PHYTATE RELATED RESPONSE OF MAIZE SEED TO PHOSPHORUS AND TEMPERATURE

NAFABUANGA MIREILLE ASANZI

BSc. Agric. (University of KwaZulu-Natal)

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE
(Crop Science)

in the
School of Agricultural Sciences and Agribusiness
Faculty of Science and Agriculture
University of KwaZulu-Natal
Pietermaritzburg

December 2006

DECLARATION

I, Nafabuanga Mireille Asanzi, hereby declare that the work presented in this thesis is the result of my own investigations and has not been submitted for any previous application for a degree. All sources of information have been acknowledged by reference to the authors.

Nafabuanga Mireille Asanzi

I, Albert Thembinkosi Modi, supervised the above candidate in conduct of her research study.

Professor A.T. Modi (Supervisor)

ACKNOWLEDGEMENTS

The author wishes to record her sincere appreciation for the assistance given by the following:
Professor A.T. Modi, associate professor in department of Crop Science University of Kwazulu-Natal and Professor J. van Staden Research Centre for Plant Growth and Development Director, who respectively supervised and co-supervised the work.

Thanks to the staff of the Centre for Electron Microscopy at the University of Kwazulu-Natal for their guidance in using the environmental scanning electron microscopy and transmission electron microscopy. I gratefully acknowledge Dr Collins Southway for opening his laboratory to me for sugar analysis and for his support during this study. I also thank Alfred Odindo for being my companion and adviser during the carbohydrate analysis study. Thanks to Q. Tharatt and K. Pillay for their technical and logistical support, respectively. Thanks to the cohort of postgraduate students during my study period (Alfred Odindo, Molipa Mosoeunyane, Rorisang Mare, Tracy Campbell and Samson Tesfay) for their emotional and moral support.

Furthermore, I would like to thank my father Dr Christoph Asanzi and mother Jeannette Musitu for their love and financial support. Thank you to my brothers Philippe, Patrick and Alson and my sisters Ange and Esperance for their encouragement and support.

Above all, the Almighty God gave me life, energy and ability to conduct this study.

ABSTRACT

The aim of the study was to determine the effect of day/night temperatures (22/16°C, 27/21°C and 33/27°C) and phosphorus levels (0, 0.12 and 1.2g per 20 kg soil) on seedling establishment and seed viability during three stages of seed development (15, 22 and 33 days after flowering) for seed of normal and quality protein maize cultivars. Soluble carbohydrate accumulation and mineral element content were determined using environmental scanning electron microscopy (ESEM) in relation to seed phytate levels and seed germination capacity at different stages of development. Leaf emergence rate and plant height during seed development were significantly ($P < 0.05$) influenced by temperature and phosphorus nutrition. Phosphorus in seed is stored primarily in the form of phytic acid, also known as phytate. Accumulation of phytate takes place during maturation phase of seed development. Phosphorus nutrition and temperature also caused a significant ($P < 0.05$) increase in seed germination at all stages of seed development. Furthermore, phosphorus nutrition and temperature influenced occurrence of soluble carbohydrates in seeds. Myo-inositol, the sugar alcohol that forms the basic structure of phytate, was increased by P nutrition and increasing growth temperature. Whereas, QPM maize was generally found to perform poorly than normal maize, with respect to phytate content, seed germination and seedling establishment, both cultivars displayed the same responses to phosphorus nutrition and temperature. In both cultivars, globoids, the sites of phytate synthesis and storage, were found only in the embryonic axis. Subsequently, there were significantly low levels of mineral elements (P, Mg and K) found in the endosperm, compared with embryonic axis. This finding suggested that the embryonic axis plays a major role in seed performance, through its effects emanating from phyate metabolism. Myo-inositol plays a role in membrane biogenesis during stress conditions such as temperature by maintaining the integrity of the cell wall and minimizes the leaching of cations essential during germination.

Myo-inositol, although it occurs in small concentrations, could be used to indicate seed quality in maize, because its accumulation was found to be associated with enhanced phyate levels and better seed germination in a wide range of temperatures. Low vigour seeds are associated with high electrolyte leakage during imbibition. Mineral elements form a significant portion of the imbibition leachate, which causes seeds to lose nutrients for early seedling growth. This study provided evidence that phosphorus nutrition can alleviate poor seed vigour of maize by improving phytate levels.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1 LITERATURE REVIEW

1.1. Introduction

Phosphorus in seeds is stored primarily in the form of phytic acid, also known variously as phytate, myo-inositol hexakis phosphoric acid and Ins P6, which are derivatives of inositol. During seed development, phytic acid is deposited in spherical inclusions known as globoids, which are structures containing seed storage proteins in protein bodies (BEECROFT & LOTT, 1996). During germination, the stored phytate is hydrolyzed to myo-inositol and inorganic phosphate by phytase enzymes (HEGEMAN *et al.,* 2001).
Phytin is a mixture of myo-inositol hexakisphosphoric acid salts of magnesium, potassium and calcium, which can be extracted from mature seeds. Phytic acid and phytate refer to the free acid and any salt of myo-inositol 1 and isomers 2, 3, 4, 5 and 6 (COSGROVE, 1966; LOEWUS, 1990).

