects under standard conditions but these showed positive SCA effects under accelerated aging conditions (CM 31 x QPM 7, CM 31 x T 2, CM 34 x P 12, CM 34 x T 2, QPM 3 x QPM 6, QPM 3 x T 3, QPM 7 x T 3) showing stress tolerance to accelerated aging conditions. Due to the predominance of SCA effects for seed germination and vigour traits, breeding methods that exploit SCA effects can be used, e.g. recurrent selection for specific combining ability. Inbred lines can be developed which show SCA effects when combined with other inbreds.

The reason is not clear why there is higher seedling vigour after accelerated aging conditions and needs further testing. It is only the inherently vigorous seeds which were capable of producing healthy and normal seedlings under these conditions. The low vigour seeds were able to germinate but were unable to grow in adverse accelerated aging conditions and weak seedlings were produced. This was also found in a study with maize under accelerated aging conditions (Basu *et al.,* 2004).

The LPA lines exhibited lower germination and vigour in general especially under accelerated aging conditions. The germination percentage under normal conditions was between 54-88% which was reduced to 11-49% under accelerated aging conditions. The germination percentage of these lines needs to be improved to >90% for use in certified seed production. In the study comparing the maize LPA mutant (*lpa241*) with its wild type, under standard germination conditions, the mutant also showed lower germination than the wild type which decreased further after the accelerated aging test with 38% more loss in the mutant than the wild type (9%) (Doria *et al.,* 2009). Line CM 32 showed very little reduction in average root length (1%) and vigour index (4%) under accelerated aging conditions, showing high stress tolerance for these traits. Studies on maize with the accelerated aging test showed different reductions with 94.7% under standard conditions to 36.3% (Noli *et al.,* 2008), 87.6% to 76.3% (Byrum and Copeland, 1995) and 91.9% to 84% (Lovato *et al.,* 2005). The varying results are due to the genotype effectiveness to the stress provided by the accelerated aging test, probably due to their genetically determined vigour traits (Gutierrrez *et al.,* 1993). The shape of the seed is also found to influence germination and vigour in maize where round seeds germinated faster than flat seeds (Tekrony *et al.,* 2005).

The findings in this study are that generally all traits showed reductions under accelerated aging conditions which was also found in other studies in maize where the traits, seedling length and dry weight were shown to have a greater response to accelerated aging than standard germination (Santipracha *et al.,* 1997; Basu *et al.,* 2004). In this study the accelerated aging test was able to measure a wide range of seed vigour (0-100%) and this was also found by Tekrony *et al. (*2005) with a range from 0% to 93%. The correlation between phytate-P content and seed vigour was confirmed with finding that the higher the phytate-P content, the higher the seed vigour (Modi and Asanzi, 2008). Revilla *et al.* (2009) found inbreds having heavier kernels would be better seed producing parents for hybrids with better early vigour and earlier flowering dates.

The QPM lines performed well compared to the normal tropical lines showing significant improvement in QPM breeding for vigour over the last few years. The line QPM 7 was very stress tolerant for all traits except average shoot length, showing increased values under

accelerated aging conditions. Line QPM 6 also exhibited stress tolerance by having increased values for germination percentage, seedling dry weight, average root length and vigour index under accelerated aging conditions. The tropical normal line, P 12 showed increased values for germination percentage, average root length and vigour index with no change for seedling dry weight under accelerated aging conditions. These lines showed high stress tolerance and should be used in breeding programmes.

The average root length trait of the six groups of crosses (LPA x QPM, LPA x Nm, Nm x Nm, QPM x Nm and QPM x QPM) showed higher average root lengths under AA conditions compared to standard germination conditions. Other studies have found similar results in different crops (Chun *et al.,* 2005; Kausar and Shahzad, 2006; Radhouane, 2007; Khan *et al.,* 2010). This could be as a result of the plant being able to access more nutrients and resources quickly by having longer root lengths to overcome the stress factors. Further research is needed to determine the exact causes of this observation. The germination percentage, average root length, average shoot length and vigour index of the LPA x QPM class of crosses was higher than the LPA x LPA class under both standard and accelerated aging conditions. It seems there are some interactions in increasing the germination percentage with the combination of LPA and QPM adaptations, with the SCA effects for all these traits significant at the 1% level. This is very promising in breeding for improved nutrition for lower phytic acid and increased levels of lysine and tryptophan in maize.

A survey of literature did not yield any information regarding previous studies of combining ability between LPA and QPM for germination and vigour. There are no other breeding programmes that have both LPA and QPM material except in Brazil, making this a novel study for breeding with LPA and QPM. There is a general opinion that the combination between LPA and QPM has serious problems due to low germination and vigour exhibited by both classes. However our results do not support this opinion. The LPA x QPM crosses showed higher average shoot length and vigour index and similar average root length compared to Nm x Nm class.

Conclusions

The following conclusions could be drawn from this study:

- Both additive and non-additive gene action was significant for both germination and vigour index and its component traits under normal and accelerated aging conditions, with specific combining ability (SCA) effects generally superior to general combining

ability (GCA) effects for all traits, indicating that genes with non-additive effects were more important for both germination and vigour traits. Due to both additive and non-additive gene action; lines can be selected for breeding that have good combining ability for these traits. Also due to the predominance of non-additive gene effects; this material can also be useful in a breeding programme to develop hybrids.

- The LPA line, CM 31 displayed the desired positive GCA effects under standard and accelerated aging conditions and CM 32 showing increased SCA values under accelerated aging conditions thereby showing stress tolerance. Hence it would be useful source of breeding material for improved germination and vigour in other LPA germplasm.
- QPM 7 was the only QPM line that had positive GCA effects for all the traits under both standard and accelerated aging conditions showing stress tolerance.
- There were nine crosses that performed well under both standard and accelerated aging conditions which included, one QPM x Nm, two LPA x QPM, two LPA x LPA and four LPA x Nm crosses thereby showing stress tolerance for seed germination and vigour traits. Breeding methods which exploit SCA effects can be used to improve these traits, such as recurrent selection for specific combining ability.
- The LPA lines and their hybrid combinations exhibited low germination (14-39%) with the QPM lines and normal tropical lines showing high germination rates under both experimental conditions; therefore they were more viable and more stress tolerant than the LPA lines.

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Appendix 18: Means of germination, abnormal and dead percentages of 10 parental lines under standard (Std) and accelerated aging (AA) conditions

Entry	Germination %		Abnormal %		Dead %	
	Std	AA	Std	AA	Std	AA
CM 31	69.00	16.00	16.00	74.00	15.00	10.00
CM 32	68.00	32.00	16.00	55.50	16.00	12.50
CM 33	88.00	49.00	8.00	43.00	4.00	8.00
CM 34	54.00	11.00	25.00	87.00	21.00	2.00
QPM 3	93.00	85.00	5.00	6.00	2.00	7.00
QPM 6	91.00	84.00	0.00	9.00	9.00	0.00
QPM 7	97.00	100.00	2.00	0.00	1.00	0.00
P 12	89.00	98.00	3.00	2.00	8.00	9.00
T 2	68.00	66.00	24.00	31.00	8.00	3.00
T 3	95.00	94.00	1.00	6.00	4.00	0.00
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.0040	0.2616
Overall mean	81.20	63.50	10.00	31.35	8.80	5.15
CV (%)	5.45	9.55	22.11	11.07	40.23	103.41
R^2 (%)	95.92	98.46	97..41	99.42	87.99	61.47

Chapter Five

Combining ability between temperate low phytic acid (LPA) and tropical normal endosperm inbred lines for seed vigour and grain yield components

Abstract

Maize is one of the most important cereal crops in the world but has high levels of phytic acid which inhibits the absorption of essential minerals such as iron and zinc. There have been low phytic acid (LPA) mutants identified with reduced levels of phytic acid but also having reduced levels of seed germination, vigour and yield. The gene action and combining abilities were determined from an eight parent diallel, including two low phytate lines and six normal tropical endosperm inbred lines. Field evaluation of the F_1 hybrids was over two seasons, with two replications at five locations. The standard and accelerated aging tests were conducted using F_2 seeds for assessment of seed vigour. The traits tested were germination percentage, seedling dry weight, average root length, average shoot length, vigour index, yield, mid-pollen shed (DMP) and silk emergence (DMS) dates, grain moisture content (%) and ears plant^{-1} traits. There was significant additive and non-additive gene action for both germination and vigour index and its component traits under normal and stress conditions. The SCA effects were generally superior to GCA effects for all seed vigour traits, indicating that genes with non-additive effects were predominant for both germination and vigour. Generally the LPA lines and their hybrid combinations had lower germination and vigour under both experimental conditions than the normal tropical lines and their hybrid combinations. The LPA line, CM 32 showed increased GCA values under stress conditions for germination and seedling dry weight thereby showing stress tolerance. The normal lines, Kenyan, P 1, P 5 and P 7 had positive GCA effects for all seed germination and vigour traits under both standard and stress conditions. There were eight crosses that performed well for the seed germination and vigour traits under both standard and stress conditions which included two LPA x Nm and six Nm x Nm crosses that are stress tolerant. The traits, DMS, DMP, Yield and EPP had both additive and non-additive gene action being important with only additive gene action being present for GMC. The ratio of GCA/SCA sum of squares was in favour of GCA showing predominance of additive gene action present for all yield and associated traits. Both the LPA lines showed negative GCA effects for all yield and associated traits, with the normal lines; Kenyan, P 1, P 5 and P 6 having positive GCA effects for all yield and associated traits. There were three LPA x Nm crosses that showed improvements to the means of germination and vigour and yield traits (CM 32 x Kenyan, P 6

x CM 32, CM 32 x T 4). The cross CM 32 x P 6 showed negative SCA effects for DMS and GMC, positive SCA effects for yield, EPP, germination percentage, average root length, average shoot length and vigour index. Due to predominance of additive gene action, breeding strategies that exploit GCA effects should be used, such as selection of lines with positive and high GCA effects. There were positive correlations between the seed germination and vigour traits, with yield being significantly correlated to DMS, DMP and GMC implying high yield is associated with longer growing cycles.

Keywords: low phytic acid, GCA, SCA, germination, vigour, accelerated aging test, yield

Introduction

Maize is one of the most important cereal crops as it is the most widely grown crop in the world. It is a primary staple food in many developing countries especially in Africa, providing food for both humans and animals. Maize breeders are therefore always striving to improve the yield and nutritional quality of this crop. Maize has high levels of phytic acid which inhibits the absorption of essential minerals such as iron and zinc in monogastric consumers and this has a negative impact on the nutritional status of maize-based diets in sub-Saharan Africa.

It has been reported that deficiencies in micronutrients are due to more than two billion people consuming less diverse diets than 30 years ago, especially iron, zinc, selenium, iodine and vitamin A (Genc *et al.,* 2005). Iron deficiency is one of the major nutritional problems in cereal based-diets of low income countries in the developing world mainly caused by poor iron content, low bioavailability of iron, or both (Brown and Solomons, 1991). Iron and zinc deficiencies in developing countries are estimated to affect 40-45% of school age children who are anaemic, of which about 50% is due to iron deficiency (Zimmerman and Hurrell, 2002). Food components such as phytates, tannins and selected dietary fibres, which bind iron in the intestinal lumen, can impair iron absorption. Phytate has probably the greatest effect on iron status as there are a large variety of plants with high phytate content, thereby limiting absorption of iron (Mendoza *et al.*, 2001). A sustainable, cost-effective alternative to conventional supplementation is to breed staple crops with an enhanced fortification with micronutrients (Genc *et al.,* 2005).

Phytic acid is the major phosphorus containing compound found in seeds. In mature seeds, 75% of the total phosphorus is found as phytic acid (Raboy, 1997), which represents a significant amount of all phosphorus removed from the soil by grain and legume crops. It is a strong chelator of positively charged mineral cations such as calcium, iron and zinc. Once

the phytic acid is ingested, it can also bind to other seed-derived minerals, as well as other endogenous minerals that are present in the digestive tract (Raboy, 2000).

Dietary phytate can have a negative global impact by contributing to mineral depletion and deficiency in populations that rely on whole grains and legume based products as staple foods (Brown and Solomons, 1991). The limitations in the uptake of minerals due to phytic acid is a serious concern and needs to be addressed in order to increase the nutritional content of staple crops especially in the developing world. Present breeding studies aim to achieve a significant decrease (50%) and not elimination of existing levels of phytic acid, resulting in an increased uptake of the vital minerals and iron. A recent study has confirmed the antioxidant properties of phytic acid (Doria *et al.,* 2009).

Progress in plant genetics has led to the identification and successful breeding of grains and legumes that are homozygous for allelic variants at a single gene that alters the phytate content of the legume or grain. Low phytic acid (LPA) mutants controlled by a single recessive mutation have been identified in maize (Raboy *et al.*, 2000; Pilu *et al*., 2003) and rice (Larson *et al.*, 2000). Wild type maize has a total inositol P content of 3.4 mg/g with the LPA mutants showing lower levels, i.e. *lpa1-1* has 1.1 mg/g (Raboy *et al*., 2000). The whole grain concentrations of potassium (K), magnesium (Mg), iron (Fe), zinc (Zn) and manganese (Mn) were found to be comparable or higher in *lpa1-1* grains compared to wild type grains, with calcium (Ca) being lower in *lpa1-1* mutants (Lin *et al.*, 2005). These low phytic acid mutants of maize have also been shown to have normal levels of seed total phosphorus but reduced levels of phytic acid phosphorus.

The use of LPA mutants in plant breeding has been limited due to non-germination of genotypes with homozygous *lpa1* alleles (Raboy, 2000), reduced seed weight of *lpa1-1* (Raboy *et al.,* 2000), and the lower vegetative growth rate and impaired seed development due to the *lpa241* mutation (Pilu *et al.,* 2005). These LPA mutants have inferior agronomic and seed viability than their wild-type (WT) parents, leading to yield reduction (Raboy *et al.,* 2000) due to reduced seed weights and low vegetative growth. The yields of the maize LPA lines have been reduced to between 5 and 15% compared to the highest yielding commercial varieties (Raboy, 2000). The maize *lpa1-1* mutant lines have shown yield reductions compared to the WT parent (Ertl *et al.,* 1998), also observed in *lpa1* barley mutants (Bregitzer and Raboy, 2006). In rice, the LPA mutations were found to most likely be the causative factor of grain yield reduction (12.5-25.6%) (Zhao *et al.,* 2008). The wheat LPA mutants had delayed development and reduced grain yield (8-25%) partly due to reduced kernel size in a high yielding environment (Guttieri *et al.,* 2006).

It has been observed that LPA mutants can show 30% decrease in germination rate when compared to the wild type (Pilu *et al*., 2003). There is no previous germination and vigour studies on the *lpa1-1* mutation lines. The *lpa241* mutant is allelic to *lpa1-1* (Pilu *et al*., 2005) with 30% decrease in germination rate compared to the WT (Pilu *et al*., 2003) and 72±15% germination under standard conditions which decreased to 45±14% germination under accelerated aging conditions (Doria *et al.,* 2009). The *lpa241* mutant has negative pleiotropic effects which are related to embryo development and size, germination rate, seedling growth rate and ear size (Pilu *et al.,* 2005). A study on rice found all LPA mutants showed reduced seed viability (Zhao *et al.,* 2008).

The LPA mutant lines also show significantly lower field emergence than their WT parents in rice (Zhao *et al.,* 2008), wheat (Oltmans *et al.,* 2005) and soybean (Meis *et al.,* 2003). This could be as a result of seed maturation in tropical environments at high temperatures to which the LPA genotypes have low stress tolerance, thereby reducing germination and emergence (Raboy, 2007). Low field emergence is an important issue for LPA genotypes once lines have been developed and need to be adopted for commercial production. Field emergence and not germination is important for seedling establishment.

Seed viability and vigour are two characteristics of seeds that determine their value and utility of seeds. There are many different factors that determine and influence seedling germination and vigour. The vigour of a seed can be determined by using different parameters such as root length, shoot length and dry weight of the seedling. Seed vigour is not only dependent on the size of kernel but also on the speed of germination which is an important factor. The size of the kernel is an estimate of the amount of nutrients available to the seed during germination. However, even if the seed has large kernel size but has low speed of germination, it can be classified as low vigourous. Seedling parameters such as the root length (indicator of potential to develop an adequate root system to access nutrients), shoot length (indicator of the competition ability to outgrow weeds in the field), and the dry weight of a seedling (effectiveness of the seed in producing biomass) are used to assess speed of germination and vigour. Seedling dry weight is the overall indicator of growth where the more vigourous seeds are expected to accumulate biomass faster than their less vigourous counterparts. To attain good stand of a crop, it is necessary to have a high emergence percentage followed by high seedling vigour (Fakorede and Ojo, 1981). Maize has problems in maintaining high germination standards for certified seeds (80% parental lines, 90% hybrids), especially for low vigour parental lines (Basu *et al.,* 2004).

The vigour test is a more sensitive index of seed quality than the germination test. Vigour testing has become common practice and ensures the seed will perform well across a wide

range of soil types and environmental conditions. The two most commonly used are the cold test and the accelerated aging test. The germination test is considered to be well standardized but does not detect many seed weaknesses, thereby increasing the need for vigour testing to act as quality control tool and marketing aid. Different vigour tests measure different aspects of seed quality under different soil conditions; therefore a combination of several vigour tests is able to provide information on the quality of a seed lot as well as its potential field performance (Byrum and Copeland, 1995). Seed storage under high relative humidity and/or high temperature and damage by insects are primary causes of poor seed quality.

The accelerated aging test was developed to predict seed storability (Delouche and Baskins, 1973). In maize it has been used to determine relative field emergence (Lovato *et al.,* 2005; Noli *et al.,* 2008), assess seed quality (Santipracha *et al.,* 1997; Munamava *et al.,* 2004); seedling vigour (Fakorede and Ojo, 1981), as well as assess seed storability (Basu *et al.,* 2004). The vigour and viability of maize inbred lines is reduced by aging due to non-inheritable degenerative changes (Revilla *et al.,* 2009).

In addition to contributing to breeding for LPA in tropical materials, the temperate lines can be used to introgress earliness in tropical materials. The *lpa1-1* gene results in low germination and vigour, with LPA lines showing low yield potential and are highly susceptible to drought and stress. As a result this germplasm is difficult to maintain and cannot be sustained in commercial production. As a result the LPA germplasm can be used to introgress this LPA trait into tropical and mid-altitude genetic backgrounds. The temperate germplasm is also able to contribute genes for ultra-early flowering and early physiological maturity, which is lacking in tropical germplasm. Ultra-early germplasm is desired in managing drought as these varieties will flower and mature before the onset of drought.

Diallel crosses are widely used as mating designs to study the genetic properties of inbred lines in plant breeding programmes, by providing information on the average performance of the individual inbred lines in crosses to select lines to hybridise. Griffing (1956) defines the diallel cross in terms of genotypic values where the sum of general combining abilities (GCAs) for the two gametes is the breeding value of the cross (i,j). The specific combining ability (SCA) represents the dominance deviation value if epistatic deviation is excluded. It is important to estimate combining abilities as the choice of an efficient breeding method depends on the major component of genetic variation present. In terms of genetic variance, GCA represents additive and additive x additive epistatic gene action, and SCA the non-additive types of variances, mainly dominance and epistasis. Selection is more effective with traits with a high additive genetic variance than high dominance variance, as dominance is

due to intralocus gene interaction (Muraya *et al.,* 2006). In maize, diallel mating designs have been widely used for different traits such as seed vigour and germination (Barla-Szabo *et al.,* 1990), heterosis (Xingming *et al.,* 2001; Muraya et al., 2006; Abdel-Moneam *et al.,* 2009), yield and associated traits (Malik *et al.,* 2004; Ünay *et al.,* 2004; Glover *et al.,* 2005; Akbar *et al.,* 2008; Bello and Olaoye, 2009; Vivek *et al.,* 2009), disease resistance (da Silva and Moro, 2004) and aflatoxin accumulation (Gardner *et al.,* 2007) to estimate GCA and SCA effects.

In breeding programme, the selection of parents and the determination of general and specific combining ability and gene action are important. To eliminate undesirable inbred lines and select the most desirable ones to constitute various hybrid combinations, the general and specific combining ability of the inbred lines and their crosses needs to be available. The problem of reduced plant vigour in the temperate LPA mutants needs to be overcome. The amount of vigour is often difficult to assess, but knowledge of the linkage between LPA and vigour can aid in developing a selection strategy.

The objectives of this study were to:

- determine the gene action and combining abilities controlling inheritance of early vigour and germination, yield, days to mid-pollen shed (DMP), days to silk emergence (DMS), ears per plant (EPP) and grain moisture content (GMC) in crosses involving tropical normal endosperm and temperate low phytate germplasm,
- determine correlations between the seed germination and vigour traits and yield and associated traits.

Materials and Methods

Germplasm

There were two temperate *lpa1-1* (LPA) lines and six tropical normal (Nm) inbred maize lines used in this study (Table 5.1). The LPA lines were obtained from Dr V. Raboy (Iowa, U.S.A.) and all contain the *lpa1-1* gene. There were 28 F_1 crosses generated from an eight x eight half diallel mating design with no reciprocals and selfs. The F_1 hybrids were advanced to the F_2 generation by selfing in pots in the greenhouse. The F_2 seeds were used for seed germination and vigour testing. The F_1 hybrids were evaluated for yield and associated components.