The negatively charged sites of phytic acid (Figure 1.1) bind mainly K^+ and Mg^{++}, but may also form salts with other cations, including Ca^{++}, Mn^{++}, Zn^{++}, Ba^{++} and Fe^{+++} (LOTT, 1984; LOTT &WEST, 2001). Phytic acid is a powerful chelator, especially of polyvalent cations, because they can be bound more strongly than monovalent cations (GRAF, 1986). Phytate commonly forms one to several percent of the dry weight of many seeds and in many cases, accounts for 50-80% of the total P in seeds (LOTT, 1984; RABOY, 1997). The aleurone of cereal grains contains two types of inclusions (a) globoids containing high amounts of phytate and (b) protein carbohydrate bodies. Maize has more than 80% of its phytic acid concentrated in the embryo whilst, wheat and rice store about 90% of the caryopsis phytate P in the aleurone layer and about 10% in the embryo (LOTT *et al.*, 1995).

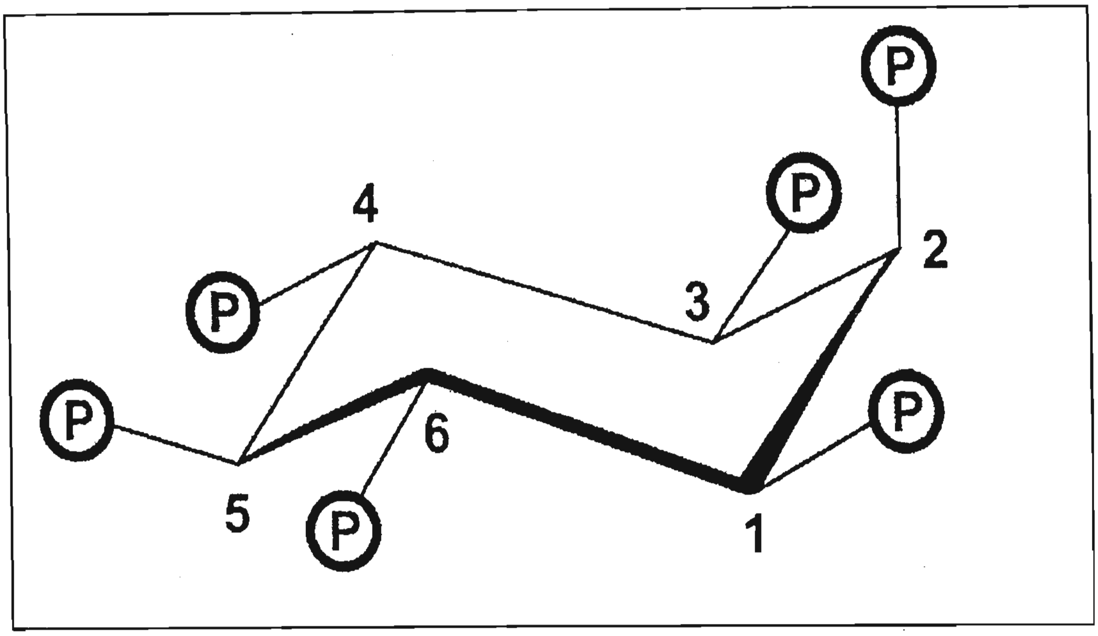


Figure 1.1. Molecular model of phytic acid. Each P circle represents a phosphate group, which has a negative charge and binds to positive cations (Ca^{++}, Mn^{++}, Zn^{++}, Ba^{+++}, Fe^{+++}, K^{+} and Mg^{++}) (GREENWOOD, 1989).

The role of myo-inositol in plants is many-fold: it participates in the phosphatidylinositol signaling pathway, auxin storage and transport, phytic acid biosynthesis, cell wall biosynthesis and production of stress related molecules (LOEWUS & MURTHY, 2000). An illustration of the role of myo-inositol in plant metabolism is presented in (Figure 1.2.). Myo-inositol is synthesized from glucose-6-phosphate by the enzyme inositol 1-phosphate synthetase (INO1). Myo-inositol is further phosphorylated into phytate P as a storage source of phosphate in seeds (LOTT *et al.*, 2000). During abiotic stress conditions, such as water and temperature stress, myo-inositol fuels the production of compounds correlated with stress protection such as verbascose and stachyose, which are vegetative storage carbohydrates (PETERBAUER *et al.*, 2001). The stress protection mechanism regulates the facilitation of water permeability which allows the leaves to regain turgor in time course of the accumulation of pinitols (VERNON & BOHNERT, 1992). Furthermore, myo-inositol plays a role in membrane biogenesis during stress conditions such as temperature by maintaining the integrity of the cell wall. This minimizes the leaching of cations essential for germination (BONHERT *et al.*, 1995).

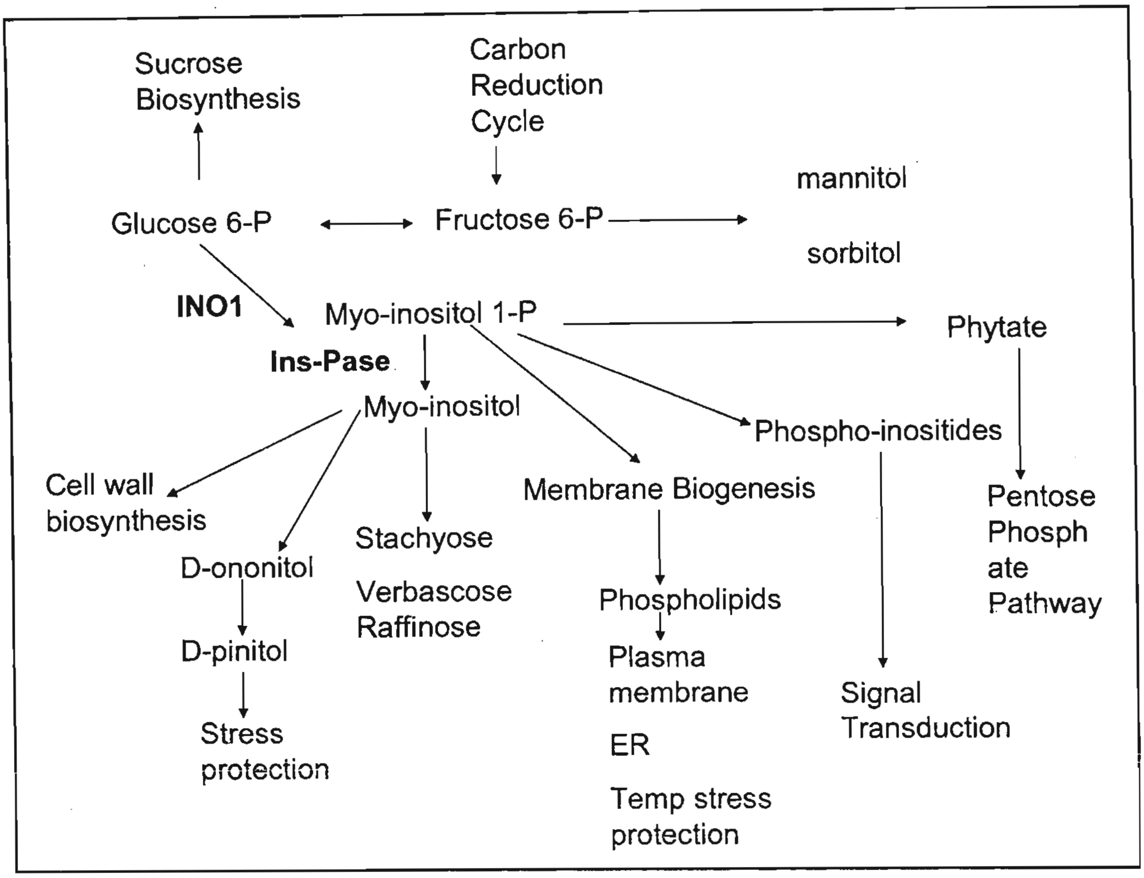


Figure 1.2. An illustration of the role of myo-inositol in plants.

Myo-inositol accumulates in plant tissues and organs that accumulate nutrient stores for subsequent redistribution, such as pollen, roots and tubers. The abundance of inositol in seeds, pollen and other plant tissues may also be ascribed to the abundance organic phosphorus in soils (RABOY, 2001).

1.1.1. Physiological and environmental considerations for phytate in seed biology

On a broad level, phytate synthesis can be regulated in two ways. Firstly, by the amount of photoassimilates and phosphorus translocated to the grain (COELHO *et al.*, 2002).

Secondly, by the partitioning of these substrates among different pools and competing metabolic pathways in the developing grain (COELHO *et al.*, 2002). As an example of the first case, it has been shown that phytate levels are correlated with the supply of P to the plant and with the content of inorganic phosphorus in leaves (RABOY & DICKINSON 1984; RABOY & DICKINSON, 1993), which ultimately leads to increased translocation of P to the grain. Another important factor seems to be the protein content of grains, since a correlation between phytate and protein contents has frequently been found (RABOY *et al.*, 1991). This is not entirely surprising given the association between protein and phytate in protein storage bodies. The regulatory mechanisms are still entirely unknown.

The accumulation of phytate takes place during the maturation phase of seed development, the period of rapid cell expansion and reserve synthesis and accumulation (LOTT *et al.*, 1995). In seeds, the protein bodies in which phytic acid is found are generally of vacuolar origin. For example, those found in the embryonic and endospermic tissues of dicotyledonous seeds and in the embryonic tissues and aleurone layer of the endosperm in monocotyledonous seeds (LOTT *et al.*, 1995). Furthermore, phytic acid appears to accumulate only in cells that remain alive throughout the quiescent phase of seed maturation and into germination. For example, in the cereal starchy endosperm, neither the high prolamine (glutelin) containing protein bodies of ER origin nor globulin-containing protein bodies of vacuolar origin contains phytic acid (O'DELL *et al.*, 1972; SIMON *et al.*, 1990). Cells of the starchy endosperm of cereals die during the maturation phase of seed development. Cells of the aleurone layer of the endosperm, however, remain alive throughout the quiescent phase and do contain relatively large amounts of phytic acid (LIN *et al.*, 2005).

It was hypothesized that phytic acid in the cytoplasm, possibly in association with the endoplasmic reticulum, accumulates initially in the ER cisternae, and is then transported to the vacuoles (developing protein bodies). Thus, following the same path as storage proteins. This hypothesis was based on light and electron microscopic observations of developing castor bean endosperm (GREENWOOD & BEWLEY 1984).