The parental inbred lines were divided into two groups: temperate LPA and tropical Nm. The means of each group was calculated and compared between the different groups. There were three groups of crosses generated between the two groups of parental lines: LPA x LPA, LPA x Nm, Nm x Nm. The means of each group was calculated and compared between the different groups. The groups were defined as: LPA group has 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g P_i (66% of total P), tropical normal has 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g P_i (7% of total P) (Raboy *et al.*, 2000).

Table 5.1: Pedigrees of inbred lines used in diallel mating design

Line	Pedigree	Endosperm type	Adaptation
CM 32	JUG 248 LPA1-1	*lpa1-1*	temperate
CM 35	Ex-USDA- not available	*lpa1-1*	temperate
Kenyan	Not available	normal	tropical
P 1	CML 202	normal	tropical
P 5	CZL 99027	normal	tropical
P 6	CZL 99028	normal	tropical
P 7	CZL 99029/CML 444	normal	tropical
T 4	Not available	normal	tropical

Standard seed testing

The standard germination test used was the between-paper method. The seed sample was randomly sampled for two replicates of 50 seeds each. The germination bed used was rolled paper towel of size 54 cm x 30 cm (Anchor Paper, Agricol (Pty) Ltd, South Africa) (ISTA, 1999). Each sheet had 50 seeds (1 replication) placed equidistant on the upper half of the moistened paper towel with the radical end of the seed pointed toward the bottom end of the paper and the embryo end side up. Paper towels were folded in the middle to cover the seeds completely. The paper towels were rolled up and placed upright individually in polythene bags in a germination chamber. Approximately 2-3 cm distance was maintained between the seeds to reduce mutual influence and bacterial infection. The containers were labelled with the date, seed name and replicate number and were covered appropriately to prevent evaporation. The containers were placed into germination chambers (Labcon growth chamber). The between-paper method used a temperature 25±1°C and the seed germination measurements taken after seven days. During the period of seed germination,

the chamber was checked daily to ensure suitable conditions for germination and the paper towel was maintained at the appropriate moisture and temperature for seven days before evaluation and counting. Seeds were visually assessed according to the International Seed Testing Association rules for percentage germination (ISTA, 1999).

Accelerated aging test

The 100 maize seeds were placed in a petri-dish with moistened filter paper. The seeds were incubated in closed petri-dishes in the germination chamber at 42°C (~100% RH) for 96 hours with regular checking to ensure sufficient moisture in the petri dish was maintained (ISTA, 1999). At the end of the aging period, the seeds were evaluated as per standard germination test, with two replications of 50 seeds each. The seeds were rated as normal and abnormal seedlings and dead seeds. A normal seedling has well developed root and shoot systems. An abnormal seedling has any abnormality in their root or shoots system. Seeds which were neither hard, fresh or have produced seedlings were classified as dead seeds (ISTA, 1999).

Data collection and analysis

All dead and defective seedlings were counted. The germinated seedlings were counted and weighed. The percentage germination was calculated as follows: (# of normal seedlings / # of seeds in sample) x 100. The percentage germinated, dead and abnormal was calculated for all the F_2 seeds tested. At the counting stage, 25 normal seedlings in each replicate were selected at random and the length of the root measured and mean values calculated. The 25 normal seedlings that were selected for the root length measurement in each replicate was again used for shoot measurements and mean values were calculated. These values were used to calculate a vigour index. The vigour index of 1-week old seedlings was calculated as VI = [(shoot length (mm) + root length (mm)] x percentage germination (Dhindwal *et al.,* 1991). The fresh weight of the germinated seedlings were taken at the counting stage and the seedling mass was subjected to 80°C for 24 hours to dry before the seedling dry weight was measured. General analysis of variance was performed for all data using the SAS programme version 9.1 (SAS Institute, 2002). Combining ability estimates were calculated for each seedling trait using the Diallel-SAS05 programme (Zhang *et al.*, 2005) in SAS.

Experimental design and analysis

The F_1 hybrids were evaluated for grain yield and associated components at the Cedara Agricultural Institute (Cedara) (1076 masl; 29°31’ S, 30°17’ E), Baynesfield Research Farm (758 masl; 29°46’ S, 30°21’ E) in South Africa and Rattray Arnold Research Station (RARS)

(1341 masl, 17°40' S, 31°13' E) and Kadoma Research Centre (KRC) (1149 masl; 18°19' S, 29°17' E) in Zimbabwe. Twenty-eight F_1 hybrids were evaluated with 12 commercial hybrids (checks) in two seasons at five locations. The trial was replicated twice in a randomized block design, with the experimental unit being one row for each entry, 75 cm apart and plant to plant distance of 25 cm. In the 2007/2008 season planting was at Cedara and in the 2008/2009 season at Cedara, Baynesfield, RARS and KRC. Standard cultural practices, fertilization and weed control were accomplished according to normal field practices. The hybrid checks (PAN) were obtained from Pannar Seed Co (Greytown, South Africa) and AGRI from Afgri (Pietermaritzburg, South Africa).

Yield (t ha^{-1}) of shelled grain (adjusted to 12.5% H_2O) was measured on the whole row basis, and relative yield for each hybrid was calculated as a percentage of the mean of the checks (Relative yield) and a percentage of the early maturing check (PAN6114) as relative yield (early). Mid-pollen shed (DMP) and silk emergence (DMS) dates were estimated as number of days from planting to when 50% of the plants were shedding pollen and had silks emerged, respectively. Prolificacy or number of ears $plant^{-1}$ (EPP) was determined as the number of ears averaged over number of plants $plot^{-1}$. Grain moisture content (GMC) was measured at harvest (Dickey-John moisture meter) using shelled grain. General analysis of variance was performed for all hybrid data including check hybrids using the SAS programme version 9.1 (SAS Institute, 2002). Combining ability estimates were calculated for each yield and associated traits using the Diallel-SAS05 programme (Zhang *et al.*, 2005) in SAS. Pearson's correlation analysis was performed with the PROC CORR procedure in SAS programme version 9.1 (SAS Institute, 2002).

Results

Gene action

Under both standard germination and accelerated aging conditions, the entry, GCA and SCA effects were significant (P≤0.001) for all the seed traits of germination percentage, seedling dry weight, average root length, average shoot length and vigour index (Table 5.2).

For yield and associated traits, the environment and entry effects were significant (P≤0.001). The GCA, Environment*Entry and GCA*Environment effects were significant (P≤0.001) for DMS, DMP and yield. EPP had significant GCA, SCA and GCA*Environment effects (P≤0.005). GMC showed significant effects at the 1% level for GCA*Environment and SCA*Environment effects and at the 5% level for GCA effects. Both DMS and DMP had significant SCA effects at the 1% level with yield at the 5% level (Table 5.3).

The ratio of GCA/SCA sum of squares was in favour of SCA for all seed germination and vigour traits under both environments, showing predominance of non-additive gene action (Table 5.4). The ratio of GCA/SCA sum of squares was in favour of GCA for yield and associated traits, showing predominance of additive gene action (Table 5.5).

Combining ability

General combining ability

Positive GCA effects were desired for all seed germination and vigour traits as well as yield and EPP traits. For DMS, DMP and GMC, negative GCA effects were required. Line CM 32 showed positive GCA effects under standard conditions for all seed germination and vigour traits except dry weight (Table 5.4). There were negative GCA effects for average root length and vigour index under accelerated aging conditions. However for germination percentage and seedling dry weight, the GCA effects increased under accelerated aging conditions, showing stress tolerance for these two traits. Line CM 35 showed negative GCA effects for all seed germination and vigour traits under both conditions except for average shoot length. Both P 6 and T 4 showed negative GCA effects for all seed germination and vigour traits under both conditions. The inbred lines Kenyan and P 1 showed positive and increased GCA effects for germination percentage, seedling dry weight and average root length. Line P 1 showed a negative GCA effect under standard conditions which increased to positive under accelerated aging conditions for average shoot length and vigour index. Line Kenyan had similar GCA effects under both conditions for average shoot length and vigour index. Lines P 5 and P 7 showed decreased GCA effects for germination percentage and seedling dry weight under accelerated aging conditions, however the lines showed positive increased GCA effects for average root length, average shoot length and vigour index.

The LPA lines showed negative GCA effects for all yield and associated traits, especially CM 32 with both negative and significant GCA effects for all traits (Table 5.5). Line CM 35 showed lower negative SCA effects for all traits and same SCA effect for GMC. The normal lines generally showed positive GCA effects with the exceptions of negative GCA effects of P 7 for EPP and T4 for yield and GMC traits. The lines, Kenyan, P1, P5 and P6 had positive GCA effects for all yield and associated traits. Line P 7 showed positive GCA effects for all yield and associated traits except GMC. Line T 4 showed negative GCA effects for yield and GMC.

Table 5.2: Mean square for seed traits of an eight parent diallel evaluated under standard germination conditions (Std) and accelerated aging conditions (AA)

Source	df	Germination percentage		Seedling dry Weight (g)		Average Root Length (mm)		Average Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
Rep	1	50.16	30.02	0.12	0.00	172.90	3542.52	174.31	1917.63	1282.95	7687.38
Entry	27	1237.82**	1516.89**	30.51**	39.68**	2063.75**	3648.89**	1808.98**	1936.69**	7949.09**	10809.00**
GCA	7	1300.07**	2037.97**	43.02**	61.75**	2330.36**	3845.89**	2169.78**	2623.74**	10720.05**	13028.12**
SCA	20	1216.04**	1334.51**	26.13**	31.95**	1970.43**	3579.94**	1682.71**	1696.22**	6979.26**	10032.323**
Error	27	114.68	84.54	2.26	1.83	397.48	500.87	208.65	320.87	872.11	1041.77

Table 5.3: Mean square for days to silk emergence (DMS), days to mid-pollen shed (DMP), yield (t ha^{-1}), number of ears per plant (EPP) and grain moisture content (GMC) traits of an eight parent diallel evaluated in 5 locations over two seasons

Source	df	DMS (d)	DMP (d)	YIELD (t ha^{-1})	EPP	GMC (%)
Env	4	4140.41**	5212.49**	597.01**	0.18**	739.99**
Rep(Env)	5	1.80	1.74	8.69	0.006	1.40
Entry	27	278.01**	262.71**	56.74**	0.12**	17.20**
GCA	7	1009.18**	955.38**	184.83**	0.41*	66.89*
SCA	20	22.10**	20.27**	11.92*	0.08*	4.40
Env x Entry	108	14.38**	16.21**	8.17**	0.02	3.88**
GCA x Env	28	38.49**	45.82**	15.43**	0.04*	7.12**
SCA x Env	80	5.94	5.84	5.63	0.02	3.50
Error	135	4.54	4.30	4.04	0.01	2.06

** P≤0.001, * P≤0.005

Table 5.4: Percent contribution of GCA and SCA sum of squares to entry sum of squares and GCA effects for seed traits under standard germination (Std) and accelerated aging conditions (AA)

	Endosperm Classification	Germination percentage		Seedling dry weight (g)		Average Root Length (mm)		Average Shoot Length (mm)		Vigour Index	
		Std	**AA**	**Std**	**AA**	**Std**	**AA**	**Std**	**AA**	**Std**	**AA**
Percent contribution of GCA and SCA sum of squares to entry sum of squares											
GCA		27	35	37	40	29	27	31	35	35	31
SCA		73	65	63	60	71	73	69	65	65	69
GCA effects for seed testing traits											
CM 32	LPA	1.23	2.50	-0.04	0.09	15.43	-1.36	10.67	3.96	18.23	-4.01
CM 35	LPA	-2.58	-3.88	-0.95	-0.98	-8.32	-1.37	1.54	3.63	-16.23	-6.97
Kenyan	Nm	4.98	11.75**	0.80	1.51**	4.92	9.79	10.55	10.88	23.71	22.24
P 1	Nm	3.36	12.44**	1.81**	2.99**	0.19	17.53	-4.62	4.57	-8.47	19.44
P 5	Nm	4.36	3.00	0.25	-0.23	6.72	11.75	6.62	11.84	26.13	32.52*
P 6	Nm	-3.70	-2.38	-0.10	0.28	-15.16	-11.07	-17.98**	-17.90**	-26.71**	-27.93
P 7	Nm	8.73*	4.13	1.10	0.62	8.31	9.70	2.98	6.48	15.00	20.44
T 4	Nm	-16.39**	-27.56**	-2.87**	-4.28**	-12.10	-34.97**	-9.75	-23.46**	-31.65**	-55.72**

** P≤0.001, * P≤0.005

Table 5.5: Percent contribution of GCA and SCA sum of squares to entry sum of squares and GCA effects for days to silk emergence (DMS), days to mid-pollen shed (DMP), yield (t ha^{-1}), number of ears per plant (EPP) and grain moisture content (%) (GMC) traits

	Endosperm Classification	DMS (d)	DMP (d)	YIELD (t ha^{-1})	EPP	GMC (%)
Percent contribution of GCA and SCA sum of squares to entry sum of squares						
GCA		94	94	84	65	78
SCA		6	6	16	35	12
GCA effects for grain yield and associated traits						
CM 32	LPA	-7.7**	-7.6*	-2.7**	-0.1**	-1.4**
CM 35	LPA	-5.1**	-4.9	-1.2**	0.0	-1.4**
Kenyan	Nm	1.6*	1.7	1.2**	0.1	0.4
P 1	Nm	2.3**	2.2	0.9**	0.1	0.7
P 5	Nm	2.8**	2.8	0.1	0.0	0.9*
P 6	Nm	1.4*	1.4	0.3	0.0	0.9*
P 7	Nm	3.6**	3.4	1.6**	-0.1	0.8
T 4	Nm	1.0	1.1	-0.3	0.1**	-0.9*

** P≤0.001, * P≤0.005

Specific combining ability effects

Positive SCA effects were desired for all seed germination and vigour traits as well as yield and EPP traits. For DMS, DMP and GMC, negative SCA effects were required. The LPA x LPA cross showed increased SCA effects for all traits with negative SCA effects for seedling dry weight, average shoot length and vigour index under standard conditions which became positive under accelerated aging conditions (Table 5.6).

There were two LPA x Nm crosses that showed positive SCA effects under both conditions (CM 32 x P 7; CM 35 x P 1) and these effects increased under stress conditions. One LPA x Nm cross had positive SCA effects under standard conditions, but became negative SCA effects under accelerated aging conditions (CM 32 x P 5). The cross CM 32 x P 6 showed positive increased SCA effects for germination percentage and average shoot length, with positive decreased effects for average root length and vigour index. The cross CM 35 x T 4 showed increased SCA effects for all traits although average root length and vigour index had negative SCA effects under standard conditions. Generally there were eight LPA x Nm crosses and nine Nm x Nm crosses that showed negative SCA effects for all traits under both conditions.

There were four Nm x Nm crosses that had positive and increased SCA effects for both conditions (Kenyan x P 5; Kenyan x P 6; Kenyan x P 7; P 1 x P 5). Two crosses (P 5 x P 7; P 7 x T 4) had positive SCA effects for all traits under both non-stress and stress conditions.

The LPA x LPA cross showed significant positive SCA effects for DMS and DMP but negative SCA effects for yield, EPP and GMC (Table 5.7). In the LPA x Nm group, there were eight crosses with positive SCA effects for yield (CM 32 x Kenyan; CM 32 x P 5, CM 32 x P 6, CM 32 x Kenyan, CM 35 x Kenyan, CM 35 x P 5, CM 35 x P 6, CM 35 x P 7). Four of these crosses also had negative SCA effects for DMS and GMC (CM 32 x P 5; CM 32 x P 6; CM 35 x P 6; CM 35 x P7). The normal group of crosses all showed positive SCA effects for DMP, with seven crosses having positive SCA effects for yield (P 1 x P 5, P 1 x P 7, P 1 x T 4, P 5 x T 4, P 6 x P 7, P 7 x Kenyan, T 4 x Kenyan). The crosses P1 x P5, P1 x T4 and P 6 x P 7 showed the highest SCA effects for yield. The crosses, P 1 x T 4 and T 4 x Kenyan showed negative SCA effects for DMS, DMP and GMC and positive SCA effects for yield and EPP.

The LPA x Nm crosses showing positive SCA effects for germination and vigour traits also had negative SCA effects for yield. The cross CM 32 x P 6 showed negative SCA effects for DMS and GMC, positive SCA effects for yield, EPP, germination percentage, average root length, average shoot length and vigour index.

Table 5.6: SCA effects of crosses under standard germination conditions and accelerated aging conditions for germination percentage, seedling dry weight and average root length, average shoot length and vigour index traits

Pedigree	Endosperm Classification	Germination percentage		Seedling dry Weight (g)		Average Root Length (mm)		Average Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
CM 32 x CM 35	LPA x LPA	0.20	3.19	-0.13	0.37	10.32	17.05	-8.36	4.87	-8.35	4.70
CM 32 x Kenyan	LPA x Nm	-6.86	-17.44	0.07	-1.52	-16.96	-25.84	-11.47	-10.61	-48.23	-49.73
CM 32 x P 1	LPA x Nm	-14.73	-23.63**	-2.04	-4.14**	-12.17	-22.16	-23.38	-16.10	-60.53	-50.07
CM 32 x P 5	LPA x Nm	9.27	-1.19	1.12	-0.18	5.97	-7.24	26.14	-3.46	23.44	-23.35
CM 32 x P 6	LPA x Nm	5.33	5.69	-0.33	-0.09	8.72	5.65	13.90	16.10	15.47	9.60
CM 32 x P 7	LPA x Nm	7.89	20.69**	1.77	3.57**	6.73	36.40	13.60	31.28	50.54	90.02*
CM 32 x T 4	LPA x Nm	-63.88**	-63.94**	-6.77**	-7.83**	-27.52	-43.74	23.12	-12.58	-49.14	-73.01
CM 35 x Kenyan	LPA x Nm	-10.05	-10.56	-2.28	-2.40	-17.17	-22.44	10.65	-13.79	-1.95	-28.46
CM 35 x P 1	LPA x Nm	10.08	22.75**	3.12	5.32**	32.90	40.43	15.01	21.70	38.92	55.88
CM 35 x P 5	LPA x Nm	-21.92	-39.81**	-3.28	-4.96**	-15.72	-60.38	-18.64	-39.42	-48.49	-83.98
CM 35 x P 6	LPA x Nm	-13.36	-14.44	-2.67	-2.82	-19.68	-30.60	-6.70	-7.59	-24.85	-30.99
CM 35 x P 7	LPA x Nm	-22.30	-31.94**	-3.12	-4.26**	-15.56	-43.68	-19.33	-26.12	-54.85	-75.88
CM 35 x T 4	LPA x Nm	1.31	12.69	2.43	4.35	-27.90	17.30	8.47	22.14	-45.80	19.98
Kenyan x P 1	Nm x Nm	-15.48	-28.88**	-2.53	-4.27**	-9.85	-55.12	9.44	-26.72	-30.22	-82.13
Kenyan x P 5	Nm x Nm	10.52	11.56	3.17	3.05*	12.93	21.95	13.29	26.52	13.25	18.67
Kenyan x P 6	Nm x Nm	32.58**	31.94**	4.48**	5.14**	37.63	51.93	13.07	17.55	65.96	84.25
Kenyan x P 7	Nm x Nm	20.14	20.44	2.18	2.25	1.83	29.05	20.17	20.53	39.30	59.13
Kenyan x T 4	Nm x Nm	-50.63**	-24.69	-2.53	-0.76	-77.49	-67.24	-34.11	-67.00**	-104.34	-146.42**
P 1 x P 5	Nm x Nm	30.14**	31.88**	6.02**	5.82**	1.00	36.77	18.69	23.47	49.83	76.70
P 1 x P 6	Nm x Nm	-8.80	-6.75	-2.73	-2.44	11.48	-3.53	13.33	0.62	3.38	-9.35
P 1 x P 7	Nm x Nm	-16.23	-5.25	0.02	1.22	-28.76	-21.21	-31.58	-20.44	-69.05	-54.35
P 1 x T 4	Nm x Nm	-43.25**	-31.00	-4.47	-3.53	-61.90	-24.30	-41.34	-9.39	-128.64	-63.89
P 5 x P 6	Nm x Nm	-61.80**	-53.31**	-8.17***	-7.42**	-91.70**	-92.05**	-88.96**	-69.52**	-160.99**	-148.82**
P 5 x P 7	Nm x Nm	14.77	13.19	3.08	1.99	45.92	57.45	30.40	35.08	92.27**	99.23**
P 5 x T 4	Nm x Nm	-35.25	-40.94**	0.03	-0.91	-26.35	-40.13	-1.40	-10.26	-72.02	-69.53
P 6 x P 7	Nm x Nm	-5.17	-0.44	-1.17	-1.07	-5.06	-4.87	2.47	0.75	-22.91	-27.54
P 6 x T 4	Nm x Nm	-37.31*	-48.81**	-8.78**	-10.74**	-39.36	-65.85	-38.19	-41.62	-107.94	-114.08
P 7 x T 4	Nm x Nm	22.13	41.19**	0.47	2.50	90.50**	31.93	60.73*	19.92	116.35	57.66

** P≤0.001, * P≤0.005

Table 5.7: SCA effects of crosses for days to silk emergence (DMS), days to mid-pollen shed (DMP), yield (t ha^{-1}), number of ears per plant (EPP) and grain moisture content (GMC) traits

Pedigree	Classification	DMS(d)	DMP (d)	YIELD (t ha^{-1})	EPP	GMC (%)
CM 32 x CM 35	LPA x LPA	3.59**	12.50**	-0.46	-0.12	-2.05
CM 32 x Kenyan	LPA x Nm	0.51	5.90**	1.14	-0.01	-0.54
CM 32 x P 1	LPA x Nm	-1.26	5.40**	-1.40	-0.12	-1.49
CM 32 x P 5	LPA x Nm	-0.49	4.80*	0.47	-0.01	-1.52
CM 32 x P 6	LPA x Nm	-1.89	6.20**	0.60	0.08	-1.36
CM 32 x P 7	LPA x Nm	-1.39	4.20*	-1.00	-0.08	0.15
CM 32 x T 4	LPA x Nm	1.11	6.50**	0.71	0.04	-1.87
CM 35 x Kenyan	LPA x Nm	0.06	3.20*	0.22	-0.13*	-1.66
CM 35 x P 1	LPA x Nm	0.09	2.70*	-0.50	-0.06	-0.10
CM 35 x P 5	LPA x Nm	0.46	2.10	0.14	0.05	-0.49
CM 35 x P 6	LPA x Nm	-0.24	3.50*	0.70	-0.03	-0.97
CM 35 x P 7	LPA x Nm	-1.94	1.50	0.70	0.05	-1.78
CM 35 x T 4	LPA x Nm	-1.84	3.80*	-0.86	-0.12	-1.56
P 1 x Kenyan	Nm x Nm	0.01	-3.90*	-0.95	-0.17*	1.68
P 1 x P 5	Nm x Nm	1.01	-5.00**	1.51	-0.06	1.89
P 1 x P 6	Nm x Nm	0.31	-3.60*	-0.23	-0.06	1.86
P 1 x P 7	Nm x Nm	0.81	-5.60**	0.68	-0.04	1.09
P 1 x T 4	Nm x Nm	-0.79	-3.30*	0.98	0.16*	-0.47
P 5 x Kenyan	Nm x Nm	-0.43	-4.50**	-0.23	-0.14*	1.34
P 5 x P 6	Nm x Nm	0.57	-4.20*	-0.97	0.01	2.17
P 5 x P 7	Nm x Nm	-1.63	-6.20**	-0.65	0.06	1.87
P 5 x T 4	Nm x Nm	0.97	-3.90*	0.13	-0.12	-0.07
P 6 x Kenyan	Nm x Nm	-0.43	-3.10	-0.08	-0.10	1.33
P 6 x P 7	Nm x Nm	1.27	-4.80**	0.90	0.07	2.11
P 6 x T 4	Nm x Nm	0.87	-2.50	-0.56	0.00	0.26
P 7 x Kenyan	Nm x Nm	1.77	-5.10**	0.06	0.02	1.63
P 7 x T 4	Nm x Nm	1.27	-4.50**	-0.50	0.06	-0.14
T 4 x Kenyan	Nm x Nm	-1.13	-2.20	0.17	0.15*	-1.34

** P≤0.001, * P≤0.005

Performance of parent inbred lines and classes

Generally the LPA lines showed the greatest reduction under stress conditions for germination percentage and seedling dry weight (Table 5.8). Line CM 35 had a lower percentage of abnormal seedlings compared to CM 32, but the same percentage of dead seedlings under standard conditions (Appendix 19). However under accelerated conditions, CM 32 showed lower percentages of abnormal seedlings to CM 35, with similar percentages for dead seedlings (Appendix 19). CM 32 showed lower reductions for average root length, average shoot length and vigour index than CM 35.