LOTT (1980) suggested that during seed development, phytate is deposited in globoids (discrete regions of cellular organelles which are usually referred to as protein bodies or aleurone particles) or dispersed evenly in the proteinaceous matrix of protein bodies as protein-phytate complexes or K-phytate in some species (e.g., pea and soybean). It appears that globoids are not static storage structures but act as ion exchange "beads" that can change composition during seed formation and seedling growth (LOTT *et al.*, 2000). In general, an increase in seed K content is correlated with a Ca^{2+} decline in the globoids (OGAWA *et al.*, 1979; LOTT *et al.*, 2000). During seedling growth the K in globoids declines rapidly and elements with divalent or trivalent cations (Mg, Ca, Fe, and Zn) tend to increase significantly (LOTT *et al.*, 1995; LOTT *et al.*, 2000). It has also been found that during seedling growth, in certain cotyledonary cells, globoids actually expand to about four times their diameter in the dry seed (PITT & LOTT, 1996).

Formation of phytate was suggested to be restricted to these subcellular regions (OGAWA *et al.*, 1979; LIU *et al.*, 2004). The immobility of phytate, a quality similar to that of starch or certain reserve polysaccharides, oils, and proteins of the seed, invites a consideration of processes which lead to formation and accumulation of phytate reserves as well as to the utilization of breakdown products (LOEWUS & LOEWUS, 1983). The importance of inositol in general metabolism has been the subject of intense studies in animal and plant systems in recent years. Myo-inositol, myo-inositol phosphates and inositol-phosphate-containing membrane lipids function in the transfer of information from cell surface to cell interior, i.e., the phosphoinositide signal transduction mechanism. LOTT *et al.* (1995) proposed a mechanism by which the inositol released by the action of phytase becomes incorporated into the protein body or vacuolar membrane lipids. It was suggested that following germination, there may be a turnover of the protein body membrane within the cells. As the reserve proteins are mobilized, protein bodies fuse to form the large central vacuole common to the parenchyma cells.

1.1.2. The effect of phosphorus nutrition on phytic acid content in seeds

Phytic acid and P concentrations in seeds of a given species may vary because of many factors, including cultivar, soil fertility status and climatic factors (HORVATIC & BALINT, 1996; MILLER *et al.*, 1980; RABOY *et al.*, 1991).

Phosphorus metabolism in developing soybean seeds is characterized by two distinct phases (RABOY & DICKINSON, 1987). The period of cell division and growth during the first three weeks after pollination is devoted to the synthesis of the P compounds needed for the synthesis of nucleic acids, membrane phospholipids, etc., and only a trace of reserve P (phytic acid) is found. Then phytic acid accumulation begins while the accumulation rate of the other P compounds declines to negligible amounts during a brief transition period. Thereafter, a steady rate of phytic acid accumulation is maintained until very late in seed maturation (RABOY & DICKSON, 1987).

An experiment by RABOY & DICKSON (1984), indicated that the phytic acid level, and to a lesser Pi, of mature soybean seeds were very responsive to altered concentrations of nutrient P, but there was little or no change in seed protein, Zn, or the other major P-containing fractions. Phytic acid levels varied over a 4-fold range as a result of the different nutrient P treatments, and at a standard level of nutrient Zn (0.05 mg l^{-1}). These two variables (P and Zn) displayed a linear relationship between 2 and 50 mg nutrient Pl^{-1}. The increase in seed Pi that accompanied high nutrient P may have been related to the observed increase in phytic acid accumulation.

Furthermore, RABOY & DICKSON (1987) discovered that the rate of phytic acid accumulation was linear with time in seeds developing on plants grown with varying levels of nutrient P. The rate increased with each increase of P in the nutrient solution. Furthermore, the duration of phytic acid accumulation was shortened as nutrient P was increased above 10 mg L^{-1}. This reduction was apparently due to rapid seed maturation as evidenced by an earlier cessation of growth.

1.1.3. Physiological roles of phytin

Results from energy dispersive X-ray spectroscopy (EDS) of unfixed, freeze-dried sections of rice tissue demonstrated that Mg^{2+} and K^{+} were mobilized from starchy endosperm to the aleurone layer, the site of phytin accumulation. In combination with chemical analysis, which demonstrated that the available cations of Mg^{2+} and K^{+} balanced the negative charge of phytic acid P. These data strongly suggested that phytin is indeed the principal storage form of Mg^{2+} and K^{+} in the rice grain (GREENWOOD, 1989; LIU *et al.*, 2004).

Following germination, phytin is digested by the action of phytase, a phosphatase. The released phosphate, cations, and inositol are then mobilized to the growing portions of the seedling to be used in support of anabolic metabolism (LOTT *et al.*, 1995). Another role for phytin in seeds may be in the control of inorganic phosphate homeostasis in both developing seeds and seedlings (STROTHER, 1980; RABOY, 1997). Although studies of this nature are limited, it appears that the level of inorganic phosphate remains relatively constant, either on a per gram fresh mass basis in seedlings, where the fresh mass: dry mass ratio would remain relatively constant, or on a per gram water content basis in developing seeds, where the fresh mass: dry mass ratio declines dramatically. The constancy in inorganic phosphate concentration is maintained *via* a carefully controlled mobilization or synthesis, respectively, of phytin. Similarly, the availability of phosphorus to the maternal plant directly influences both the amount of phytin synthesized in the developing seed and the number of seeds produced (RABOY & DICKINSON, 1987).

Accumulation of cyclic polyols such as myo-inositol and its methylated derivatives has been correlated with tolerance to drought and salinity (LOEWUS & DICKSON, 1982). Polyols seem to function in two ways that are difficult to separate: osmotic adjustment and osmoprotection. In osmotic adjustment, they act as osmolytes, facilitating the retention of water in the cytoplasm and allowing sodium sequestration to the vacuole or apoplast (BOHNERT *et al.*, 1995). Those polyols that are non-reducing sugars may also store excess carbon under environmental stress conditions (PAUL & COCKBURN, 1989). The importance of altered metabolism under abiotic stress, for example, the division of carbon to polyol biosynthesis, is exemplified by the metabolic reactions originating from the glucose-6-phosphate pool. Myo-inositol, polyols, and their metabolism outline the inositol biosynthetic pathway catalyzed by inositol-1-phoshate synthase (INO1) and inositol monophosphatase as well as pathways that originate from inositol and inositol-1-phosphate. There are several interesting aspects of the inositol-1-phosphates (LOEWUS & DICKINSON, 1982). Inositol and inositol-1-phosphate also stimulate the production of other compounds that have been correlated with stress tolerance, (e.g., gums, cell wall-located carbohydrates, carbohydrates in glycoproteins, and mucilages). Plants use inositol to synthesize vegetative storage carbohydrates such as stachyose and verbascose, which are stress-induced in some species. Yet another product of this pathway is phytate, inositol-hexakisphosphate, which serves as a storage form of phosphate in seeds (BOHNERT *et al.*, 1995).

1.1.4. Determination of phytate and phosphorus

Phytate can be extracted and measured in several ways including ferric precipitation, paper chromatography, thin layer chromatography, gel chromatography, gas liquid chromatography, ion exchange chromatography, HPLC and P-NMR (COSGROVE, 1980). The last two methods listed are very useful for separating the numerous isomers of inositol phosphates (IP) (LOTT *et al.*, 2000). Some of these procedures cannot distinguish phytic acid (IP6) from other inositol phosphates such as IP4 and IP5. Thus, total phytate analysis that includes inositol phosphates with varying degrees of phosphorylation, can give misleading information with regard to mineral availability in animals, since only IP5 and IP6 effectively inhibit Ca and Zn uptake in animals (LONNERDAL *et al.*, 1989). In mature seeds, the myo-inositol phosphates are almost exclusively in the IP6 form.

One way in which mineral nutrient reserves have been studied extensively is with energy dispersive X-ray (EDX) analysis. With this procedure an electron beam is placed on a localized area of a specimen, for example a globoid, and the elements present are determined. It is important to note that the actual compounds involved in the mineral storage cannot be determined by EDX analysis. Various procedures have been developed to retain phytate in place such as cryogenic preparation, freeze-dried tissue powders, and low-water-content (LOTT *et al.*, 1984). Such studies have shown that globoids generally have considerable P, Mg, and K, and on occasion other elements such as Ca, Mn, Zn, Ba and Fe. It is likely that these elements occur as salts of phytic acid (RABOY, 2002).

1.1.5. Raffinose family oligosaccharides as a stress-induced vegetative storage carbohydrates

The cofactor role of myo-inositol in the biosynthesis of a higher raffinose family of oligosaccharides has long been established (PETERBAUER & RITCHER, 2001). The concentration of myo-inositol substrate for the synthesis of galactinol could also affect the accumulation of the raffinose family of oligosaccharides in seeds. The reduction of the content of myo-inositol in tubers of transgenic potato (*Solanum tuberosum*.L) resulted in strongly reduced levels of galactinol and raffinose (KELLER *et al.*, 1998).

The raffinose family of oligosaccharides (RFO) has multiple functions in plants. They serve as transport carbohydrates in the phloem and as storage reserves and cryoprotectants in frost-hardy plant organs (AYRE *et al.*, 2003; SPRENGER & KELLER, 2000; PENNYCOOKE *et al.*, 2003). Raffinose family oligosaccharides accumulate in maturing seeds, where they may play a role in the acquisition of desiccation tolerance and storability (HORBOWICZ & OBENDORF, 1994). The biosynthesis of raffinose proceeds by the reversible addition of galactose units from galactinol (0-α-D-galacto pyranosyl – (1-1)-L-Myo-inositol) to sucrose. Chain elongation is catalysed by the consecutive action of raffinose synthase and stachyose synthase (PETERBAUER *et al.*, 2001). Some stachyose synthases are able to add two galactose units to raffinose, yielding the pentasaccharide verbascose (PETERBAUER *et al.*, 2003).

Physiologically, RFO's in seeds may be regarded as storage carbohydrates. They make up as much as 16.2% of dry mass in soybeans (MUZQUIZ *et al.*, 1999), but more typically are in the range of 2-10% (HORBOWICZ & OBENDORF, 1994). Raffinose family oligosaccharides are broken down during the early stages of germination and thus provide readily available energy and substrates to support growth. These oligosaccharide solutions may promote the formation of a vitreous (glassy) state that protects macromolecular structures during desiccation (LEOPOLD *et al.*, 1994). Galactosyl cyclitols and RFOs accumulate late in seed development, starting at about the beginning of seed fill and continuing into maturation drying. They are deposited in all parts of the seed; including the endosperm, embryo and the seed coat, although the levels of individual α-galactosides may vary considerably in these tissues (HORBOWICZ & OBENDORF, 1994). Raffinose family of oligosaccharides is known to increase during cold acclimation in vegetative tissues (BACHMANN & KELLER, 1995). In cucumber, lupin and soybean seeds the maturation temperature had little effect on the concentration of RFOs (OBENDORF *et al.*, 1998). An exception seems to be buckwheat, which contained three times as much stachyose in the seeds grown at 18°C compared with seeds grown at 25°C (HORBOWICZ *et al.*, 1998). In contrast, pinitol and the respective galactopinitols, remained more or less unchanged by high maturation temperature in soybean (OBENDORF *et al.*, 1998), but were more than doubled in lupin (GORECKI *et al.*, 1996).