The normal lines, P 7 and T 4 showed increased values for all traits except average shoot length under stress conditions (Table 5.8 and 5.9). The LPA group showed distinct decreased values for all traits under accelerated conditions (Figure 5.1 to 5.5). The Normal group of lines showed slight decreased values for germination percentage, average shoot length and vigour index, with increased values for seedling dry weight and average root length under stress conditions. The Nm x Nm group of lines only showed slightly higher average root lengths and seedling dry weight under accelerated aging than the standard germination conditions compared to the LPA group of lines which showed a major decrease under accelerated aging conditions. Under accelerated aging conditions, germination percentage and seedling dry weight were significant (P≤0.001).

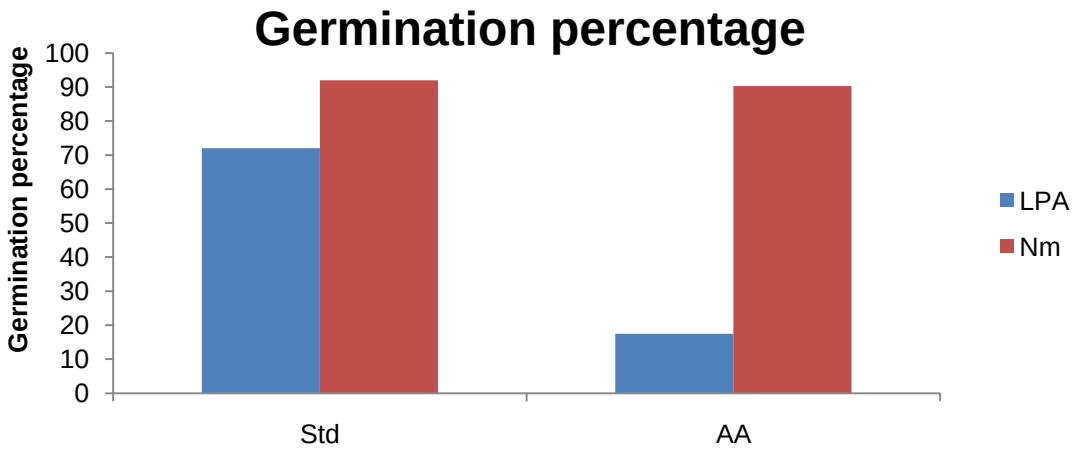

Figure 5.1: Averages of low phytic acid (LPA) and normal (Nm) groups of parental lines for germination percentage under standard germination and accelerated aging test conditions

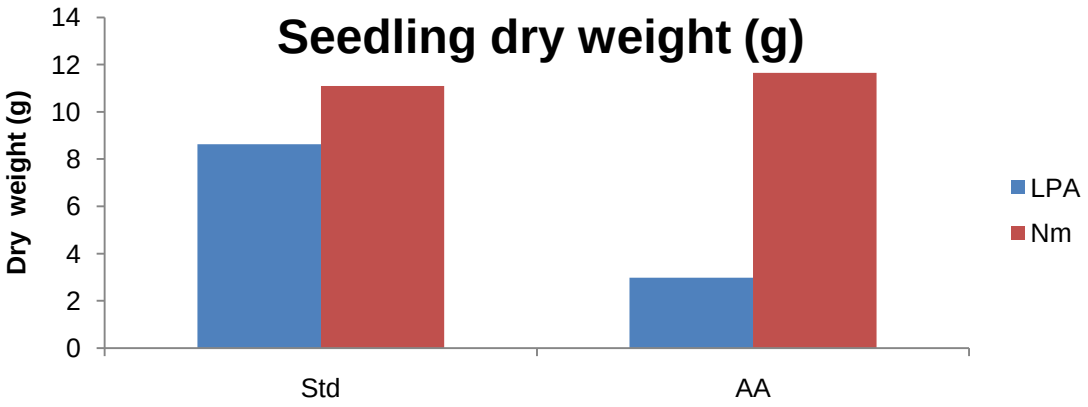

Figure 5.2: Averages of low phytic acid (LPA) and normal (Nm) groups of parental lines for seedling dry weight under standard germination and accelerated aging test conditions

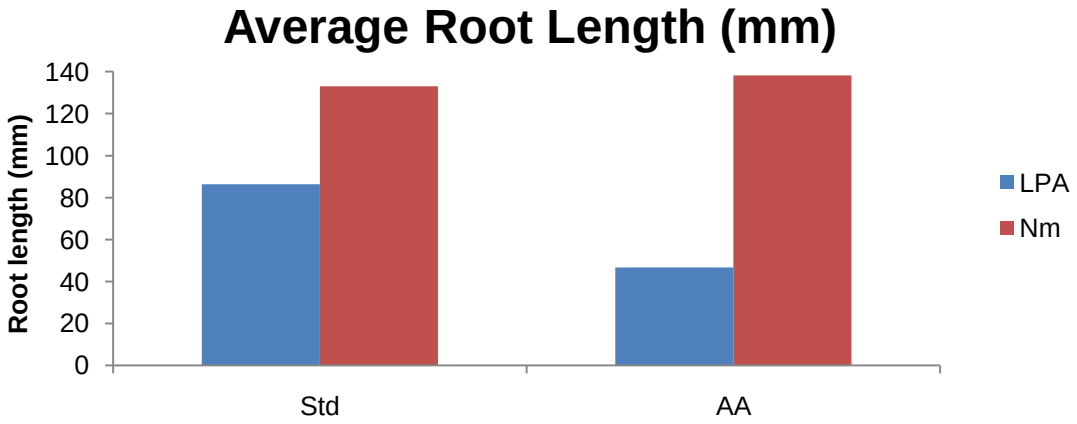

Figure 5.3: Averages of low phytic acid (LPA) and normal (Nm) groups of parental lines for average root length under standard germination and accelerated aging test conditions

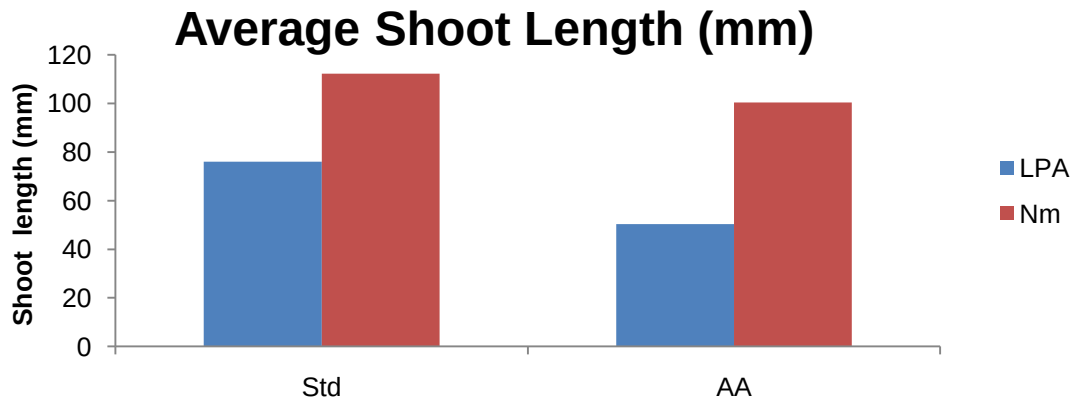

Figure 5.4: Averages of low phytic acid (LPA) and normal (Nm) groups of parental lines for average shoot length under standard germination and accelerated aging test conditions

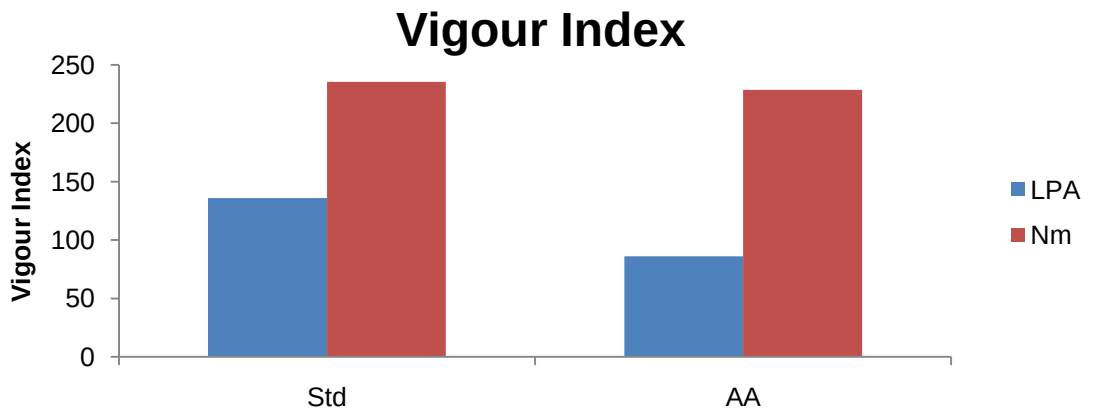

Figure 5.5: Averages of low phytic acid (LPA) and normal (Nm) groups of parental lines for the vigour index trait under standard germination and accelerated aging test conditions

Performance of crosses

The one LPA x LPA cross generally showed a decrease under accelerated aging conditions for all traits (Table 5.10). In the LPA x Nm group, there were six crosses with less than 50% germination percentage under standard conditions. There were three crosses with >73% germination (P 6 x CM 32; CM 32 x T 4; CM 32 x P 1) under both conditions. The cross P 1 x CM 35 showed very low germination under standard conditions which was further reduced under stress conditions. The crosses of P 6 x CM 35 and P 7 x CM 32 showed an increase in germination under accelerated aging conditions although it was still low. For seedling dry weight, there were three crosses that showed an increase under accelerated aging conditions (P 6 x CM 35; P 7 x CM 32; CM 32 x P 1). There were six crosses showing an increase for average root length under accelerated aging conditions (P 5 x CM 35; P 6 x CM 32; P 6 x CM 35; P 7 x CM 32; P 7 x CM 35; CM 32 x P 1). There were no crosses in the LPA x Nm group that showed increases for average shoot length under accelerated aging conditions. For the vigour index, there were six crosses with high and or increasing means under accelerated aging conditions (P 5 x CM 35; P 6 x CM 32; P 7 x CM 32; P 7 x CM 35; CM 32 x P 1; CM 32 x T 4).

In the Nm x Nm group, there were crosses that showed increased or retained germination, seedling dry weight and average root length under stress conditions (P 5 x P 7; P 6 x Kenyan; T 4 x P 7). There was one cross (T4 x Kenyan) that showed zero percent germination as it produced 100% abnormal seedlings under both standard and stress conditions (Appendix 20). The normal line T 4 also showed high negative and mostly significant GCA effects for all traits under both conditions (Table 5.4).

There were two crosses that showed very high germination percentage (>94%) with corresponding positive and high SCA effects for all other traits (P 6 x Kenyan; T 4 x P 7). There were significant P values at the 1% level for all seed vigour traits under both conditions except average root length under standard conditions. The following four crosses had very good means for all seed traits and can be recommended from the LPA x Nm group (P 6 x CM 32; P 7 x CM 32; CM 32 x P 1; CM 32 x T 4). For yield and its associated traits, the LPA x LPA cross performed lower for all traits compared to the LPA x Nm and Nm x Nm groups of crosses (Table 5.11). The relative yield was only 34% of the early maturing check (PAN 6114) and 29% of the mean of all the hybrid checks. The LPA x LPA cross had earlier flowering dates, lower ears per plant, lowest yield and low grain moisture content than other crosses. The check hybrid, PAN 6114 was selected as an early check due to its low DMS and DMP as well as low GMC. The LPA x Nm crosses were generally slightly higher than the LPA x LPA cross for all traits but lower than the Nm x Nm crosses and the check hybrids. The cross CM 32 x P 6 showed early flowering dates with increased yield compared to the LPA x LPA cross.

In the LPA x Nm group, there were four crosses (CM 35 x P 7, CM 35 x Kenyan, CM 35 x P 6, CM 32 x Kenyan) that performed above the yield of the early check, with only one cross performing better than the relative average yield of all the checks (CM 35 x P 7). In the Nm x Nm group, all the crosses performed the same or better than the early check (99-164%). There were only two hybrid checks that performed lower than the early check (2-8%). The entry and environment*entry effects were significant for all traits (P≤0.001) except for EPP.

Phenotypic Correlations

There were significant positive correlations between the seed germination and vigour traits (P≤0.001) except between seedling dry weight (AA) and average root length (Standard) which was positive but not significant (Table 5.12). All the yield and associated traits had positive correlations between them. DMS showed positive correlations with yield and other associated traits but was only significant for DMP, GMC and yield (P≤0.001). DMP showed similar trend with significant correlations with GMC and yield. GMC showed significant and positive correlation with yield (P≤0.001). The correlations between the seed vigour traits and yield traits were mostly low negative and low positive values. There was a very high correlation between DMS and DMP (1.00).

Table 5.8: Means of eight parental inbred lines under standard germination (Std) and accelerated aging (AA) for germination percentage, seedling dry weight (g) and average root length (mm) traits

Entry	Germination percentage			Seedling dry weight (g)			Average Root Length (mm)		
	Std	**AA**	**% reduction[+]**	**Std**	**AA**	**% reduction[+]**	**Std**	**AA**	**% reduction[+]**
LPA group mean	**72.00**	**17.50**		**8.63**	**2.98**		**86.35**	**46.63**	
CM 32	68.00	32.00	53%	8.60	5.50	36%	68.95	68.25	1%
CM 35	76.00	3.00	96%	8.65	0.45	95%	103.75	25.00	76%
Nm group mean	**92.00**	**90.33**		**11.09**	**11.65**		**133.06**	**138.30**	
Kenyan	98.00	91.00	7%	17.50	17.65	-1%	118.80	138.90	-17%
P 1	95.00	96.00	-1%	11.10	12.45	-12%	143.45	108.60	24%
P 5	84.00	74.00	12%	9.10	7.60	16%	109.90	129.80	-18%
P 6	81.00	85.00	-5%	8.35	9.65	-16%	144.50	144.80	0%
P 7	97.00	98.00	-1%	12.95	14.35	-11%	134.15	151.00	-13%
T 4	97.00	98.00	-1%	7.55	8.20	-9%	147.55	156.70	-6%
Overall Mean	87.00	72.13		10.48	9.48		121.38	115.38	
P value	0.0017	<0.0001		0.0003	<0.0001		0.1016	0.0403	
CV (%)	5.21	9.26		9.74	7.77		18.85	27.93	
R^2 (%)	92.91	98.27		95.59	99.07		74.31	81.14	

[+] =100 - (standard germination – accelerated aging)

Table 5.9: Means of eight parental inbred lines under standard germination (Std) and accelerated aging (AA) for average shoot length (mm) and vigour index traits

Entry	Average Shoot Length (mm)			Vigour Index		
	Std	**AA**	**% reduction[+]**	**Std**	**AA**	**% reduction[+]**
LPA group mean	**76.00**	**50.40**		**135.87**	**85.98**	
CM 32	68.05	58.05	15%	115.73	110.98	4%
CM 35	83.95	42.75	49%	156.00	60.98	61%
Nm group mean	**112.23**	**100.44**		**235.40**	**228.53**	
Kenyan	109.90	81.65	26%	224.09	206.85	8%
P 1	104.90	78.75	25%	236.42	182.12	23%
P 5	112.85	101.95	10%	201.79	199.67	1%
P 6	110.90	124.10	-12%	242.10	261.05	-8%
P 7	111.00	98.15	12%	242.78	246.71	-2%
T 4	123.80	118.05	5%	265.20	274.80	-4%
Overall Mean	103.17	87.93		210.51	192.89	
P value	0.0148	0.0017		0.0314	0.0165	
CV (%)	10.06	12.75		15.89	22.58	
R^2 (%)	88.96	92.93		82.85	86.09	

[+] =100 - (standard germination – accelerated aging)

Table 5.10: Means for F_2 seeds generated under standard germination (Std) and accelerated aging (AA) conditions for germination percentage, seedling dry weight (g) and average root length (mm) traits

Pedigree	Classification	Germination percentage		Seedling dry weight (g)		Average Root Length (mm)		Average Shoot Length (mm)		Vigour Index	
		Std	AA	Std	AA	Std	AA	Std	AA	Std	AA
CM 32 x CM 35	LPA x LPA	65.00	41.50	5.10	3.10	139.05	55.50	116.05	48.00	185.44	92.48
P 5 x CM 32	LPA x Nm	45.00	21.00	5.05	2.75	84.55	56.05	85.50	59.60	105.49	81.82
P 5 x CM 35	LPA x Nm	71.50	63.00	9.60	8.65	78.20	113.50	110.05	97.00	140.57	170.89
P 6 x CM 32	LPA x Nm	95.00	89.00	12.10	11.75	115.20	139.90	134.00	113.50	239.57	246.04
P 6 x CM 35	LPA x Nm	9.00	21.00	2.15	6.34	50.00	59.25	56.50	41.41	82.40	36.68
P 7 x CM 32	LPA x Nm	57.00	64.00	10.95	12.20	79.85	97.40	67.10	66.20	99.04	129.76
P 7 x CM 35	LPA x Nm	34.00	27.00	3.80	3.55	60.00	74.20	64.50	60.75	90.96	107.66
T 4 x M 35	LPA x Nm	41.00	26.50	4.45	3.25	102.40	69.85	106.05	72.50	135.39	117.04
Kenyan x CM 32	LPA x Nm	61.00	54.00	7.85	7.20	88.25	85.15	87.80	64.90	126.94	109.21
Kenyan x CM 35	LPA x Nm	48.00	17.00	5.95	2.35	82.50	49.15	79.95	34.45	111.21	64.97
CM 32 x P 1	LPA x Nm	79.00	80.00	10.85	11.65	130.60	136.10	127.60	117.30	245.33	250.68
CM 32 x T 4	LPA x Nm	89.00	73.00	12.45	9.75	161.10	170.30	140.35	128.95	294.96	296.42
P 1 x CM 35	LPA x Nm	13.50	2.00	1.50	0.64	88.40	56.15	107.60	73.41	137.41	57.40
LPA x Nm		**53.58**	**44.79**	**7.23**	**6.67**	**93.42**	**92.25**	**97.25**	**77.50**	**150.77**	**139.05**
P 1 x P 5	Nm x Nm	60.00	54.50	6.90	6.85	117.55	105.70	104.15	88.05	155.22	137.95
P 5 x P 7	Nm x Nm	72.00	84.00	12.00	14.70	124.90	148.00	112.25	105.50	175.79	212.58
P 5 x T 4	Nm x Nm	41.00	12.00	4.05	1.20	82.85	41.35	89.85	51.65	122.99	85.80
P 5 x Kenyan	Nm x Nm	41.50	32.00	4.30	3.85	56.95	48.30	77.15	53.70	93.77	78.35
P 6 x P 1	Nm x Nm	60.50	49.50	8.85	7.45	103.50	73.95	110.10	79.80	155.27	112.73
P 6 x P 7	Nm x Nm	54.00	48.00	8.10	7.60	95.40	63.55	115.70	64.30	146.59	103.78
P 6 x Kenyan	Nm x Nm	95.00	94.00	13.20	14.30	127.55	142.05	105.95	86.10	224.53	222.79
P 7 x Kenyan	Nm x Nm	52.00	56.00	7.00	8.20	96.65	94.30	91.05	62.85	129.77	126.39
P 1 x P 7	Nm x Nm	51.00	44.00	7.75	6.30	103.55	85.35	83.00	68.00	110.79	109.59
T 4 x P 6	Nm x Nm	81.00	79.00	12.25	11.70	124.70	134.85	130.80	124.80	224.06	217.66
T 4 x P 7	Nm x Nm	99.00	100.00	16.10	15.95	108.05	157.45	121.00	115.45	229.06	272.88
T 4 x Kenyan	Nm x Nm	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P 1 x T 4	Nm x Nm	76.00	57.00	9.35	7.05	128.25	94.55	143.75	87.95	229.37	149.38
P 1 x Kenyan	Nm x Nm	64.00	58.50	7.55	7.65	109.15	84.60	106.90	77.75	168.55	121.90
P 5 x P 6	Nm x Nm	53.50	50.00	5.60	5.50	79.55	77.35	123.05	76.30	167.09	131.04
Nm x Nm		**60.03**	**54.57**	**8.20**	**7.89**	**97.24**	**90.09**	**100.98**	**76.15**	**155.52**	**138.85**
P value		<0.0001	<0.0001	<0.0001	<0.0001	0.0014	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Overall Mean		57.45	49.91	7.81	7.61	100.69	94.71	103.62	79.49	154.58	137.28
CV (%)		18.64	18.42	19.59	14.46	20.17	22.55	14.20	19.39	19.10	23.51
R^2 (%)		91.53	94.72	92.61	96.79	77.21	86.69	83.42	85.52	90.17	91.42

Table 5.11: Means of F_1 hybrids and hybrid checks for grain yield and secondary traits evaluated over five locations over two seasons with relative yield to mean of checks and early check (PAN6114)

Pedigree	Classification	DMS	DMP	EPP	GMC	Yield	Relative yield to mean of checks (%)	Relative yield to early check (%)
CM 32 x CM 35	LPA x LPA	61.00	62.80	0.81	14.52	2.01	29%	34%
CM 35 x P 7	LPA x Nm	66.80	68.10	0.98	14.79	7.47	107%	128%
CM 35 x Kenyan	LPA x Nm	66.80	68.80	1.00	14.91	6.59	94%	113%
CM 35 x P 6	LPA x Nm	66.30	67.70	1.00	15.60	6.17	88%	106%
CM 32 x Kenyan	LPA x Nm	64.60	66.20	1.02	16.03	6.01	86%	103%
CM 35 x P 1	LPA x Nm	67.50	69.40	1.07	16.47	5.57	79%	95%
CM 35 x P 5	LPA x Nm	68.40	69.90	1.08	16.08	5.41	77%	93%
CM 32 x P 6	LPA x Nm	62.00	64.70	1.01	15.21	4.57	65%	78%
CM 32 x P 7	LPA x Nm	64.70	66.20	0.75	16.71	4.27	61%	73%
CM 32 x P 5	LPA x Nm	64.80	65.70	0.92	15.05	4.24	60%	73%
CM 32 x T 4	LPA x Nm	64.60	65.90	1.07	14.70	4.08	58%	70%
CM 35 x T 4	LPA x Nm	64.30	65.80	1.01	15.01	4.01	57%	69%
CM 32 x P 1	LPA x Nm	63.50	65.10	0.91	15.08	3.17	45%	54%
	LPA x Nm	**65.36**	**66.96**	**0.99**	**15.47**	**5.13**		
P 1 x P 7	Nm x Nm	76.90	78.30	0.99	17.65	9.55	136%	164%
P 7 x Kenyan	Nm x Nm	77.20	78.40	1.05	18.19	9.23	132%	158%
P 6 x P 7	Nm x Nm	76.50	77.10	1.00	18.67	9.17	131%	157%
P 1 x P 5	Nm x Nm	76.30	77.60	1.07	18.45	8.88	127%	152%
P 1 x T 4	Nm x Nm	72.70	74.50	1.39	16.10	7.95	113%	136%
P 6 x Kenyan	Nm x Nm	72.80	74.00	1.03	17.89	7.79	111%	133%
P 1 x Kenyan	Nm x Nm	74.10	75.50	1.06	18.24	7.52	107%	129%
P 5 x Kenyan	Nm x Nm	74.20	75.60	0.99	17.90	7.44	106%	127%
T 4 x Kenyan	Nm x Nm	71.70	73.60	1.38	15.23	7.44	106%	127%
P 5 x P 7	Nm x Nm	75.00	76.90	0.99	18.43	7.42	106%	127%

Pedigree	Classification	DMS	DMP	EPP	GMC	Yield	Relative yield to mean of checks (%)	Relative yield to early check (%)
P 1 x P 6	Nm x Nm	74.20	74.60	1.07	18.42	7.34	105%	126%
P 7 x T 4	Nm x Nm	76.10	77.40	1.09	16.43	7.17	102%	123%
P 5 x T 4	Nm x Nm	75.00	76.20	1.01	16.50	6.30	90%	108%
P 6 x T 4	Nm x Nm	73.50	75.40	1.13	16.83	5.81	83%	99%
P 5 x P 6	Nm x Nm	75.00	76.80	1.04	18.73	5.80	83%	99%
	Nm x Nm	**74.75**	**76.13**	**1.09**	**17.58**	**7.65**		
PAN6227		69.20	70.80	1.40	16.60	8.76	125%	150%
PAN7M-97		72.10	74.80	1.17	18.28	8.74	125%	150%
AGRI		72.50	74.10	1.03	17.15	8.23	117%	141%
PAN8M-95		75.10	78.40	0.93	19.25	7.53	107%	129%
PAN77		73.60	74.30	1.10	17.56	7.39	105%	127%
PAN6777		71.20	72.70	1.12	18.16	7.35	105%	126%
PAN67		73.30	74.80	1.08	18.38	6.80	97%	116%
PAN6757		70.90	72.20	1.13	18.02	6.22	89%	107%
PAN6611		72.40	68.50	1.28	16.33	6.16	88%	105%
PAN6114		64.80	65.70	0.98	14.00	5.84	83%	100%
PAN6017		70.10	72.30	1.08	14.89	5.71	81%	98%
PAN6243		73.50	74.80	1.26	16.34	5.39	77%	92%
	Hybrid Checks	**71.56**	**72.78**	**1.13**	**17.08**	**7.01**		
P value (entry)		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**		
P value (env*entry)		**<0.0001**	**<0.0001**	**0.0031**	**<0.0001**	**<0.0001**		
Overall Mean		**70.63**	**72.04**	**1.06**	**16.72**	**6.56**		
CV (%)		**2.83**	**4.93**	**13.99**	**9.02**	**24.41**		
R^2 (%)		**97.55**	**94.08**	**76.49**	**92.58**	**86.77**		

Table 5.12: Pearson's phenotypic correlation coefficients for germination percentage (Germ %), seedling dry weight (g) (DW), average root length (mm) (ARL), average shoot length (ASL) and vigour index (VI) seed traits under standard (Std) and accelerated aging conditions (AA) and days to silk emergence (DMS), mid-pollen shed (DMP), yield (t ha^{-1}), number of ears per plant (EPP) and grain moisture content (GMC) traits

	Germ % Std	**Germ % AA**	**DW Std**	**DW AA**	**ARL Std**	**ARL AA**	**ASL Std**	**ASL AA**	**VI Std**	**VI AA**	**DMS**	**DMP**	**EPP**	**GMC**	**Yield**
Germ % Std	1.00														
Germ % AA	0.92**	1.00													
DW Std	0.93**	0.95**	1.00												
DW AA	0.80**	0.95*	0.92**	1.00											
ARLStd	0.79**	0.66*	0.68**	0.54	1.00										
ARL AA	0.86**	0.92**	0.90**	0.89**	0.73**	1.00									
ASL Std	0.76**	0.63*	0.63*	0.48	0.86**	0.67**	1.00								
ASLAA	0.81**	0.81**	0.82**	0.75**	0.76**	0.92**	0.82**	1.00							
VI Std	0.86**	0.77**	0.77**	0.64*	0.89**	0.82**	0.91**	0.87**	1.00						
VI AA	0.90**	0.90**	0.90**	0.83**	0.73**	0.96**	0.72**	0.92**	0.88**	1.00					
DMS	0.02	0.11	0.10	0.14	-0.07	0.00	0.00	0.01	-0.08	-0.03	1.00				
DMP	0.03	0.12	0.11	0.15	-0.07	0.01	0.01	0.02	-0.07	-0.02	1.00**	1.00			
EPP	-0.09	-0.09	-0.08	-0.13	-0.18	-0.08	-0.04	-0.02	0.01	-0.05	0.39	0.42	1.00		
GMC	0.11	0.25	0.17	0.28	0.06	0.09	0.14	0.11	-0.01	0.01	0.82**	0.82**	0.06	1.00	
Yield	-0.12	-0.01	-0.02	0.04	-0.22	-0.09	-0.21	-0.17	-0.26	-0.19	0.84**	0.84**	0.44	0.67*	1.00

** P≤0.001, * P≤0.005

Discussion

In this study there was predominately non-additive gene action present for germination percentage, seedling dry weight, average root length, average shoot length and vigour index which was the same findings of other germination and vigour studies (Barla-Szabo *et al.,* 1990; Akram *et al.*, 2007; Chapi *et al.,* 2008) while other studies on other crops have found mainly additive gene action (Cho and Scott, 2000; Sadeghian and Khodaii, 1998) for these traits. Due to significant environment and entry effects for all yield and associated traits and GCA x Environment interactions for all traits, some hybrids were performing differently in the environments suggesting the need to validate these hybrids performance over many locations over more seasons. Due to the predominance of non-additive gene action, superior SCA effects can be exploited for hybrid production.

This study found significant GCA and SCA effects for DMS, DMP ($P \leq 0.001$), EPP ($P \leq 0.005$) and yield ($P \leq 0.001$, $P \leq 0.005$) therefore both additive and non-additive gene action was present for these traits. GMC only showed significant GCA effects ($P \leq 0.005$) and therefore only additive gene action was present for this trait. Other studies on maize have found DMP and DMS to be additive gene action (Glover *et al.,* 2005; Bello and Olaoye, 2009) which confirms the results found in this study. Malik *et al.* (2004) found significant GCA effects for EPP ($P \leq 0.001$) and less significant SCA effects ($P \leq 0.05$) suggesting that there is both additive and non-additive gene action with predominance of additive gene effects. However in this study, EPP was found to have significant GCA and SCA effects ($P \leq 0.001$) showing both additive and non-additive gene action for this trait. GMC was found to be under additive gene action also found by Malik *et al.* (2004). The traits DMS, DMP, EPP and yield can be improved by either selection of lines with superior GCA effects or selection of crosses that show superior SCA effects. Due to GMC having only additive gene action, the breeding strategy would be the selection of lines with positive and high GCA effects.

This study also found yield to be under both additive and non-additive gene action, however there was predominance of additive gene action which was also found by studies on maize (Muraya *et al.,* 2006; Ahsan *et al.,* 2007; Derera *et al.,* 2008; Vivek *et al.,* 2009). Other studies on maize have found both additive and non-additive gene action present for this yield (Malik *et al.,* 2004; Ünay *et al.,* 2004; Glover *et al.,* 2005; Muraya *et al.,* 2006; Ahsan *et al.,* 2007; Akbar *et al.,* 2008; Derera *et al.,* 2008; Bello and Olaoye, 2009; Vivek *et al.,* 2009). For maize under stress conditions such as drought and high temperatures, yield showed significant GCA effects indicating predominance of additive gene action (Derera *et al.,* 2007; Akbar *et al.,* 2008).

The LPA line, CM 32 showed stress tolerance for germination percentage and seedling dry weight. Kenyan, P 1, P 5 and P 7 had almost all positive GCA effects for all traits under both standard and accelerated aging conditions; hence these four normal tropical lines and one LPA line can be used as sources in breeding for improved germination and vigour. There were two LPA x Nm crosses that showed positive SCA effects under both conditions (CM 32 x P 7; CM 35 x P 1) and these effects increased under accelerated aging conditions. These two crosses show stress tolerance and can be used for the development of inbred lines by using breeding strategies that exploit SCA effects, e.g. recurrent selection for specific combining ability. The LPA x LPA cross showed increased SCA effects for all traits with negative SCA effects for seedling dry weight, average shoot length and vigour index under standard conditions which became positive under accelerated aging conditions, showing some degree of stress tolerance.

Generally the LPA lines showed the greatest reduction under accelerated aging conditions for germination percentage and seedling dry weight. Only the Nm group of lines showed a slight increase in average root length and seedling dry weight under accelerated ageing conditions. Similar observations were reported for maize (Chun *et al.,* 2005; Kausar and Shahzad, 2006), pearl millet (Radhouane, 2007) and wheat (Khan *et al.,* 2010). These lines could be showing an adaptative response to the stress conditions. However, further studies are required to determine the exact cause of this observation. Other studies have also found that the speed of germination, seedling length and dry weight had greatest response to accelerated aging than standard germination (Santipracha *et al.,* 1997; Basu *et al.,* 2004) as seen with the LPA lines in this study. The soybean LPA mutants showed reduced field emergence than the WT with the emergence of lines significantly influenced by the environment in which the seed was produced for planting (Meis *et al.,* 2003).

Both these LPA lines showed negative GCA effects for all yield and associated traits, and can not be recommended for improvement of yield and associated traits. The normal lines Kenyan, P1, P5 and P6 had positive GCA effects for all yield and associated traits and can be recommended for breeding for improved yield and associated traits. The LPA x LPA cross showed significant and positive SCA effects for DMS and DMP with negative SCA effects for yield, EPP and GMC and can be used to improve flowering dates. In the LPA x Nm group, there were eight crosses with positive SCA effects for yield (CM 32 x Kenyan; CM 32 x P 5, CM 32 x P 6, CM 32 x Kenyan, CM 35 x Kenyan, CM 35 x P 5, CM 35 x P 6, CM 35 x P 7). Four of these crosses also had negative SCA effects for DMS and GMC (CM 32 x P 5; CM 32 x P 6; CM 35 x P 6; CM 35 x P7) that can be used in breeding programmes by selecting inbred lines with positive and high GCA effects.

The selection of negative SCA effects for DMS and DMP imply selection for early flowering and negative SCA effects for GMC imply quick drying of the grain and therefore lower moisture content in the grain at harvest. In the group of Nm x Nm crosses, P 1 x T 4 and T 4 x Kenyan and showed negative SCA effects for DMS, DMP and GMC and positive SCA effects for yield and EPP and show SCA effects for earlier flowering dates, quick dry down of grain, improvement of yield and increased prolificy of ears. The breeding strategy would be the selection of lines with positive and high GCA effects.

The cross CM 32 x P 6 showed the desired negative SCA effects for DMS and GMC, positive SCA effects for yield, EPP, germination percentage, average root length, average shoot length and vigour index and should be included in breeding programme to improve yield and seed germination and vigour while retaining earliness and quick dry down of grain. Breeding strategies that exploit GCA effects and SCA effects should be used such as selection of lines with positive and high GCA effects and recurrent selection for specific combining ability.

It was observed that there was one cross (T4 x Kenyan) that showed zero percent germination as it produced 100% abnormal seedlings under both standard and stress conditions (Appendix 20). The one parental line T 4 showed high negative and mostly significant GCA effects for all traits under both conditions (Table 3). There seems to be some negative interaction between T 4 and Kenyan lines resulting in germination of abnormal seedlings.

Generally the LPA lines showed the greatest reduction of means of germination and vigour traits under accelerated aging conditions. The germination of the LPA lines are very inferior compared to the threshold for certified seed (>90%) and needs to be significantly improved. While there have been no accelerated aging tests on the *lpa1-1* mutant lines, there have been two studies on LPA mutant line *lpa241* which is allelic to *lpa1-1* (Pilu *et al.,* 2005) for germination and vigour that confirm the lower germination rate. Pilu *et al*. (2003) found 30% decrease in germination rate while Doria *et al.* (2009) found 72±15% germination under standard conditions (26% reduction compared to WT). The accelerated aging test was able to further emphasize the low vigour of the LPA line by finding the germination percentage decreased to 45±14% (38% reduction compared to the wild type of 9%) (Doria *et al.,* 2009). It was also found in this study that *lpa241* seed had higher moisture content than the wild type and this resulted in faster aging of the LPA seed, which may explain the differences in germination capabilities between *lpa241* and wild type seed (Doria *et al.,* 2009).

The accelerated aging test has been shown to have varying reductions (84% to 36.3%) of germination in maize inbred lines (Byrum and Copeland, 1995; Lovato *et al.,* 2005; Noli *et al.,* 2008). The varying results could be due to the genotype efficiency to the stress test, probably due to their genetically determined vigour traits (Gutièrrrez *et al.,* 1993). However this test seems to effective for determining seed vigour of maize, rice and soybean LPA lines (Meis *et al.,* 2003; Spear and Fehr, 2007; Zhao *et al.,* 2008; Doria *et al.,* 2009). Studies on soybean LPA lines found the accelerated aging test effective in identifying lines with inferior field emergence (Spear and Fehr, 2007) and also effective in differentiating the field emergence potential of soybean LPA lines (Meis *et al.,* 2003). The aging tests evaluated storability of the rice LPA mutants and found that the LPA mutants lost their ability to germinate faster than their WT parents, therefore there should be special requirements for LPA seed storage (Zhao *et al.,* 2008).

This study found larger yield reduction for the LPA x LPA cross (relative yield of 34% of the early check (PAN 6114) and 29% of the mean of all the hybrid checks) than previously reported. Previous studies on yield assessment on maize LPA lines showed a reduction of 5 and 15% compared to the highest yielding commercial varieties (Raboy, 2000) while in rice grain yield was reduced to 12.5-25.6% (Zhao *et al.,* 2008) and wheat LPA mutants had reduced grain yield of between 8-25% (Guttieri *et al.,* 2006). This LPA x LPA cross however showed earlier flowering dates, high ears per plant, lowest yield and low grain moisture content than other crosses. The LPA x Nm cross CM 32 x P 6 showed early flowering dates with increased yield compared to the LPA x LPA cross and the breeding strategy would be the selection of lines with positive and high GCA effects due to the additive effects of DMS, DMP and yield.

There were three LPA x Nm crosses identified in this study that showed relatively high means for both the seed germination and vigour traits and the yield and associated traits. The cross CM 32 x Kenyan had 103% relative yield to the early check with 61% germination under standard conditions which decreased to 54% under accelerated aging conditions but retained seedling dry weight and average root length and slight decreases for average shoot length and vigour index under accelerated aging conditions. There were two crosses that had high percentages of the relative yield of the early check with high germination percentages (P 6 x CM 32: 95% standard, 89% AA, 78% relative yield of early check; CM 32 x T 4: 89% standard, 73% AA, 70% relative yield of early check). These two LPA x Nm crosses show improvements to the means of the seed germination and vigour and yield traits compared to the LPA x LPA cross. These crosses can be used to improve seed germination and vigour as well as yield using recurrent selection for specific combining

ability in a breeding programme by developing lines which show SCA effects when combined with other inbreds.

There were significant positive correlations between the seed germination and vigour traits and between the yield and associated traits. The high correlation between DMS and DMP (1.00) implies that only of one of these traits can be used to predict both traits. The high and significant correlations of yield with DMS, DMP and GMC imply that high yield is associated with longer growing cycles. In this study positive correlations were found between yield and EPP (Yousuf and Saleem, 2001; Broccoli and Byrak, 2004; Derera *et al.,* 2007), DMS and GMC (Odiyi, 2007), DMP and DMS with EPP (Yousuf and Saleem, 2001) yield with DMP (Samanci, 1996; Yousuf and Saleem, 2001) and DMS with yield (Yousuf and Saleem, 2001) which correlates with the findings of other studies on maize.

This study confirmed the lower germination and vigour of the LPA lines compared to normal inbred lines. The advantages of the temperate LPA lines were also shown i.e. early flowering and low moisture content at harvest. Due to their low yield capacity, these LPA lines cannot be used directly in the tropical environment due to their temperate adaptability. The flowering of the crosses with LPA parent was earlier than crosses with Nm and QPM parents. The LPA x LPA cross also showed the lowest grain yield compared to LPA x Nm and Nm x Nm crosses, confirming the low yield capacity of LPA x LPA. The LPA x LPA crosses also exhibited the lowest GMC, thereby confirming their quick dry down of the grain at harvest. There were some promising LPA x Nm crosses identified with high germination and vigour traits as well as high yield and associated traits. Due to the predominance of SCA effects for seed germination and vigour traits, breeding methods that exploit SCA effects can be used, e.g. recurrent selection for specific combining ability. Inbred lines can be developed which show SCA effects when combined with other inbreds. This will aid in breeding for improved germination and yield with retaining the earliness and quick dry down of grain at harvest of the temperate material.