1.1.5.1. Metabolism of raffinose family of oligosaccharide during germination

Upon imbibition, RFOs disappear rapidly in soybeans and their degradation may be almost completed before radicle protrusion (MODI *et al.*, 2000). Yet, the germination of seeds containing no or little RFOs does not differ from that of seeds containing high RFO levels (BLACKMAN *et al.*, 1992). Thus, it seems that RFOs are not an indispensable storage reserve for germination. Particularly seeds such as fenugreek (*Trigonella foenum-graecum*) and guar (*Ceratonia siliqua*); contain RFOs as well as high levels of galactomannan. In these plants, α-galactosidases activity, although already present in dormant seeds, increases during germination and the first days of seedling growth by *de novo* synthesis (BUCKERIDGE & DIETRICH, 1996). However, a correlation between RFO breakdown and α-galactosidases was only observed in the endosperm, which contains both the RFOs and galactomannan, and not in the embryo, which is devoid of the latter (SEILER, 1977). These results imply that the α-galactosidases synthesized are involved in the degradation of galactomannan, while pre-existing α-galactosidases appear to be responsible for RFO hydrolysis. Indeed, the suppression of α-galactosidase synthesis by cycloheximide inhibited the breakdown of galactomannan, but the breakdown of RFOs was almost unaffected (SEILER, 1977). The mechanism triggering the breakdown of RFOs is unknown. As already discussed above, acidification of protein storage vacuoles could activate α-galactosidases within these organelles (SWANSON *et al.*, 1998). Alternatively, release of α-galactosidases from protein storage vacuoles could be responsible for the breakdown of the bulk of RFOs located in the cytoplasm (SEKHAR & DEMASON, 1990).

1.2. Research proposal

1.2.1. Justification

In the context of evidence that myo-inositol is an important nutrient for plant tissues, the existing question of its physiological function takes on renewed significance. Based on previous discussions, plant cells must have inositol to build into phospholipid molecules, and to use these molecules in the synthesis of cell wall polysaccharides. Inositol also serves as a phosphate acceptor in phytate synthesis. It is known that both the phospholipids and phytate are actively involved in the regulation and catalysis of cellular events. Future research should disclose the details of these involvements and perhaps additional roles of inositol as such, or in the form of other derivatives.

Due to a fundamental lack of observational and experimental studies into the cellular mechanisms of phytic acid accumulation, there have been no unequivocal demonstrations of how phytic acid is deposited into protein bodies. One suggestion is that the protein bodies are the sites of phytic acid biosynthesis (TANAKA *et al.*, 1976), and thus the mechanism of deposition would be self-evident. The study of TANAKA *et al.* (1976) followed the incorporation of labeled myo-inositol and ATP into myo-inositol phosphates in an incubation system employing isolated protein bodies. Although labeled phytic acid was obtained, the purity of the protein body isolated was neither established nor maintained. The main criticism of this study is that only labeled myo-inositol-1-phosphate, not labeled phytic acid, was obtained (LOTT *et al.*, 1995). Phytic acid can be quantified *via* indirect methods, such as HPLC, which identifies the inorganic phosphorus; and the EDX analysis, which is based on identifying phosphorus in the globoids.

The interaction of phosphorus nutrition and environmental factors, with respect to phytate accumulation in seeds, has been explored (RABOY & DICKSON, 1993). Temperature is generally known for its correlation with plant development and it effect on accumulation of reserves in seed tissues (FAGERIA, 1992). Hence, it is expected that myo-inositol accumulation will be influenced by temperature during seed development and maturation. Phosphorus nutrition is expected to enhance soluble carbohydrate, and hence myo-inositol accumulation. Previous studies have shown that low temperature stress during seed development hastens the formation of phytic acid (HORVATIC & BALINT, 1996), as well as the increase in supply of inorganic phosphorus (RABOY & DICKINSON, 1987). However, the effect of temperature stress interaction with phosphorus nutrition in phytic acid accumulation during seed development stages has not been reported. Furthermore, the effect of temperature stress and phosphorus nutrition on soluble carbohydrate with respect to seed quality has not been investigated.

1.2.2 Hypothesis and objective

This study tested the hypothesis that seed quality as determined by vigour is not affected by phytic acid levels. Furthermore, phosphorus nutrition and temperature stress do not impact on myo-inositol, other soluble carbohydrates and phytate content of maize caryopses during seed development. The objective of this study was designed to examine the effect of phosphorus nutrition and controlled environment temperature on phytate, soluble carbohydrate and germination of normal maize and quality protein maize (QPM) cultivars.

References

AYRE, B.G., KELLER, F., & TURGEON, R. 2003. Symplastic continuity between companion cells and the translocation stream. Long-distance transport is controlled by retention and retrieval mechanisms in the phloem. *Plant Physiology* 131: 1518–1528.

BACHMANN, M., & KELLER, F. 1995. Metabolism of the raffinose family oligosaccharides in leaves of Ajuga reptans L. Cold acclimation, translocation, and sink to source transition: Discovery of a chain elongation enzyme. *Plant Physiology* 105: 1335-1345.

BEECROFT, P., & LOTT, J.N.A. 1996. Changes in the element composition of globoids from *Cucurbita maxima* and *Cucurbita andreana* cotyledons during early seedling growth. *Canadian Journal of Botany* 74: 838-847.