Conclusions

The following conclusions could be drawn from this study:

- Both additive and non-additive gene effects were significant with SCA effects generally superior to GCA effects, indicating that genes with non-additive effects were more important for both seed germination and vigour.

- For days to mid-silking (DMS), days to mid-pollen shed (DMP), yield and ears per plant (EPP) traits, there were both additive and non-additive gene action present, with additive gene action present for grain moisture content (GMC), with predominance of additive gene action present for all yield and associated traits.
- For traits showing predominant additive gene action; lines can be selected for breeding that have for good combining ability for these traits and for traits showing predominance of non-additive gene effects; this material can also be useful in a breeding programme to develop hybrids.
- The LPA line, CM 32 showed increased SCA values under accelerated aging conditions for germination and seedling dry weight thereby indicating stress tolerance, while normal lines, Kenyan, P 1, P 5 and P 7 had positive GCA effects for all seed germination and vigour traits hence they would be useful sources of breeding for improved germination and vigour.
- In the LPA x Nm group, there were four crosses with the desired positive SCA effects for yield and negative SCA effects for DMS and GMC (CM 32 x P 5; CM 32 x P 6; CM 35 x P 6; CM 35 x P7), and three LPA x Nm crosses that showed improvements to the means of germination and vigour and yield traits (CM 32 x Kenyan, P 6 x CM 32, CM 32 x T 4). The inbred lines can be selected for breeding due to the additive gene action of yield and associated traits. Also due to the non-additive gene action of the seed germination and vigour traits, inbred lines can be developed that show SCA effects when combined with other inbreds.
- There were positive correlations between the seed germination and vigour traits, with yield positively and significantly correlated to DMS, DMP and GMC therefore high yield was associated with long growing cycles.

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Appendix 19: Means of germination, abnormal and dead percentages of eight parental lines under standard (Std) and accelerated aging (AA) conditions

Entry	Standard conditions			Accelerated aging conditions		
	Germination %	Abnormal %	Dead %	Germination %	Abnormal %	Dead %
CM 32	68.00	16.00	16.00	32.00	55.50	12.50
CM 35	76.00	7.00	17.00	3.00	87.00	10.00
Kenyan	98.00	0.00	2.00	91.00	1.00	8.00
P 1	95.00	0.00	5.00	96.00	1.00	3.00
P 5	84.00	6.00	10.00	74.00	12.00	14.00
P 6	81.00	14.00	5.00	85.00	12.00	3.00
P 7	97.00	2.00	1.00	98.00	1.00	1.00
T 4	97.00	1.00	2.00	98.00	2.00	0.00
P value	0.0017	0.0001	0.0289	<0.0001	<0.0001	0.0689
Overall Mean	87.00	5.75	7.25	72.13	21.44	6.44
CV (%)	5.21	29.40	57.11	9.26	18.78	65.23
R^2 (%)	92.91	96.62	82.93	98.27	99.23	78.34

Appendix 20: Mean values for germination, abnormal and dead percentages under standard (Std) and accelerated aging (AA) conditions for crosses between low phytic acid and normal inbred lines

	Standard conditions			Accelerated aging conditions		
Pedigree	**Germination %**	**Abnormal %**	**Dead %**	**Germination %**	**Abnormal %**	**Dead %**
P 1 x P 5	60.00	10.00	30.00	54.50	17.50	28.00
P 5 x P 7	72.00	2.00	26.00	84.00	0.00	16.00
P 5 x T 4	41.00	31.00	28.50	12.00	80.00	8.00
P 5 x Kenyan	41.50	28.50	30.50	32.00	45.00	23.00
P 5 x CM 32	45.00	17.00	38.00	21.00	50.00	29.00
P 5 x CM 35	71.50	3.00	25.00	63.00	18.00	19.00
P 6 x P 1	60.50	12.00	27.00	49.50	23.50	26.50
P 6 x P 7	54.00	14.00	32.00	48.00	32.00	20.00
P 6 x Kenyan	95.00	1.00	4.00	94.00	4.00	2.00
P 6 x CM 32	95.00	1.00	4.00	89.00	8.00	3.00
P 6 x CM 35	9.00	69.00	22.00	21.00	62.00	17.00
P 7 x Kenyan	52.00	18.00	30.50	56.00	24.00	20.00
P 7 x CM 32	57.00	8.00	35.00	64.00	15.00	21.00
P 7 x CM 35	34.00	38.00	28.00	27.00	53.00	20.00
P 1 x P 7	51.00	10.00	39.00	44.00	29.00	27.00
T 4 x P 6	81.00	7.00	12.00	79.00	5.00	16.00
T 4 x P 7	99.00	1.00	0.00	100.00	0.00	0.00
T 4 x Kenyan	0.00	100.00	0.00	0.00	100.00	0.00
T 4 x CM 35	41.00	25.00	34.00	26.50	56.00	17.50
P 1 x T 4	76.00	8.00	16.00	57.00	24.00	19.00
Kenyan x CM 32	61.00	12.00	27.00	54.00	19.00	27.00
Kenyan x CM 35	48.00	21.00	31.00	17.00	57.00	26.00
CM 32 x P 1	79.00	16.00	5.00	80.00	19.00	1.00
CM 32 x T 4	89.00	9.00	2.00	73.00	26.00	1.00
CM 32 x CM 35	65.00	7.00	28.00	41.50	47.00	11.50
P 1 x Kenyan	64.00	14.00	22.00	58.50	16.00	25.50
P 1 x CM 35	13.50	57.50	29.00	2.00	82.00	16.00
P 5 x P 6	53.50	27.50	19.00	50.00	35.00	15.00
P value	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	0.0013
Overall mean	57.45	20.27	22.30	49.91	33.82	16.25
CV (%)	18.64	31.43	37.21	18.42	34.64	45.09
R^2 (%)	91.53	96.15	80.92	94.72	90.81	76.91

Chapter Six

Grain yield and associated traits analysis in diallel crosses among normal endosperm, low phytic acid (LPA) and quality protein maize (QPM) inbred lines

Abstract

Maize is a staple crop in many countries in the world. Due to its food security importance, not only does the yield need to be improved, but also the nutritional status of the crop. Maize contains high levels of phytic acid and low levels of amino acids lysine and tryptophan. Monogastric consumers such as humans and pigs have the problem of phytic acid inhibiting absorption of essential minerals and not being able to produce the amino acids, lysine and tryptophan. There are low phytic acid (LPA) mutant lines which contain 66% reduction in phytic acid (*lpa1-1*) and quality protein maize (QPM) lines which contain twice the amount of lysine and tryptophan found in normal lines. A 10 x 10 half diallel was made between four temperate LPA, three tropical QPM and three tropical normal inbred lines. The F_1 hybrids were evaluated over two seasons and six locations for field evaluation. There were both additive and non-additive gene action effects for NCLB, GLS and PLS and for DMS, DMP, EPP, GMC and yield, there is predominance of additive gene action. The LPA lines were early flowering and having quick dry down but all showed negative and significant GCA effects for yield. The QPM and normal lines all showed positive GCA effects for yield. There were five crosses that showed desired negative SCA effects for DMS, DMP and GMC, negative SCA effects for at least one of the three diseases and positive SCA effects for EPP and yield (CM 31 x T 3; CM 33 x T2; CM 31 x QPM 6; CM 33 x QPM 7; T 2 x T 3). The cross CM 31 x T 3 also showed negative SCA effects for all three diseases and was the most resistant of the crosses. The yield of the LPA x LPA, LPA x Nm and LPA x QPM group of crosses is much lower than the check hybrids (-67% to -32%) showing the need for yield improvement of the LPA combinations, with comparable yields for the QPM and Nm groups to the check hybrids. The groups with LPA combinations showed the highest EPP ratio and lowest GMC compared to the other groups and checks, retaining the earliness and quick dry down of grain at harvest of the LPA temperate background. The LPA x LPA, LPA x Nm and LPA x QPM groups did not show much difference in disease scores to GLS and PLS compared to the other groups, however there was slightly more susceptibility to NCLB compared to the other groups. Phenotypic correlations showed a very high and significant positive correlation between DMS and DMP showing the ability to only use of these traits for selection and it would be an accurate indicator of the other trait. Yield was negatively

correlated with all three diseases showing that increased incidence of diseases lead to decreased yield. Yield was positively associated with later maturity, higher ear prolific ratio and higher GMC therefore higher yield is associated with longer growing cycles.

Keywords: grain yield, low phytic acid, quality protein maize

Introduction

Maize is a subsistence crop in many parts of the world. There are millions of people, especially in rural communities that are dependent on maize as a staple crop with little or no other supplements. Due to its food security importance, not only does the yield need to be improved, but also the nutritional status of the crop. Maize contains high levels of phytic acid which inhibits absorption of essential minerals (iron, zinc, calcium, potassium and magnesium) in monogastric consumers. Much of the phosphorus (P) in the grain is in the form of phytates, complex of phytic acid and other minerals (Raboy, 1997) of which >80% of phytates are found in the germ with the remainder in the aleurone (Raboy *et al.,* 2001). Monogastric consumers and animals do not have the enzyme phytase and therefore cannot degrade phytate P (Ertl *et al.,* 1998). Their diets have to be supplemented with either P or the enzyme phytase to degrade phytic acid. Thus diets high in phytic acid may lead to nutrient deficiencies (Brown and Solomons, 1991).

The leading source of P pollution from agricultural sources is animal waste which is applied as manure/fertilizer to the soil to enhance fertility for crops. In some cases, the P application can exceed the crop's ability to take up the added P, and this can result in an accumulation of P in the soil, resulting in run-off and leaching into surface waters (Ertl *et al.,* 1997). The reduction of phytic acid in grains can help improve the bio-availability of essential minerals to monogastric consumers and also reduce the excretion of P by animals and thereby help reduce environmental pollution by P.

In the maize *lpa1-1* mutants show a 66% reduction in phytic acid P (Raboy, 2000). There is 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g P_i (66% of total P) in the *lpa1-1* mutant compared to the normal maize of 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g P_i (7% of total P) (Raboy *et al.,* 2000). The yields of these lines have also been reduced to between 5 and 15% of the highest yielding commercial varieties (Raboy, 2000). These LPA mutants have inferior agronomic and seed viability than their wild-type (WT) parents, leading to yield reduction (Ertl *et al.,* 1998; Raboy *et al.,* 2000) due to reduced seed weights and low vegetative growth. The reduced yield shown by maize *lpa1-1* mutant lines

has also been observed in other LPA mutants in barley (Bregitzer and Raboy, 2006), rice (Zhao *et al.,* 2008) and wheat (Guttieri *et al.,* 2006).

Quality protein maize (QPM) lines contain higher levels of lysine (4.2 g/100 g protein) and tryptophan (0.9 g/100 g protein) compared to normal maize levels of lysine (2.6 g/100 g protein) and tryptophan (0.4g/100g protein) (Vivek *et al.,* 2008), however these lines have low germination and yield. A study comparing normal and QPM maize found normal maize has higher levels of phytate P and inorganic P and lower myo-inositol levels compared to QPM maize (Modi and Asanzi, 2008). High myo-inositol concentrations indicate poor phytate synthesis associated with poor seed performance. The yield of eight QPM maize lines was found to be relatively low with 3.53 to 4.00 t ha^{-1} in 2003/2004 (Akande and Lamidi, 2006). There have been QPM cultivars developed for adaptation to sub-Saharan Africa conditions with comparable yields to the normal lines (Krivanek *et al.,* 2007). There have also been early maturing QPM hybrid varieties developed with the same yield potential (Gupta *et al.,* 2009).

The foliar diseases northern corn leaf blight (NCLB), grey leaf spot (GLS) and *Phaeosphaeria* leaf spot (PLS) commonly occur and regularly cause yield losses. Successful maize cultivars need to be able to produce high yield under pressure from these diseases.

The objectives of this study were to:

- determine the gene action controlling inheritance of yield, days to mid-pollen shed (DMP), silk-emergence days (DMS), ears per plant (EPP), grain moisture content (GMC), and resistance to northern corn leaf blight (NCLB), grey leaf spot (GLS) and *Phaeosphaeria* leaf spot (PLS) diseases in crosses involving tropical normal endosperm, temperate low phytate and tropical QPM germplasm, and
- to test combining ability of LPA, QPM and normal tropical inbred lines for yield, DMP, DMS, EPP, GMC, NCLB, GLS and PLS disease traits, and
- determine phenotypic correlations between yield, DMP, DMS, EPP, GMC, and resistance to NCLB, GLS and PLS diseases.

Materials and Methods

Germplasm

There were four temperate *lpa1-1* lines, three tropical QPM lines and three tropical normal endosperm (Nm) inbred maize lines used in this study (Table 6.1). The pedigree information for the LPA lines is not available but the inbred lines were obtained from Dr V. Raboy (Iowa.

U.S.A.). There were 45 F_1 crosses generated from a 10 x 10 half diallel mating design with no reciprocals and selfs.

Table 6.1: Pedigrees of inbred lines used in diallel mating design

Line	Pedigree	Endosperm type	Adaptation
CM 31	TS3 LPA1-1	*lpa1-1*	temperate
CM 32	JUG 248 LPA1-1	*lpa1-1*	temperate
CM 33	Ex-USDA-actual pedigree unavailable	*lpa1-1*	temperate
CM 34	CO63 LPA1-1	*lpa1-1*	temperate
QPM 3	CML 176	QPM	tropical
QPM 6	CZL 01006	QPM	tropical
QPM 7	OBATANPA-SRC1F3#-MALE	QPM	tropical
P 12	CZL 00008	Normal	tropical
T 2	PN7-2B	Normal	tropical
T 3	PN8-B	Normal	tropical

The parental inbred lines were divided into three groups: temperate LPA, tropical QPM and tropical normal endosperm. There were six groups of crosses generated between the three groups of parental lines: LPA x LPA, LPA x Nm, LPA x QPM, QPM x QPM, Nm x Nm and QPM x Nm. The means of each group was calculated and compared between the different groups. The groups were defined as: LPA group has 1.1 mg/g total inositol P (23% of total P) and 3.1 mg/g Pi (66% of total P), tropical normal and QPM group has 3.4 mg/g total inositol P (76% of total P) and 0.3 mg/g Pi (7% of total P) (Raboy *et al*., 2000).

Experimental design and analysis

The F_1 hybrids were evaluated for grain yield and associated components at the Cedara Agricultural Institute (Cedara) (1076 masl; 29°31’ S, 30°17’ E), Baynesfield Research Farm (758 masl; 29°46’ S, 30°21’ E) in South Africa and Rattray Arnold Research Station (RARS) (1341 masl, 17°40’ S, 31°13’ E) and Kadoma Research Centre (KRC) (1149 masl; 18°19’ S,

29°17' E) in Zimbabwe. Forty-five F_1 hybrids were evaluated with five commercial hybrids (checks) in two seasons at six locations. The trial was replicated twice in a randomized complete block design, with the experimental unit being one row for each entry, 75 cm apart and plant to plant distance of 25 cm. In the 2007/2008 season planting was at Cedara (early and late planting) and in the 2008/2009 season at Cedara, Baynesfield, RARS and KRC. Standard cultural practices, fertilization and weed control were accomplished according to normal field practices. The hybrid checks (PAN) were obtained from Pannar Seed Co (Greytown, South Africa), AGRI from Afgri (Pietermaritzburg, South Africa) and N3/MP72 from Prof P. Tongoona (ACCI, Pietermaritzburg, South Africa).

Yield (t ha^{-1}) of shelled grain (adjusted to 12.5% H_2O) was measured on the whole row basis, and relative yield for each hybrid was calculated as a percentage of the mean of the checks (Relative yield) and a percentage of the early maturing check (PAN 6114) as relative yield (early). Days to mid-pollen shed (DMP) and silk emergence (DMS) dates were estimated as number of days from planting to when 50% of the plants were shedding pollen and had silks emerged, respectively. Prolificacy or number of ears $plant^{-1}$ (EPP) was determined as the number of ears averaged over number of plants $plot^{-1}$. Grain moisture content (GMC) was measured using shelled grain at harvest (Dickey-John moisture meter). Disease severity was assessed at grain hard dough stages using a rating scale of 1 (resistant) to 9 (susceptible). NCLB was present in all six of the locations used, however GLS and PLS was only present in five of the locations and absent from KRC, Zimbabwe.

General analysis of variance was performed for all hybrid data including check hybrids using the SAS programme version 9.1 (SAS Institute, 2002). Combining ability estimates were calculated for yield and associated traits using the Diallel-SAS05 programme (Zhang *et al.*, 2005) in SAS. Pearson's correlation analysis was performed with the PROC CORR procedure in SAS programme version 9.1 (SAS Institute, 2002).

Results

Gene action

The environment and entry effects were significant for all traits (P≤0.001), except for entry effects for EPP (P≤0.005) (Table 6.2 and 6.3). Both GCA and SCA effects were significant for DMS, DMP, Yield, NCLB, GLS and PLS (P≤0.001). EPP showed significant GCA effects at the 1% level and SCA effects at the 5% level. Only GCA effects were significant for GMC (P≤0.001).

The environment*entry, GCA*environment and SCA*environment interactions were significant for all traits (P≤0.001) with lower significance for environment*entry effects of EPP and SCA*environment effects of GLS (P≤0.005).

For DMS, DMP, EPP, GMC and yield, the ratio of GCA and SCA sum of squares to the entry sum of squares was in favour of GCA, showing predominance of additive gene action for these traits (Table 6.4). The ratio of GCA and SCA sum of squares to the entry sum of squares was in favour of both GCA and SCA for NCLB, GLS and PLS disease traits, showing both additive and non-additive gene action that were significant.

Combining ability

General combining ability effects

Positive GCA effects for grain yield and EPP were desired and negative GCA effects for DMS, DMP and GMC traits. The LPA lines showed negative GCA effects for DMS, DMP, EPP, GMC and yield (Table 6.4). There were positive GCA effects for the disease traits except for PLS with CM 31 and CM 33.

The QPM and normal lines all showed positive GCA effects for yield with the highest and significant GCA effects shown by QPM 3 and T3 (P≤0.001). Generally there were positive GCA effects for the QPM and normal lines for DMS, DMP, EPP and GMC except negative GCA effects for DMS by QPM 6 and GMC by T 3. QPM 7, T 2 and T 3 showed negative GCA effects for GLS, PLS and NCLB. There were negative GCA effects by QPM 6 for only PLS and NCLB, P12 for GLS and QPM 3 for NCLB.

To improve grain yield and EPP traits, QPM 3, T 2, QPM 6 and QPM 7 can be recommended. Lines QPM 7, T 2 and T 3 all showed negative GCA effects for all three diseases and can be recommended for disease resistance breeding. Line T 3 was the only normal endosperm line showing negative GCA effect for GMC and can be recommended for breeding for quick dry down rate of grain at harvest. For breeding for earliness, QPM 6 can be used as it showed negative GCA effects for DMS. Depending on the aim of the breeding programme, various suitable inbred lines can be selected for improvement of the trait of interest.

Table 6.2: Mean square for days to silk emergence (DMS), days to mid-pollen shed (DMP), number of ears per plant (EPP), grain moisture content (GMC) and grain yield (t ha^{-1}) traits of an 10 parent diallel evaluated in six locations over two seasons

Source	df	DMS	DMP	EPP	GMC	YIELD
Env	5	7886.74**	6515.56**	8.11**	1131.08**	380.14**
Rep(Env)	6	4.90	6.92	2.05	4.10	7.28
Entry	44	154.49**	169.55**	1.48*	17.53**	38.50**
GCA	9	619.71**	710.32**	1.10**	66.13**	156.88**
SCA	35	34.86**	32.81**	2.51*	4.47	7.94**
Env x Entry	220	9.70**	8.51**	2.07**	3.99**	4.00**
GCA x Env	45	17.59**	15.01**	0.06	6.32**	6.96**
SCA x Env	175	7.67**	2.72**	0.03	1.78**	3.62**
Error	264	2.56	2.51	0.72	2.33	1.93

Table 6.3: Mean square for grey leaf spot (GLS), *Phaeosphaeria* leaf spot (PLS) and northern corn leaf blight (NCLB) diseases of 10 parent diallel

Source	df	GLS	PLS	df	NCLB
Env	4	244.65**	201.03**	5	243.09**
Rep(Env)	5	1.64	4.42**	6	1.76
Entry	44	2.50**	2.93**	44	3.05**
GCA	9	6.64**	7.71**	9	6.79**
SCA	35	1.44	1.70*	35	2.08**
Env x Entry	176	1.49**	2.01**	220	2.17**
GCA x Env	36	2.24**	2.86**	45	4.94**
SCA x Env	140	1.29*	1.80**	175	1.46**
Error	220	0.75	0.72	264	0.67

** P≤0.001, * P≤0.005

Table 6.4: Percent contribution of GCA and SCA sum of squares to entry sum of squares and GCA effects for days to silk emergence (DMS), days to mid-pollen shed (DMP), number of ears per plant (EPP), grain moisture content (GMC), grain yield (t ha^{-1}), grey leaf spot (GLS), *Phaeosphaeria* leaf spot (PLS) and northern corn leaf blight (NCLB) diseases traits

	Endosperm classification	DMS	DMP	EPP	GMC	Yield	GLS	PLS	NCLB
Percent contribution of GCA and SCA sum of squares to entry sum of squares									
GCA		82	85	76	79	83	54	54	46
SCA		18	15	24	21	17	46	46	54
GCA effects									
CM 31	LPA	-3.66**	-3.92**	-0.09**	-0.63	-1.77**	0.26	-0.01	0.50
CM 32	LPA	-2.93	-2.96*	-0.07	-0.87	-1.01**	0.07	0.09	0.10
CM 33	LPA	-0.01	-0.76	-0.06	-0.62	-0.66	0.18	-0.12	0.00
CM 34	LPA	-3.17*	-3.60**	-0.10**	-1.19	-1.98**	0.31	0.46	0.36
QPM 3	QPM	2.41	2.46	0.27**	0.19	1.64**	0.21	0.38	-0.31
QPM 6	QPM	-0.16	0.86	0.02	0.77	0.42	0.04	-0.06	-0.01
QPM 7	QPM	1.41	2.03	0.01	1.05	0.43	-0.25	-0.38	-0.16
P 12	Nm	3.23*	3.32**	-0.01	0.89	0.86	-0.03	0.27	0.03
T 2	Nm	2.81	2.49	0.07	0.77	0.68	-0.12	-0.12	-0.19
T 3	Nm	0.06	0.08	-0.04	-0.36	1.40**	-0.66*	-0.51	-0.31

** P≤0.001, * P≤0.005

Specific combining ability effects

There were five crosses that showed negative SCA effects for DMS, DMP and GMC, negative SCA effects for at least one of the three diseases and positive SCA effects for EPP and yield (Table 6.5). CM 31 x T 3 also showed negative SCA effects for resistance scores to all three diseases. CM 33 x T2 showed negative SCA effect for NCLB and CM 31 x QPM 6 showed negative SCA effects for GLS and NCLB. CM 33 x QPM 7 showed negative SCA effects for PLS and T 2 x T 3 showed negative SCA effect for GLS. There were five crosses that showed negative SCA effects for resistance scores to all three diseases (CM 31 x CM 32; CM 31 x T 3; CM 33 x QPM 3; P 12 x T 3; QPM 3 x T 2).

In the LPA x LPA group of crosses, there were three crosses that showed positive SCA effects for yield and EPP (CM 31 x CM 32; CM 31 x CM 34; CM 32 x CM 33) with varying reactions to the diseases. Of the six LPA x LPA crosses, four of them showed negative SCA effects for DMS and or DMP.

There were four LPA x Nm crosses with positive SCA effects for yield (CM 31 x T 3; CM 33 x P 12; CM 33 x T 2; CM 34 x T 3). CM 31 x T3 showed negative SCA effects for DMS, GMC and reaction to all three diseases. CM 33 x P 12 showed negative SCA effects for PLS only, while CM 33 x T 2 showed negative SCA effects for DMS, DMP, GMC and NCLB and positive SCA effects for EPP. CM 34 x T 3 showed positive SCA effects for EPP and negative SCA effects for GMC and NCLB. There were seven LPA x Nm crosses that showed negative SCA effects for DMS and or DMP.

In the LPA x QPM group of crosses, there were seven crosses with positive SCA effects for yield (CM 31 x QPM 6; CM 31 x QPM 7; CM 32 x QPM 6; CM 33 x QPM 3; CM 33 x QPM 7; CM 34 x QPM 3; CM 34 x QPM 7). There were eight crosses with negative SCA effects for GMC and DMS and or DMP. CM 31 x QPM 6 showed negative SCA effects for DMS, DMP, GMC, GLS and NCLB and positive SCA effects for EPP and yield. CM 32 x QPM 6 showed negative SCA effects for DMS, DMP, GLS and PLS and positive SCA effects for EPP and yield. CM 33 x QPM 3 showed positive SCA effects for EPP and negative SCA effects for all three diseases. CM 33 x QPM 7 showed negative SCA effects for DMS, DMP, GMC and PLS and positive SCA effects for EPP and yield. CM 34 x QPM 7 showed negative SCA effects for DMS, GMC, GLS and NCLB and positive SCA effects for EPP and yield.

In the Nm x Nm group of crosses, P12 x T 3 showed positive SCA effects for yield, EPP and negative SCA effects for GMC, GLS, PLS and NCLB. T 2 x T3 showed positive SCA effects for EPP and yield and negative SCA effects for DMS, DMP, GMC and GLS.

There were four QPM x Nm crosses that showed positive SCA effects for yield. P 12 x QPM 3 showed negative SCA effects for DMS, DMP, GLS and PLS. QPM 3 x T 2 showed positive SCA effects for EPP and yield and negative SCA effects for GMC, GLS, PLS and NCLB. P 12 x QPM 6 showed positive SCA effects for EPP and yield and negative SCA effect for NCLB. QPM 6 x T 3 showed negative SCA effects for GMC and NCLB and positive SCA effect for yield.

There was only one QPM x QPM cross with desired positive SCA effect for yield (QPM 3 x QPM 7) with negative SCA effects for DMS, DMP, GLS and PLS.

Performance of crosses and groups of crosses

The LPA x LPA group of crosses were the lowest yielding, earliest flowering dates, highest prolificacy ratio and lowest grain moisture content at harvest (Table 6.6).CM 31 x CM 33 showed the highest EPP ratio although showed very low yield (2.17 t ha^{-1}). The LPA x Nm group of crosses showed early flowering with higher yield than the LPA x LPA group of crosses. There were two LPA x Nm crosses that showed early flowering with almost twice the yield of the LPA x LPA group (CM 32 x T 2; CM 32 x T 3). The high yielding LPA x Nm crosses (>6 t ha^{-1}) showed medium maturity flowering dates.

In the LPA x QPM group of crosses, there were eight crosses that showed early flowering with increased yield (CM 31 x QPM 3; CM 31 x QPM 6; CM 32 x QPM 3; CM 32 x QPM 6; CM 32 x QPM 7; CM 33 x QPM 7; CM 34 x QPM 6; CM 34 x QPM 7). The LPA x QPM cross with the highest yield (7.87 t ha^{-1}) had 74 DMS and 72 DMP, almost 14 days after the earliest LPA x LPA cross.

The Nm x Nm crosses was very high yielding (7.5-8.6 t ha^{-1}) with medium flowering dates. The Nm x QPM and QPM x QPM groups showed very high yield (6.2-9.3 t ha^{-1}) with high GMC, medium flowering dates and ~1ear/plant. The highest check hybrid was N3/MP72 with grain yield of 10.32 t ha^{-1} with late flowering dates. EPP showed a high CV value (69.98%) (Table 6.6).

Generally the LPA x LPA, LPA x QPM and LPA x Nm crosses showed similar disease resistance scores for GLS and PLS and slightly higher disease scores for NCLB to the other types of crosses (Table 6.7).

Table 6.5: SCA effects of crosses for days to silk emergence (DMS), mid-pollen shed (DMP), number of ears per plant (EPP), grain moisture content (GMC), grain yield (t ha^{-1}), grey leaf spot (GLS), *Phaeosphaeria* leaf spot (PLS) and northern corn leaf blight (NCLB) diseases traits

Pedigree	Classification	DMS	DMP	EPP	GMC	Yield	GLS	PLS	NCLB
CM 31 x CM 32	LPA x LPA	5.33	5.42	0.15	1.24	1.58	-0.30	-0.03	-0.70
CM 31 x CM 33	LPA x LPA	-3.42	-3.52	-0.12	-0.43	-1.49	0.09	-0.11	0.15
CM 31 x CM 34	LPA x LPA	0.32	-0.11	0.01	-0.15	0.05	-0.33	-0.49	0.04
CM 32 x CM 33	LPA x LPA	2.02	2.02	0.02	0.30	0.82	0.18	0.19	-0.20
CM 32 x CM 34	LPA x LPA	-0.82	-1.23	-0.06	0.85	-0.41	0.15	-0.59	0.27
CM 33 x CM 34	LPA x LPA	-0.32	0.49	0.01	0.19	-0.91	-0.16	0.23	0.29
CM 31 x P 12	LPA x Nm	-1.41	-1.44	-0.08	-1.08	-0.12	0.20	-0.30	0.79
CM 31 x T 2	LPA x Nm	-1.07	-0.61	-0.07	1.03	-1.04	0.49	-0.31	0.34
CM 31 x T 3	LPA x Nm	-1.07	-0.45	0.00	-0.15	0.24	-0.37	-0.03	-0.38
CM 32 x P 12	LPA x Nm	0.53	0.68	0.06	-0.35	-0.04	0.39	0.10	-0.07
CM 32 x T 2	LPA x Nm	-1.89	-2.15	-0.06	-1.33	-0.13	-0.32	0.39	-0.26
CM 32 x T 3	LPA x Nm	0.03	-0.08	-0.02	0.20	-1.10	-0.38	-0.13	0.52
CM 33 x P 12	LPA x Nm	0.53	0.90	-0.04	0.04	0.12	0.18	-0.09	-0.63
CM 33 x T 2	LPA x Nm	-0.14	-0.51	0.06	-0.39	0.97	0.17	0.20	-0.24
CM 33 x T 3	LPA x Nm	1.28	0.81	0.04	0.70	-0.51	-0.10	-0.01	0.12
CM 34 x P 12	LPA x Nm	-0.81	-0.51	0.09	0.00	-0.29	0.15	-0.26	0.51
CM 34 x T 2	LPA x Nm	0.52	0.99	0.05	-0.19	-0.42	0.44	0.13	0.14
CM 34 x T 3	LPA x Nm	0.27	0.23	0.04	-0.08	0.80	0.38	0.01	-0.16
CM 31 x QPM 3	LPA x QPM	-0.50	-0.24	-0.05	-0.28	-0.97	0.07	0.19	0.54
CM 31 x QPM 6	LPA x QPM	-1.44	-1.98	0.05	-0.66	0.82	-0.17	0.63	-0.25
CM 31 x QPM 7	LPA x QPM	3.25	2.93	0.12	0.48	0.92	0.32	0.45	-0.52
CM 32 x QPM 3	LPA x QPM	-2.23	-2.03	-0.07	-0.86	-0.79	0.05	0.39	0.18
CM 32 x QPM 6	LPA x QPM	-0.42	-0.52	0.04	0.61	0.34	-0.28	-0.48	0.23

Pedigree	Classification	DMS	DMP	EPP	GMC	Yield	GLS	PLS	NCLB
CM 32 x QPM 7	LPA x QPM	-2.56	-2.11	-0.06	-0.66	-0.25	0.50	0.15	0.04
CM 33 x QPM 3	LPA x QPM	1.27	1.43	0.11	0.31	0.96	-0.46	-0.40	-0.21
CM 33 x QPM 6	LPA x QPM	-0.25	-0.22	-0.09	-0.09	-0.57	0.00	0.34	0.41
CM 33 x QPM 7	LPA x QPM	-0.98	-1.39	0.02	-0.63	0.60	0.09	-0.34	0.31
CM 34 x QPM 3	LPA x QPM	2.01	0.85	-0.08	0.01	0.45	-0.28	0.43	-0.74
CM 34 x QPM 6	LPA x QPM	-0.93	-0.72	-0.08	-0.16	-0.58	-0.22	0.36	0.30
CM 34 x QPM 7	LPA x QPM	-0.24	0.03	0.02	-0.47	1.30	-0.13	0.19	-0.64
P 12 x T 2	Nm x Nm	1.29	0.99	-0.03	0.59	-0.18	-0.22	0.61	-0.02
P 12 x T 3	Nm x Nm	0.54	0.48	0.02	-0.41	0.09	-0.48	-0.40	-0.24
T 2 x T 3	Nm x Nm	-1.21	-1.11	0.06	-0.38	0.37	-0.20	0.39	0.39
P 12 x QPM 3	QPM x Nm	-2.14	-1.65	-0.04	0.80	0.04	-0.65	-0.29	0.26
QPM 3 x T 2	QPM x Nm	1.36	1.35	0.19	-0.11	0.83	-0.06	-0.30	-0.19
QPM 3 x T 3	QPM x Nm	0.03	0.17	0.00	-0.42	-0.11	1.18	0.29	0.09
P 12 x QPM 6	QPM x Nm	0.51	0.53	0.11	0.20	1.15	0.32	0.75	-0.28
QPM 6 x T 2	QPM x Nm	0.84	1.28	-0.11	0.40	-0.16	0.00	-0.86	-0.40
QPM 6 x T 3	QPM x Nm	0.68	0.27	-0.07	-0.16	0.67	0.34	-0.58	-0.37
P 12 x QPM 7	QPM x Nm	0.95	0.03	-0.09	0.21	-0.76	0.10	-0.13	-0.31
QPM 7 x T 2	QPM x Nm	0.28	-0.22	-0.09	0.39	-0.24	-0.31	-0.24	0.25
QPM 7 x T 3	QPM x Nm	-0.55	-0.32	-0.07	0.69	-0.44	-0.37	0.45	0.03
QPM 3 x QPM 6	QPM x QPM	0.67	0.23	-0.03	0.20	-0.49	0.18	0.04	-0.20
QPM 3 x QPM 7	QPM x QPM	-0.48	-0.11	-0.02	0.34	0.07	-0.03	-0.34	0.28
QPM 6 x QPM 7	QPM x QPM	0.33	1.15	0.18	-0.34	-1.19	-0.17	-0.20	0.57

Table 6.6: Means of F_1 hybrids and hybrid checks for days to silk emergence (DMS), days to mid-pollen shed (DMP), number of ears per plant (EPP), grain moisture content (GMC) and grain yield (t ha-1) traits with relative yield to mean of checks and early check (PAN6114)

Pedigree	Classification	DMS	DMP	EPP #	GMC %	Yield	Relative yield to mean of checks (%)	Relative yield to early check (%)
CM 31 x CM 32	LPA x LPA	72.00	69.00	1.07	15.75	2.11	25%	35%
CM 31 x CM 33	LPA x LPA	61.67	59.08	1.98	14.90	2.17	26%	36%
CM 31 x CM 34	LPA x LPA	62.25	59.67	1.67	14.61	2.45	29%	41%
CM 33 x CM 34	LPA x LPA	65.25	63.42	1.78	14.96	2.49	30%	42%
CM 32 x CM 34	LPA x LPA	61.83	59.50	1.83	15.37	2.68	32%	45%
CM 32 x CM 33	LPA x LPA	73.00	69.50	0.96	15.15	4.51	54%	75%
LPA x LPA		**66.00**	**63.36**	**1.55**	**15.12**	**2.74**	**33%**	**46%**
CM 32 x P 12	LPA x Nm	74.50	72.00	1.07	14.50	1.45	17%	24%
CM 34 x T 2	LPA x Nm	75.00	70.50	1.10	15.84	3.28	39%	55%
CM 31 x T 2	LPA x Nm	66.83	65.25	1.86	17.75	4.07	49%	68%
CM 31 x P 12	LPA x Nm	72.00	67.00	0.90	15.32	4.28	51%	71%
CM 34 x P 12	LPA x Nm	68.00	66.50	1.08	16.28	4.45	53%	74%
CM 31 x T 3	LPA x Nm	64.08	63.00	0.97	15.44	4.85	58%	81%
CM 32 x T 3	LPA x Nm	65.92	64.33	1.01	15.54	5.27	63%	88%
CM 32 x T 2	LPA x Nm	66.75	64.67	1.37	15.15	5.75	69%	96%
CM 34 x T 3	LPA x Nm	71.50	67.00	0.97	14.62	6.13	73%	102%
CM 33 x T 3	LPA x Nm	70.08	67.42	0.85	16.30	6.23	74%	104%
CM 33 x P 12	LPA x Nm	72.50	70.75	1.59	16.89	6.35	76%	106%
CM 33 x T 2	LPA x Nm	71.42	68.50	1.10	16.34	6.97	83%	116%
LPA x Nm		**69.88**	**67.24**	**1.16**	**15.83**	**4.92**	**59%**	**82%**
CM 34 x QPM 6	LPA x QPM	64.50	63.83	1.40	16.00	3.88	46%	65%
CM 31 x QPM 7	LPA x QPM	83.00	79.50	1.12	19.25	4.48	54%	75%
CM 33 x QPM 6	LPA x QPM	68.33	67.17	0.94	16.64	4.88	58%	81%
CM 31 x QPM 3	LPA x QPM	67.00	65.58	2.75	15.86	5.21	62%	87%
CM 32 x QPM 7	LPA x QPM	64.67	64.25	1.00	16.10	5.50	66%	92%
CM 31 x QPM 6	LPA x QPM	63.50	62.25	1.27	16.07	5.74	69%	96%
CM 32 x QPM 3	LPA x QPM	66.00	64.75	1.29	15.04	5.84	70%	97%
CM 34 x QPM 7	LPA x QPM	66.75	65.75	1.04	15.98	5.90	70%	98%
CM 32 x QPM 6	LPA x QPM	65.25	64.67	0.92	17.09	5.97	71%	100%
CM 33 x QPM 7	LPA x QPM	69.17	67.17	1.00	16.38	6.62	79%	110%
CM 34 x QPM 3	LPA x QPM	70.00	67.00	1.04	15.59	6.77	81%	113%

Pedigree	Classification	DMS	DMP	EPP #	GMC %	Yield	Relative Yield to mean of checks	Relative Yield to early checks
CM 33 x QPM 3	LPA x QPM	74.00	72.00	1.39	16.44	7.87	94%	131%
LPA x QPM		**68.51**	**66.99**	**1.26**	**16.37**	**5.72**	**68%**	**95%**
P 12 x T 2	Nm x Nm	76.08	74.08	0.97	18.83	7.56	90%	126%
T 2 x T 3	Nm x Nm	73.00	69.50	1.17	16.61	8.20	98%	137%
P 12 x T 3	Nm x Nm	74.00	71.50	1.06	16.72	8.60	103%	143%
Nm x Nm		**74.36**	**71.69**	**1.07**	**17.39**	**8.12**	**97%**	**135%**
P 12 x QPM 7	Nm x QPM	74.33	72.67	0.91	18.73	6.19	74%	103%
QPM 6 x T 2	Nm x QPM	72.25	71.92	0.89	18.53	6.96	83%	116%
QPM 7 x T 2	Nm x QPM	73.25	71.58	1.03	18.8	7.19	86%	120%
QPM 7 x T 3	Nm x QPM	69.67	69.08	0.94	17.79	7.91	95%	132%
P 12 x QPM 3	Nm x QPM	72.25	71.42	1.12	18.47	8.21	98%	137%
QPM 6 x T 3	Nm x QPM	69.33	68.50	0.82	16.83	8.41	100%	140%
P 12 x QPM 6	Nm x QPM	72.33	72.00	1.02	18.44	8.94	107%	149%
QPM 3 x T 3	Nm x QPM	71.25	70.00	1.05	15.99	8.97	107%	150%
QPM 3 x T 2	Nm x QPM	75.33	73.58	1.41	17.43	9.34	112%	156%
Nm x QPM		**72.22**	**71.19**	**1.02**	**17.89**	**8.01**	**96%**	**134%**
QPM 6 x QPM 7	QPM x QPM	70.33	71.33	1.18	18.07	6.12	73%	102%
QPM 3 x QPM 6	QPM x QPM	71.67	70.83	1.15	17.74	7.51	90%	125%
QPM 3 x QPM 7	QPM x QPM	72.08	71.67	1.13	18.17	8.35	100%	139%
QPM x QPM		**71.36**	**71.28**	**1.15**	**17.99**	**7.33**	**88%**	**122%**
PAN6114		73.50	69.00	1.01	15.15	6.00	72%	100%
PAN6757		84.50	75.00	1.08	17.82	6.83	82%	114%
PAN6243		83.00	81.00	1.37	15.33	7.81	93%	130%
PAN6611		75.83	74.42	1.26	16.56	8.38	100%	140%
PAN6777		75.33	73.17	0.94	17.55	8.47	101%	141%
PAN67		82.00	81.50	1.24	17.21	9.52	114%	159%
PAN6227		73.50	71.67	1.21	16.18	9.60	115%	160%
N3/MP72		84.00	79.00	1.05	16.63	10.32	123%	172%
Hybrid Checks		**78.96**	**75.60**	**1.15**	**16.55**	**8.37**	**100%**	**140%**
P value (entry)		**<0.0001**	**<0.0001**	**0.00**	**<0.0001**	**<0.0001**		
P value(env*entry)		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**		
Overall Mean		**69.17**	**67.69**	**1.21**	**16.57**	**6.32**		
CV (%)		**2.48**	**2.47**	**69.98**	**9.2**	**23.98**		
R^2 (%)		**98.40**	**98.26**	**75.01**	**91.9**	**89.61**		

Table 6.7: Disease resistance scores of F_1 hybrids and hybrid checks for grey leaf spot (GLS), *Phaeosphaeria* leaf spot (PLS) and northern corn leaf blight (NCLB) diseases traits