BLACKMAN, S.A., OBENDORF, R.L., & LEOPOLD, A.C. 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. *Plant Physiology* 100: 225-230.

BOHNERT, J.H., NELSON, D.E., & JENSEN, R.G. 1995. Adaptations to environmental stresses. *Plant Cell* 7: 1099-1111.

BUCKERIDGE, M.S., & DIETRICH, S.M.C. 1996. Mobilization of the raffinose family oligosaccharides and galactomannan in germinating seeds of sesbania marginata Bent. (*Leguminosae-Faboideae*). *Plant Science* 117: 33-43.

COELHO, C.M.M., SANTOS, J.C.P., TSAI, S.M., &. VITORELLO, V.A. 2002. Seed phytate content and phosphorus uptake and distribution in dry bean genotypes. *Brazilian Journal of Plant Physiology* 14: 51-58.

COSGROVE, D.J. 1966. The chemistry and biochemistry of inositol polyphosphates. *Review of Pure and Applied Chemistry* 16: 209-224.

COSGROVE, D.J. 1980. Chromatography and analytical methods. *In* Inositol phosphates: their chemistry, biochemistry and physiology (Cosgrove, D.J., ed.); pp 12-25. Elsevier, Amesterdam.

FAGERIA, N.B. 1992. Maximizing crop yields. New York, Marcel Dekker, Inc.

GORECKI, R.J., BRENAC, P., CLAPHAM, W.M., WILLCOTT, J.B., & OBENDORF, R.L. 1996. Soluble carbohydrates in white lupin (*Lupinus albus* L. cv. Ultra) seeds matured at 13^0 and 28 ^{0}C. *Crop Science* 36: 1277-1282.

GRAF, E. 1986. Phytic acid chemistry and applications. Pilatus Press, Minneapolis.

GREENWOOD, J.S., & BEWLEY, J.D. 1984. Subcellular distribution of phytin in the endosperm of developing castor bean: a possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. *Planta* 160: 113-120.

GREENWOOD, J.S. 1989. Phytin synthesis and deposition: Recent advances in the development and germination of seeds. Plenum Press, New York.

HEGEMAN, C.E., GOOD, L.L., & GRABAU, E.A. 2001. Expression of D-myo-inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. *Plant Physiology* 125: 1941-1948.

HORBOWICZ, M., & OBENDORF, R.L. 1994. Seed desiccation tolerance and storability: dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. *Seed Science Research* 4: 385–405.

HORBOWICZ, M., OBENDORF, R.L., & OBENDORF, R.L. 1998. Fagopyritol B1, O-α-D-galactopyranosyl-(1-2)-D-chiro-inositol, a galactosyl cyclitol in maturing buckwheat seeds associated with desiccation tolerance. *Planta* 205: 1-11.

HORVATIC, M., & BALINT, L. 1996. Relationship among the phytic acid and protein content during maize grain maturation. *Journal of Agronomy and Crop Science* 176: 73-77.

KELLER, R., BREARLEY, C.A., TRETHEWEY, R.N., & MULLER-ROBER, B. 1998. Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-myo-inositol 3-phosphate synthase. *Plant Journal* 16: 403-410.

LEOPOLD, A.C., SUN, W.Q., & BERNAL-LUGO., I. 1994. The glassy state in seeds: analysis and function. *Seed Science Research* 4: 267-274.

LIN, L., OCKENDEN, I., & LOTT, J.N.A. 2005. The concentration and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid 1-1 (lpa 1-1) corn (*Zea mays* L.) grain and grain parts. *Canadian Journal of Botany* 83: 131-141.

LIU, J.C., OCKENDEN, I., TRUAX, M., & LOTT., J.N.A. 2004. Phytic acid-phosphorus and other nutritionally important mineral nutrient elements in grains of wild-type and low phytic acid (lpa 1-1) rice. *Seed Science Research* 14: 109-116.

LOEWUS, F.A., & DICKINSON, D.B. 1982. Cyclitols. *In*: Encyclopedia of Plant Physiology, New Series, Vol. 13 A, Plant carbohydrates I, (Loewus, F.A., Tammer, W., eds); pp. 193-216. Heidelberg, Springer.

LOEWUS, F.A., & LOEWUS, M.W. 1983. Myo-inositol its biosynthesis and metabolism. *Annual Review of Plant Physiology* 34: 137-161.

LOEWUS, F.A. 1990. Structure and occurrence of inositols in plants. (Morre, D.J., Boss, W.F & Loewus, F.A, eds); pp. 1-11. *In* Inositol metabolism in plants. New York, Wiley-Liss.

LOEWUS, F.A., & MURTHY, P.P.N. 2000. Myo-inositol metabolism in plants. *Plant Science* 150: 1-19.

LONNERDAL, B., SANDBERG, A.S., SANDSTROM, B., & KUNZ, C. 1989. Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *Journal of Nutrition* 119: 211-214.

LOTT, J.N.A. 1980. Protein bodies. *In*: Biochemistry of Plants, Volume1. The Plant Cell. (Tolbert, N.E., ed); pp. 589-623. New York press, Academic.

LOTT, J.N.A. 1984. Accumulation of seed reserves of phosphorus and other minerals. *In* Seed Physiology, volume1. (Murray, D.R., ed); pp. 139-166. Sydney Press, Academic.