Pedigree	Classification	GLS	PLS	NCLB
CM 31 x CM 32	LPA x LPA	3.40	3.40	4.83
CM 31 x CM 33	LPA x LPA	3.90	3.10	5.58
CM 31 x CM 34	LPA x LPA	3.60	3.30	5.83
CM 33 x CM 34	LPA x LPA	3.70	3.90	5.58
CM 32 x CM 34	LPA x LPA	3.90	3.30	5.67
CM 32 x CM 33	LPA x LPA	3.80	3.50	4.83
LPA x LPA		**3.72**	**3.42**	**5.39**
CM 32 x P 12	LPA x Nm	3.70	4.20	4.92
CM 34 x T 2	LPA x Nm	4.00	3.80	5.25
CM 31 x T 2	LPA x Nm	4.00	2.90	5.58
CM 31 x P 12	LPA x Nm	3.90	3.90	5.67
CM 34 x P 12	LPA x Nm	3.60	4.60	4.25
CM 31 x T 3	LPA x Nm	2.60	2.80	4.75
CM 32 x T 3	LPA x Nm	2.40	2.80	5.25
CM 32 x T 2	LPA x Nm	3.00	3.70	4.58
CM 34 x T 3	LPA x Nm	3.40	3.30	4.83
CM 33 x T 3	LPA x Nm	2.80	2.70	4.75
CM 33 x P 12	LPA x Nm	3.30	3.20	4.42
CM 33 x T 2	LPA x Nm	3.60	3.30	4.50
LPA x Nm		**3.36**	**3.43**	**4.90**
CM 34 x QPM 6	LPA x QPM	3.30	3.60	4.50
CM 31 x QPM 7	LPA x QPM	3.80	3.30	6.25
CM 33 x QPM 6	LPA x QPM	3.40	2.50	5.08
CM 31 x QPM 3	LPA x QPM	3.50	3.90	5.17
CM 32 x QPM 7	LPA x QPM	3.80	3.80	5.00
CM 31 x QPM 6	LPA x QPM	3.70	3.40	4.75
CM 32 x QPM 3	LPA x QPM	3.20	2.90	5.25
CM 34 x QPM 7	LPA x QPM	3.80	3.80	5.83
CM 32 x QPM 6	LPA x QPM	3.70	3.20	4.92
CM 33 x QPM 7	LPA x QPM	3.70	3.40	4.33
CM 34 x QPM 3	LPA x QPM	3.50	4.10	5.58
CM 33 x QPM 3	LPA x QPM	3.60	3.50	5.33
LPA x QPM		**3.58**	**3.45**	**5.17**
P 12 x T 2	Nm x Nm	3.40	3.30	4.25
T 2 x T 3	Nm x Nm	2.40	3.10	4.83
P 12 x T 3	Nm x Nm	4.10	3.50	4.42
Nm x Nm		**3.30**	**3.30**	**4.50**
P 12 x QPM 7	Nm x QPM	2.90	3.70	4.92
QPM 6 x T 2	Nm x QPM	2.70	2.60	4.83
QPM 7 x T 2	Nm x QPM	3.00	4.10	4.75
QPM 7 x T 3	Nm x QPM	2.20	2.70	4.42
P 12 x QPM 3	Nm x QPM	3.80	3.70	4.42
QPM 6 x T 3	Nm x QPM	2.10	2.90	4.50
P 12 x QPM 6	Nm x QPM	3.30	3.00	4.75
QPM 3 x T 3	Nm x QPM	3.10	2.20	4.25

Pedigree	Classification	GLS	PLS	NCLB
QPM 3 x T 2	Nm x QPM	3.30	2.30	4.33
Nm x QPM		**2.93**	**3.02**	**4.57**
QPM 6 x QPM 7	QPM x QPM	3.20	3.10	4.50
QPM 3 x QPM 6	QPM x QPM	3.00	2.70	5.33
QPM 3 x QPM 7	QPM x QPM	3.70	4.30	4.67
QPM x QPM		**3.30**	**3.37**	**4.83**
PAN6114		4.33	3.50	4.50
PAN6757		3.88	2.97	5.10
PAN6243		4.33	3.67	2.63
PAN6611		3.80	3.20	5.25
PAN6777		2.50	5.33	5.00
PAN67		4.50	2.67	3.38
PAN6227		2.50	4.08	4.67
N3/MP72		4.50	1.67	2.63
Hybrid Checks		**3.79**	**3.39**	**4.15**
P value (entry)		**<0.0001**	**<0.0001**	**<0.0001**
P value (env*entry)		**<0.0001**	**<0.0001**	**<0.0001**
Overall Mean		**3.37**	**3.34**	**4.94**
CV (%)		**25.66**	**25.48**	**16.58**
R^2 (%)		**89.18**	**89.15**	**91.22**

Figure 6.1 shows the DMS and DMP for the six groups of crosses and check hybrids. The LPA x LPA, LPA x Nm and LPA x QPM groups retains earliness and is lower than the check hybrids. The Nm x Nm, Nm x QPM and QPM x QPM groups of crosses show a later maturity. In Figure 6.2, the yield of the LPA x LPA, LPA x Nm and LPA x QPM group of crosses is clearly much lower than the check hybrids (-67% to -32%). The QPM x QPM group only has 12% less than the check hybrids while the Nm x Nm and Nm x QPM groups have very little reduction in yield (-3% to -4%). The LPA x LPA, LPA x Nm and LPA x QPM groups showed the highest EPP ratio and lowest GMC compared to the other groups and checks (Figure 6.3 and 6.4). The LPA x LPA, LPA x Nm and LPA x QPM groups did not show much difference in disease scores for GLS and PLS compared to the other groups, however there was slightly more susceptibility to NCLB compared to the other groups (Table 6.7 and Figure 6.5).

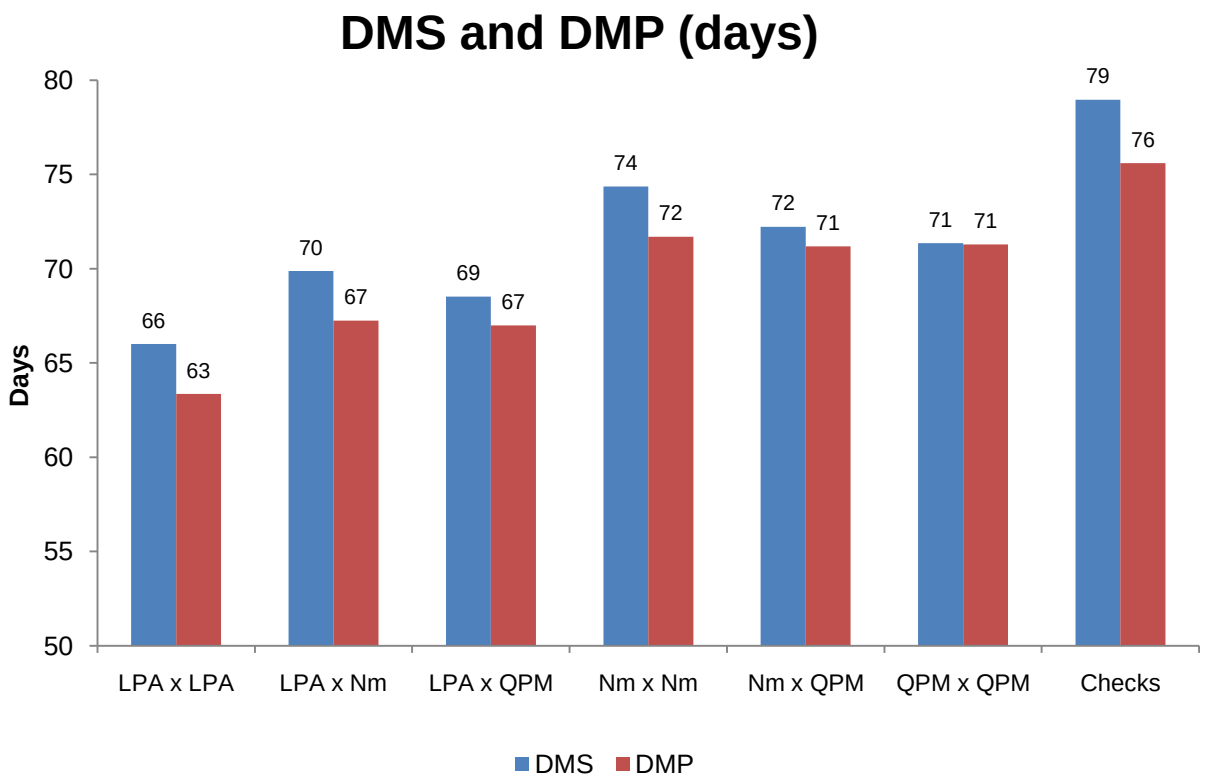

Figure 6.1: Average DMS and DMP (days) for the six classes of hybrids and the check hybrids

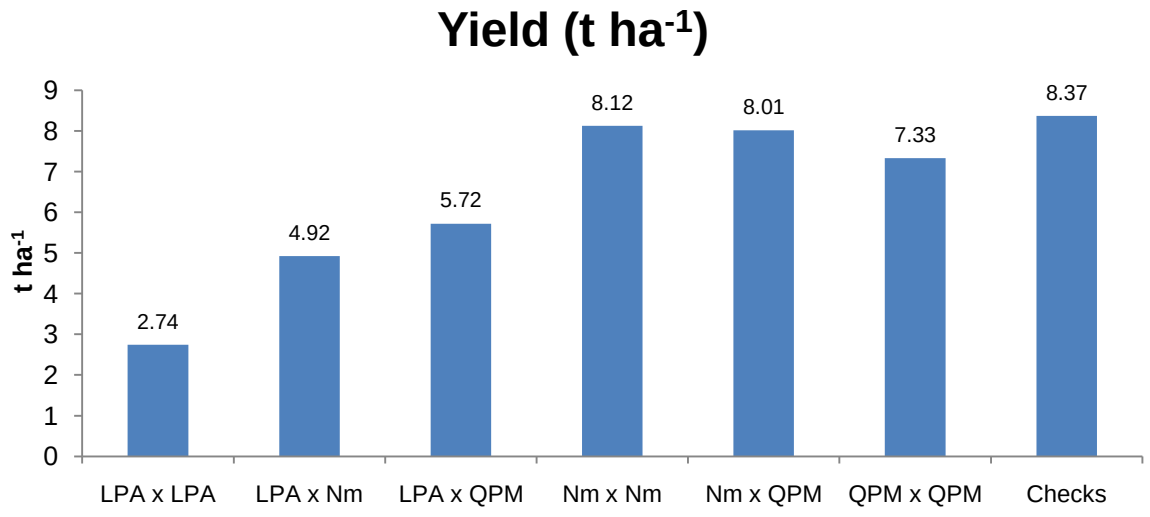

Figure 6.2: Average yield (t ha^{-1}) for the six classes of hybrids and the check hybrids

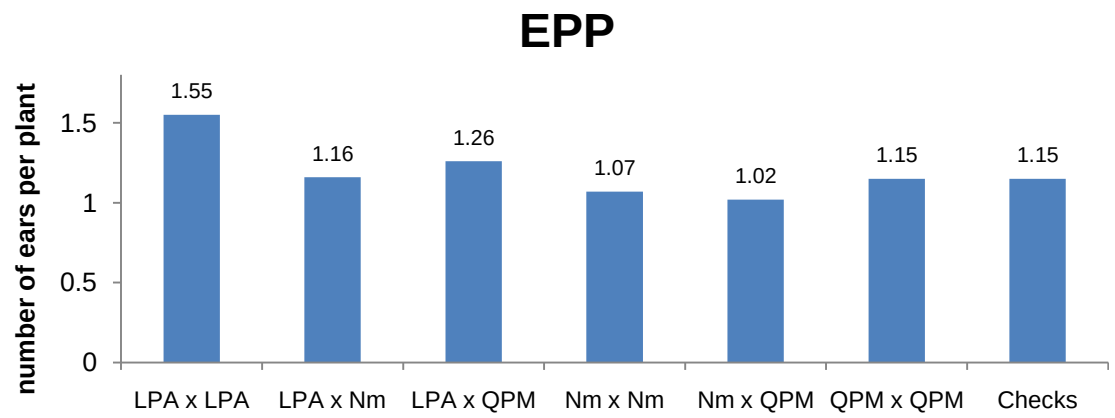

Figure 6.3: Average ears per plant (EPP) for the six classes of hybrids and the check hybrids

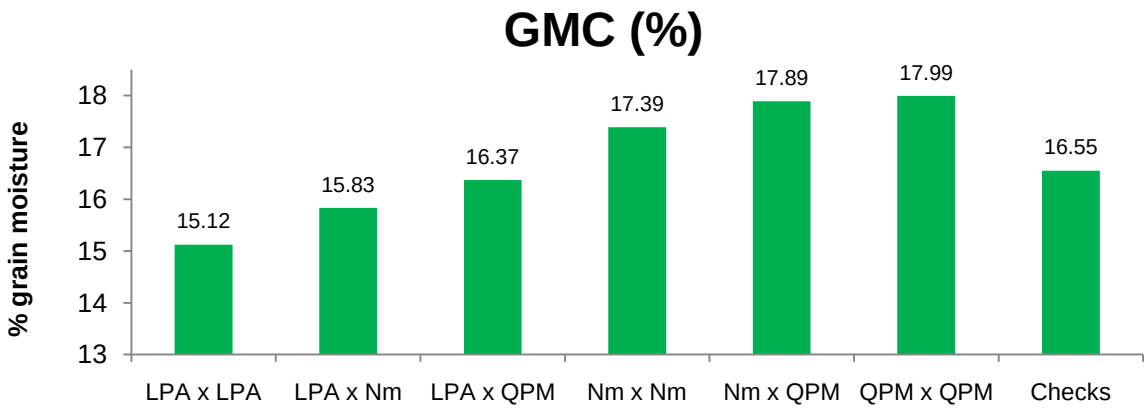

Figure 6.4: Average grain moisture content (%) for the six classes of hybrids and the check hybrids

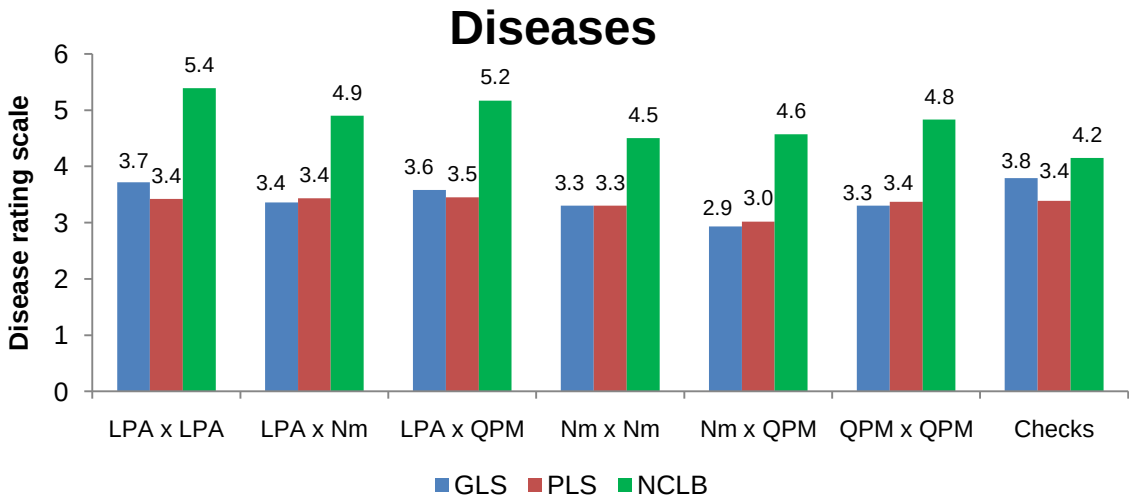

Figure 6.5: Average disease rating scores for NCLB, GLS and PLS for the six classes of hybrids and the check hybrids

Phenotypic correlations

There was a very high and significant positive correlation (P≤0.001) between DMS and DMP (Table 6.8). DMS showed negative correlations with EPP, GLS and NCLB. DMS was positively correlated with yield and significantly correlated with GMC (P≤0.005). DMP was positively and significantly correlated with GMC (P≤0.001) and yield (P≤0.005).

There were negative correlations between DMP and EPP, GLS, PLS and NCLB. EPP showed negative correlations with GMC and yield and positive correlations with GLS, PLS and NCLB disease resistance scores. GMC was positively and significantly correlated with yield (P≤0.001) and negatively correlated with GLS, PLS and NCLB. Yield was negatively correlated with all three disease scores. GLS was positively correlated with PLS and NCLB and PLS was positively correlated with NCLB scores.

Table 6.8: Pearson's phenotypic correlation coefficients among days to silk emergence (DMS), days to mid-pollen shed (DMP), number of ears per plant (EPP), grain moisture content (GMC), grain yield (t ha^{-1}), grey leaf spot (GLS), *Phaeosphaeria* leaf spot (PLS) and northern corn leaf blight (NCLB) diseases traits

	DMS	**DMP**	**EPP**	**GMC (%)**	**YIELD (t ha^{-1})**	**GLS**	**PLS**	**NCLB**
						Scores		
DMS	1.00							
DMP	0.96**	1.00						
EPP	-0.39	-0.40	1.00					
GMC	0.54*	0.69**	-0.25	1.00				
YIELD	0.38	0.50*	-0.35	0.56**	1.00			
GLS	-0.01	-0.1	0.30	-0.18	-0.38	1.00		
PLS	0.03	-0.03	0.10	-0.15	-0.31	0.47	1.00	
NCLB	-0.16	-0.27	0.32	-0.24	-0.53	0.33	0.12	1.00

** P≤0.001, * P≤0.005

Discussion

There was both additive and non-additive gene action for DMS, DMP, Yield, NCLB, GLS and PLS as indicated by significant GCA and SCA effects respectively. EPP showed significant GCA effects with lower significant SCA effects, therefore showing both additive and non-additive gene action with predominance of additive gene action. Due to only GCA effects being significant for GMC, only additive gene action was predominant. In a study on maize inbred lines, the traits of EPP yield, DMS and GMC showed the same GCA and SCA effects significance trends (Malik *et al.,* 2004). For DMS, DMP, EPP, GMC and yield, the ratio of GCA and SCA sum of squares to the entry sum of squares was in favour of GCA, showing predominance of additive gene action for these traits. Yield was found to be under both additive and non-additive gene action with predominance of genetic additive effects. This was also found by other studies on maize (Malik *et al.,* 2004; Muraya *et al.,* 2006; Ahsan *et al.,* 2007; Derera *et al.,* 2007; Vivek *et al.,* 2009). EPP, DMS and DMP were found to be under predominant additive genetic effects and GMC only under genetic additive effects, which are consistent with a previous study (Malik *et al.,* 2004).

In this study, GLS resistance was found to only show significant GCA effects; however the ratio of GCA and SCA sum of squares to the entry sum of squares was in favour of both GCA and SCA for GLS resistance, therefore showing additive gene action with some non-additive genetic effects also involved. This was also shown by other studies on GLS resistance (Derera *et al.,* 2008; Vivek *et al.,* 2009). PLS resistance showed more significant GCA effects than SCA effects but the ratio of SCA sum of squares to the entry sum of squares was in favour of both GCA and SCA, therefore both additive and non-additive gene actions are responsible for PLS resistance with more additive gene action. Other studies on PLS resistance also found the disease to be under additive gene action (da Silva and Moro, 2004; Vivek *et al.,* 2009). In this study, NCLB resistance was found to be under both additive and non-additive gene action while other studies have found mainly additive gene action (Vivek *et al.,* 2009).

For disease resistance traits, under mostly genetic additive effects that are responsible for the source of variation for resistance to this disease, emphasis should be placed on the mean performance of the line in hybrid combinations during selection for breeding programme to advance resistance to the disease.

The environment*entry, GCA*environment and SCA*environment interactions were significant for all traits with lower significance for environment*entry effects of EPP and SCA*environment effects of GLS, showing the need to validate results over different locations and seasons due to the hybrids behaving differently in some environments. Other

studies have found grain yield to have significant environments*entry interactions (Broccoli and Burak, 2004; Glover *et al.,* 2005; Derera *et al.,* 2007; Derera *et al.,* 2008; Vivek *et al.,* 2009) and significant GCA*environment and SCA*environment interactions (Derera *et al.,* 2007; Vivek *et al.*, 2009). Significant GCA*environment interactions were found for EPP, DMS and DMP (Derera *et al.,* 2007).

The significance of environment*entry and GCA*environment effects for diseases implies inbred lines resistant to disease in one location may have different reactions to the same disease in another location, such as for NCLB, GLS and PLS. The significant SCA*environment interactions imply hybrids had different resistant reactions in any location, such as for NCLB and PLS and to a lesser significance for GLS. Similar results were found with GLS showing significant environment*entry, GCA*environment and SCA*environment interactions and PLS and NCLB showing only significant environment*entry and GCA*environment interactions (Vivek *et al.,* 2009). However in a study on PLS resistance, the GCA*environment interaction was found to be not significant (da Silva and Moro, 2004). GLS scores for location*male GCA effects were significant as well as genotype*environment interactions (Derera *et al.,* 2008).

Selection of inbred lines based on GCA effects would require negative GCA effects for DMS, DMP (early flowering), GMC (quick dry down of grain at harvest) and all three diseases (disease resistance). Positive GCA effects for increasing yield and ear prolificacy would be desired. The LPA lines showed negative GCA effects for DMS, DMP, GMC and yield. Although these lines were early flowering and having quick dry down of grain, all showed negative and significant GCA effects for yield except CM 33 which only showed the least negative GCA effect for yield. These LPA lines require improvement for yield. The LPA lines, CM 31 and CM 33 also showed negative GCA effects for disease resistance scores and can be recommended for use to improve flowering, quick dry down of grain and PLS resistance.

The QPM and normal lines all showed positive GCA effects for yield with the highest and significant GCA effects shown by QPM 3 and T3. As in this study, there were positive GCA effects found for yield of three QPM lines and positive and negative GCA effects for DMS, GMC and EPP (Malik *et al.,* 2004). In a study with ten QPM inbreds, variable GCA effects were found for yield (Xingming *et al.,* 2001). To improve yield and EPP traits, QPM 3, T 2, QPM 6 and QPM 7 can be recommended. QPM 7, T 2 and T 3 all showed negative GCA effects for all three diseases and can be recommended for disease resistance breeding. T 3 was the only line showing negative GCA effect for GMC and can be recommended for breeding for quick dry down of grain at harvest. For breeding for earliness, QPM 6 can be used as it showed negative GCA effect for DMP. Depending on the aims of the breeding

programme, the inbred lines can be selected for earliness, quick dry down, ear prolificacy, yield improvement and resistance to foliar diseases.

The QPM lines in this study showed both positive and negative GCA effects for DMS and positive GCA effects for DMP, EPP, GMC and yield. Similar GCA effects were found with studies on QPM lines with high and positive GCA effects for DMP, EPP and yield with some negative and positive GCA effects for GMC (Xingming *et al.,* 2001; Malik *et al.,* 2004). High yield was observed with the QPM x QPM crosses (Xingming *et al.,* 2001) as observed in this study as well.

There were five crosses that showed desired negative SCA effects for DMS, DMP and GMC, negative SCA effects for at least one of the three diseases and positive SCA effects for EPP and yield (CM 31 x T 3; CM 33 x T2; CM 31 x QPM 6; CM 33 x QPM 7; T 2 x T 3) including two LPA x Nm, two LPA x QPM and one Nm x Nm cross. The cross CM 31 x T 3 also showed negative SCA effects for all three diseases and was the most suitable of the crosses. CM 33 x T2 showed negative SCA effect for NCLB and CM 31 x QPM 6 showed negative SCA effects for GLS and NCLB. CM 33 x QPM 7 showed negative SCA effects for PLS and T 2 x T 3 showed negative SCA effect for GLS. There were five crosses that showed negative SCA effects for all three diseases (CM 31 x CM 32; CM 31 x T 3; CM 33 x QPM 3; P 12 x T 3; QPM 3 x T 2) with different SCA effects for yield. Due to the additive nature of yield and associated traits, the breeding strategy would be the selection of lines with positive and high GCA effects.

For the DMS and DMP traits of the six groups of crosses and check hybrids, the LPA x LPA, LPA x Nm and LPA x QPM groups retains the earliness and is lower than the check hybrids. The Nm x Nm, Nm x QPM and QPM x QPM groups of crosses show a later maturity. Although the LPA lines are prolific, the LPA x LPA group of crosses showed the lowest yield due to the small size of the cobs and the early flowering due to non-adaptability to the environment. The crosses between the LPA parental lines were smaller in size and therefore cannot maintain a large cob size and weight. The yield of the LPA x LPA, LPA x Nm and LPA x QPM group of crosses is clearly much lower than the check hybrids (-67% to -32%) showing the need for yield improvement of the LPA combinations. The yield of the LPA combinations need to be increased to the levels of the check hybrids before it can be used in commercial production. The QPM x QPM group only has 12% less than the check hybrids while the Nm x Nm and Nm x QPM groups have very little reduction in yield (-3% to -4%), thereby showing their adaptability to the environmental conditions.

The groups with LPA combinations showed the highest EPP ratio and lowest GMC compared to the other groups and checks, retaining the earliness and quick dry down of grain at harvest of the LPA temperate background. The traits of earliness and low moisture

content at harvest are advantageous traits from the temperate background that can be very useful in tropical material. Although the LPA lines are more prolific but due to yield being related to lateness or long growing season, the LPA combinations groups have low biomass due to their earliness. The LPA x LPA, LPA x Nm and LPA x QPM groups did not show much difference in disease scores to GLS and PLS compared to the other groups, however there was slightly more susceptibility to NCLB compared to the other groups. This is encouraging as it shows the LPA combinations do not have a higher susceptibility to these diseases compared to the Nm and QPM groups of crosses. These diseases are endemic to southern Africa and therefore the disease reaction of these hybrids is useful in evaluating adaptability to tropical growing conditions.

Due to the very high and significant positive correlation between DMS and DMP, only one of these traits can be used to predict for anthesis dates for selection as it would be an accurate indicator of the other trait. Yield was negatively correlated with all three disease resistance scores showing that increased incidence of diseases decreased yield. Yield was positively associated with later maturity, higher ear prolific ratio and higher GMC therefore higher yield is associated with longer growing cycles. Yield was found to be positively correlated with EPP (Yousuf and Saleem, 2001; Broccoli and Byrak, 2004; Derera *et al.,* 2007) which correlates with the findings of this study. DMP was found to be negatively correlated with DMS in a study on maize (Yousuf and Saleem, 2001), DMS negatively correlated with yield (Muraya *et al.*, 2006; Odiyi, 2007) and yield negatively correlated with GMC (Odiyi, 2007) which does not compare with the findings of this study. However there have been postive correlations between DMS and GMC (Odiyi, 2007), DMP and DMS with EPP (Yousuf and Saleem, 2001) yield with DMP (Samanci, 1996; Yousuf and Saleem, 2001) and DMS with yield (Yousuf and Saleem, 2001) which correlates with the findings of this study.

Conclusions

The following conclusions can be drawn from this study:

- Both additive and non-additive gene action were significant for hybrid reaction to northern corn leaf blight (NCLB), grey leaf spot (GLS) and *Phaeosphaeria* leaf spot (PLS) diseases therefore recurrent selection methods that exploit both GCA and SCA effects can be used while additive gene action was predominant for DMS, DMP, EPP, GMC and yield, therefore selection of lines can be used to improve these traits.
- The LPA lines were early flowering and having quick dry down rate but all showed negative and significant GCA effects for yield with CM 33 showing the least negative GCA effect for yield, hence they contributed to low yield in hybrids. The LPA lines,

CM 31 and CM 33 also showed negative GCA effects for PLS disease resistance scores and can be recommended for use to improve early flowering, quick dry down rate of grain and PLS disease resistance.

- The QPM and normal lines all showed positive GCA effects for yield, hence they contributed to high yield in hybrids. Due to negative GCA effects for all three disease resistance score, QPM 7, T 2 and T 3 can be recommended for disease resistance breeding with yield improvement.
- There were four crosses involving LPA lines that showed the desired negative SCA effects for DMS, DMP and GMC, negative SCA effects for at least one of the three diseases and positive SCA effects for EPP and yield (CM 31 x T 3; CM 33 x T2; CM 31 x QPM 6; CM 33 x QPM 7). Due to the additive gene action for yield and associated traits, the selection of lines with positive and high GCA effects would be the appropriate breeding strategy.
- The yield of the LPA x LPA, LPA x Nm and LPA x QPM groups of crosses was much lower than that the check hybrids (-67% to -32%) showing the need for yield improvement of the LPA hybrid combinations.
- The LPA x LPA, LPA x Nm and LPA x QPM groups of crosses did not show much difference in disease resistance scores to GLS and PLS compared to the other groups, however they were slightly more susceptible to NCLB compared to the other groups, showing comparable levels of disease resistance to the Nm and QPM groups of crosses.

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Chapter Seven

Overview

Introduction

This chapter provides an overview of the study, re-stating the main research objectives. It summarizes the main findings. Limitations, challenges and implications of the findings and directions for future research (recommendations) are outlined.

The study had the following general objectives:

- to develop a molecular marker for use at the early vegetative stage of the plant for the detection of the *lpa1-1* gene and to validate the single nucleotide polymorphism (SNP) marker nucleotide change,
- to determine the amount of recurrent parent (P 16) genome present in each of the BC_2F_1 lines using *lpa1-1* SNP marker for foreground selection and amplified fragment length (AFLP) markers for background selection,
- to determine the gene action controlling seed germination, seedling vigour grain yield and resistance to foliar diseases in low phytic acid (LPA) x normal (Nm) and LPA x quality protein maize (QPM) crosses; and
- to determine the level of grain yield, seed germination and vigour in hybrid crosses involving LPA, Nm and QPM maize inbred lines.

Summary of the major findings

Development of a SNP marker for detection of the low phytic acid (*lpa1-1*) gene for use during maize breeding

- A SNP marker was developed and optimised that amplified a 150 bp PCR product for both the parental lines at an annealing temperature of 55°C.
- HRM analysis of an amplicon containing the *lpa1-1* SNP marker could successfully differentiate the two inbred maize lines on the basis of their melt profiles. The SNP marker was validated by forward and reverse DNA sequencing and the single nucleotide base pair change (C to T) was clearly seen between the two parental amplicon sequences.

- The *lpa1-1* SNP marker is a co-dominant marker that can be used to distinguish between homozygous dominant, homozygous recessive and heterozygous alleles of the *lpa1-1* trait based on melting curves and difference plots.
- The cost of the *lpa1-1* SNP marker with HRM analysis was 8% of the cost of DNA sequencing which is the conventional method of detecting SNP genotypes.
- Crude DNA extraction was as effective as high quality DNA extraction method for melt curve analysis.

Marker-assisted selection for low phytic acid (*lpa1-1*) with SNP marker and AFLPs for background selection in a maize backcross breeding programme

- There were 250 BC_2F_1 lines, generated from the cross P 16 x CM 32 with P 16 as the recurrent parent, screened with the *lpa1-1* SNP marker in the foreground selection and 11 homozygous recessive (LPA) and 17 heterozygous lines identified and selected for background selection using AFLP markers.
- The AFLP technique was highly polymorphic with only six *Eco*RI/*Mse*I primer combinations producing 84% polymorphic bands between the parental inbred lines.
- The percentage of recurrent parent genome recovered ranged from 62.12% to 92.15%, with 13 lines showing >83% of the recurrent parent genome and are recommended for further advancement and field assessment.

Seed germination and vigour analysis in diallel crosses among normal endosperm, low phytic acid (LPA) and quality protein maize (QPM) inbred lines under normal and accelerated aging conditions

- Both additive and non-additive gene action was significant for both germination and vigour index and its component traits under normal and accelerated aging conditions, with specific combining ability (SCA) effects generally superior to general combining ability (GCA) effects for all traits, indicating that genes with non-additive effects were more important for both germination and vigour traits. Due to both additive and non-additive gene action; lines can be selected for breeding that have for good combining ability for these traits. Also due to the predominance of non-additive gene effects; this material can also be useful in a breeding programme to develop hybrids.
- The LPA line, CM 31 displayed the desired positive GCA effects under standard and accelerated aging conditions and CM 32 showing increased SCA values under accelerated aging conditions thereby showing stress tolerance. Hence it would be

useful source of breeding material for improved germination and vigour in other LPA germplasm.

- QPM 7 was the only QPM line that had positive GCA effects for all the traits under both standard and accelerated aging conditions showing stress tolerance.
- There were nine crosses that performed well under both standard and accelerated aging conditions which included, one QPM x Nm, two LPA x QPM, two LPA x LPA and four LPA x Nm crosses thereby showing stress tolerance for seed germination and vigour traits. Breeding methods which exploit SCA effects can be used to improve these traits, such as recurrent selection for specific combining ability.
- The LPA lines and their hybrid combinations exhibited low germination (14-39%) with the QPM lines and normal tropical lines showing high germination rates under both experimental conditions; therefore they were more viable and more stress tolerant than the LPA lines.

Combining ability between temperate low phytic acid (LPA) and tropical normal endosperm inbred lines for seed vigour and grain yield components

- Both additive and non-additive gene effects were significant with SCA effects generally superior to GCA effects, indicating that genes with non-additive effects were more important for both seed germination and vigour.
- For days to mid-silking (DMS), days to mid-pollen shed (DMP), yield and ears per plant (EPP) traits, there were both additive and non-additive gene action present, with additive gene action present for grain moisture content (GMC), with predominance of additive gene action present for all yield and associated traits.
- For traits showing predominant additive gene action; lines can be selected for breeding that have for good combining ability for these traits and for traits showing predominance of non-additive gene effects; this material can also be useful in a breeding programme to develop hybrids.
- The LPA line, CM 32 showed increased SCA values under accelerated aging conditions for germination and seedling dry weight thereby indicating stress tolerance, while normal lines, Kenyan, P 1, P 5 and P 7 had positive GCA effects for all seed germination and vigour traits hence they would be useful sources of breeding for improved germination and vigour.
- In the LPA x Nm group, there were four crosses with the desired positive SCA effects for yield and negative SCA effects for DMS and GMC (CM 32 x P 5; CM 32 x P 6; CM 35 x P 6; CM 35 x P7), and three LPA x Nm crosses that showed improvements to the means of germination and vigour and yield traits (CM 32 x Kenyan, P 6 x CM

32, CM 32 x T 4). The inbred lines can be selected for breeding due to the additive gene action of yield and associated traits. Also due to the non-additive gene action of the seed germination and vigour traits, inbred lines can be developed that show SCA effects when combined with other inbreds.

- There were positive correlations between the seed germination and vigour traits, with yield positively and significantly correlated to DMS, DMP and GMC therefore high yield was associated with long growing cycles.

Grain yield and associated traits analysis in diallel crosses among normal endosperm, low phytic acid (LPA) and quality protein maize (QPM) inbred lines

- Both additive and non-additive gene action were significant for hybrid reaction to northern corn leaf blight (NCLB), grey leaf spot (GLS) and *Phaeosphaeria* leaf spot (PLS) diseases therefore recurrent selection methods that exploit both GCA and SCA effects can be used while additive gene action was predominant for DMS, DMP, EPP, GMC and yield, therefore selection of lines can be used to improve these traits.
- The LPA lines were early flowering and having quick dry down rate but all showed negative and significant GCA effects for yield with CM 33 showing the least negative GCA effect for yield, hence they contributed to low yield in hybrids. The LPA lines, CM 31 and CM 33 also showed negative GCA effects for PLS disease resistance scores and can be recommended for use to improve early flowering, quick dry down rate of grain and PLS disease resistance.
- The QPM and normal lines all showed positive GCA effects for yield, hence they contributed to high yield in hybrids. Due to negative GCA effects for all three disease resistance score, QPM 7, T 2 and T 3 can be recommended for disease resistance breeding with yield improvement.
- There were four crosses involving LPA lines that showed the desired negative SCA effects for DMS, DMP and GMC, negative SCA effects for at least one of the three diseases and positive SCA effects for EPP and yield (CM 31 x T 3; CM 33 x T2; CM 31 x QPM 6; CM 33 x QPM 7). Due to the additive gene action for yield and associated traits, the selection of lines with positive and high GCA effects would be the appropriate breeding strategy.
- The yield of the LPA x LPA, LPA x Nm and LPA x QPM groups of crosses was much lower than that the check hybrids (-67% to -32%) showing the need for yield improvement of the LPA hybrid combinations.
- The LPA x LPA, LPA x Nm and LPA x QPM groups of crosses did not show much difference in disease resistance scores to GLS and PLS compared to the other groups, however they were slightly more susceptible to NCLB compared to the other

groups, showing comparable levels of disease resistance to the Nm and QPM groups of crosses.

Breeding implications

The results have the following implications for breeding:

- A co-dominant *lpa1-1* SNP marker was developed and optimised for detection of LPA genotypes. The marker was validated by DNA sequencing. It is the first SNP marker available for LPA genotypes detection with HRM analysis. It is non-destructive, quicker, reliable and cost-effective than the conventional method of detecting phytic acid (Chen *et al.,* 1956) and can be used to screen segregating material in a breeding programme.
- There was both successful foreground selection with *lpa1-1* SNP marker and background selection with AFLPs markers in backcross breeding programme. There were 28 BC_2 lines identified as homozygous recessive and heterozygous for the *lpa1-1* SNP marker and were characterised for amount of recurrent parent genome using AFLP markers. These lines can be tested under field conditions as there is sufficient recovery of the recurrent parent genome. The lines which were homozygous recessive for the LPA trait would be considered as sources for breeding LPA hybrids for deployment in tropical environments.
- The germination and vigour traits were found to be under both additive and non-additive gene action with higher SCA effects than GCA effects therefore genes with non-additive effects were more important for these traits. The appropriate breeding strategy for these traits would be development of hybrids, or to use reciprocal recurrent selection procedures that emphasise both GCA and SCA effects.
- Yield and associated traits were found to be under additive gene action and therefore the appropriate breeding strategy would be to emphasise selection of inbreds with high GCA effects for yield. These lines can be used to develop hybrids and synthetic open-pollinated varieties.
- The control of resistance to diseases (NCLB, PLS, GLS) were found to be both additive and non-additive gene action, therefore reciprocal recurrent selection strategy can be employed.
- The LPA lines and their combinations showed very low germination and low yield, thereby confirming the poor agronomic and vigour performance of the LPA germplasm. The LPA lines showed variation for the vigour traits, with CM 31 and CM 32 showing stress tolerance for germination and vigour. However the LPA lines showed early flowering and quick grain dry down rate which are desired traits.

- The QPM and normal lines all showed high germination and yield, showing the improvement of breeding of QPM material. The late flowering and late drying trends shown by these lines are not desired by farmers as this would mean a late harvest of maize.
- Yield was negatively correlated with all three foliar diseases, indicating that the increased disease incidence reduced grain yield of the varieties and hence varieties with resistance to GLS, PLS and NCLB should be developed. Yield was positively correlated with later maturity, higher ear prolific ratio and higher GMC therefore higher yield is associated with longer growing cycles. There needs to be a compromise between yield and maturity, therefore maybe have medium maturity material with slightly lower yield. Also can possibly incorporate LPA trait into late maturity varieties, which provides improved germination and vigour required by LPA material.
- This is the first study on LPA x QPM crosses and there were some promising crosses identified that show the possibility of developing material with both traits at the same time using a selection index for both traits. Further field evaluations for agronomic performance and laboratory tests need to be done to ascertain both the LPA and QPM trait presence, quantity of phytic acid, lysine and tryptophan and overall performance of these crosses.

Breeding challenges

The challenges faced by breeding for low phytic acid in maize varieties are:

- Breeding for low phytic acid is hampered by low germination and reduced vigour with field evaluation of LPA hybrids showing very low yield compared to normal and QPM hybrids. Due to LPA crosses being not high yielding, recurrent selection can be used due to the recessive nature of the LPA trait. However this breeding approach requires a selfing cycle to fix lines with recessive gene or identify the lines with the recessive gene. The use of the marker to identify heterozygous genotypes can greatly reduce number of generations need to introduce the LPA trait into other high yielding germplasm.
- There are often problems with drought for small scale farmers in Africa. The earliness and quick dry down rate of the grain are valuable traits of the temperate material that can be introduced into the adapted tropical material to alleviate this problem.
- There were concerns about the effect of DNA concentration on SNP marker with HRM analysis for detection. However, due to the same method of DNA extraction and the same starting amount of leaf material was used for DNA extraction of all 250 BC_2F_1 lines; this was not a problem in this study.

- The SNP marker with HRM analysis is highly effective but to be adopted into a breeding programme, there needs to be adaptation to high-throughput methods and decreased costs. This can be addressed by optimizing the marker reaction to lower PCR volumes. Also FTA cards can be tested as an alternative to the DNA extraction. Another area of optimization is testing if chipping the seed embryo for DNA extraction would be effective in detecting the different genotypes.
- In the backcross breeding programme, markers were successfully used for both foreground and background selection. The *lpa1-1* SNP marker was ideally co-dominant thereby allowing identification of all three types of genotypes. There were some concerns about the polymorphism rate of AFLP markers, sufficient differentiation of the BC_2F_1 lines and method of detection of the markers. However, the AFLP markers were very effective in identifying lines containing greater amounts of recurrent parent genome expected in BC_2 generation with only six primer combinations and a high polymorphic rate. A DNA sequencer was used in this study for detection of AFLP markers, which is not available at all institutions, however polyacrylamide gels can also be used to detect AFLP markers. This study showed the effectiveness and potential of using MAS to track trait of interest and reduce the number of generations in a maize backcross breeding programme.

Directions for future research

The *lpa1-1* SNP marker can be refined to a high-throughput marker assay by testing embryo chipping and FTA elute cards as an alternative to the DNA extraction. The method of embryo chipping enables the germination of the seed thereby reducing the need for large numbers of plants needing to be planted, but the issues of reduced germination due to infection of the seed through the chip on the seed needs to be addressed. However, fungicides can be used to reduce infection of the seeds used for embryo chipping. The process has been well documented and published for maize (Gao *et al*., 2008).

FTA cards from which DNA can be directly eluted are available and these can be tested as an alternative to DNA extraction methods. The cost-effectiveness of these FTA cards would need to be determined. Due to the high cost of the PCR mix used for the *lpa1-1* SNP marker, lower volumes of PCR reaction mix can be tested to decrease costs, thereby making the SNP assay affordable and quick.

Field trials of 28 BC_2F_1 lines should be conducted over different seasons and locations to determine adaptability and yield potential. The homozygous recessive lines with high percentage of recurrent parent should be carefully considered as the LPA trait is already

fixed. These lines will be tested for adaptation as lines per se or in hybrid combination. The agronomic performance of these lines needs to be determined under field conditions to determine adaptability and yield performance. Another validation of the low phytic acid content would be to subject these selected lines to the colorimetric assay to confirm the actual levels of phytic acid.

Field trials of the F_1 hybrids were promising because there were some crosses that showed both good germination and vigour traits and good yield and associated traits, especially LPA x QPM hybrids. These can be further advanced for improvement of germination and vigour and yield by either selection of inbred lines or development of hybrids depending on the gene action of the traits. The SNP marker can be used to identify or track LPA genotypes in the breeding material.

Due to the information on the gene action of the seed germination, vigour and yield traits, suitable lines with high GCA effects can be selected for improvement of traits under additive gene action. For traits under predominantly non-additive gene action, inbred lines can be developed that show SCA effects when combined with other inbreds. For traits under both additive and non-additive gene action, reciprocal recurrent selection procedures that emphasise both GCA and SCA effects can be used.

References

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