LOTT, J.N.A., GOODCHILD, D.J., & CRAIG, S. 1984. Studies of mineral reserves in pea (*Pisum sativum*) cotyledons using low-water-content procedures. *Australian Journal of Plant Physiology* 69: 829-833.

LOTT, J.N.A., GREENWOOD, J.S., & BATTEN, G.D. 1995. Mechanisms and regulation of mineral nutrient storage during seed development. *In*: seed development and germination. (Kigel, J & Galili, G., eds) pp. 215-235. New York, Marcel Dekker,inc.

LOTT, J.N.A., OCKENDEN, I., RABOY, V., & BATTEN, G.D. 2000. Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research* 10: 11-33.

LOTT, J.N.A., & WEST, M.M. 2001. Elements present in mineral nutrient reserves in dry *Arabidopsis thaliana* seeds of wild type and pho1, pho2, and man1 mutants. *Canadian Journal of Botany* 79: 1292–1296.

MILLER, G.A., YOUNGS, V.L., & OPLINGER, E.S. 1980. Effect of available soil phosphorus and environment on the phytic acid concentrations in oats. *Cereal Chemistry* 57: 192-194.

MODI, A.T., MCDONALD, M.B., & STREETER, J.G. 2000. Soluble carbohydrates in soybean seeds during development and imbibition. *Seed Science and Technology* 28: 115-127.

MUZQUIZ, M., BURBANO, C., PEDROSA, M.M., FOLKMAN, W. & GULEWICZ, K. 1999. Lupins as a potential source of raffinose family oligasaccharides-preparative method for their isolation and purification. *Industrial Crops and Products* 9: 183-188.

OBENDORF, R.L., HORBOWICZ, M., DICKERMAN, A.M., BRENAC, P., & SMITH, M.E. 1998. Soluble oligosaccharides and galactosyl cyclitols in maturing soybean seeds in planta and in vitro. *Crop Science 3*8: 78-84.

O'DELL, B.L., DE BOLAND, A.R., & KOIRTYOHANN, S.R. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. *Journal of Agricultural and Food Chemistry* 20: 718-721.

OGAWA, M., TANAKA, K., & KASAI, Z. 1979. Energy-dispersive x-ray analysis of phytin globoids in aleurone particles of developing rice grains. *Soil Science and Plant Nutrition* 25: 437-448.

PAUL, M.J., & COCKBURN, W. 1989. Pinitol, a compatible solute in *mesembryanthemum crystallinum L. Journal of Experimental Botany* 40: 1093-1098.

PENNYCOOKE, J.C., JONES, M.L., STUSHNOFF C. 2003. Down-regulating-α galactosidase enhances freezing tolerance in transgenic petunia. *Plant Physiology* 133: 901–909.

PETERBAUER, T., LAHUTA, L.B., BLÖCHL, A., MUCHA, J., JONES, D.A., HEDLEY, C.L., GÒRECKI, R.J., RICHTER, A. 2001. Analysis of the raffinose family oligosaccharide pathway in pea seeds with contrasting carbohydrate composition. *Plant Physiology* 127: 1764–1772.

PETERBAUER, T., KARNER, U., MUCHA, J., MACH, L., JONES, D.A., HEDLEY, C.L., RICHTER, A. 2003. Enzymatic control of the accumulation of verbascose in pea seeds. *Plant, Cell and Environment* 26: 1385–1391.

PITT, M.W., & LOTT, J.N.A. 1996. Large globoids particles in the cotyledons of Cucurbita maxima seedlings. *Canadian Journal of Botany* 74: 1186-1189.

RABOY, V., & DICKINSON, D.B. 1984. Effect of phosphorus and zinc nutrition on soybean seed phytic acid and zinc. *Plant Physiology* 75: 1094-1098.

RABOY, V., & DICKINSON, D.B. 1987. The timing and rate of phytic acid accumulation in developing soybean seeds. *Plant Physiology* 85: 841-844.

RABOY, V., NOAMAN, M.M., TAYLOR, G.A., & PICKETT, S.G. 1991. Grain phytic acid and protein are highly correlated in winter wheat. *Crop Science* 31: 631-635.

RABOY, V., & DICKINSON, D.B. 1993. Phytic acid levels in seeds of *Glycine max* and *Glycine soja* as influenced by phosphorus status. *Crop Science*. 33: 1300-1305.

RABOY, V. 1997. Accumulation and storage of phosphate and minerals. *In*: cellular and molecular biology of plant seed development. (Larkins, B.A & Vasil, I.K, eds); pp. 441-477. Kluwer Academic, Netherlands.

RABOY, V. 2001. Seeds for a better future: low phytate grains help to overcome malnutrition and reduce pollution. *Trends in Plant Science* 10: 458-462.

RABOY, V. 2002. Progress in breeding low phytate crops. *Journal of Nutrition* 132: 503-505.

SEILER, A. 1977. Galaktomannanabbau in keimenden Johannisbrotsamen (*Ceratonia siliqua* L.). *Planta* 134: 209-221.

SEKHAR, K.N.C., & DEMASON, D.A. 1990. Identification and immunocytochemical localization of α-galactosidase in resting and germinated date palm (*Phoenix dactylifera* L.) seeds. *Planta* 181: 53-61.

SIMON, R., ALTSCHULER, Y., RUBIN, R., & GALILI, G. 1990. Two closely related wheat storage proteins follow a markedly different subcellular route in *Xenopus laevis oocytes*. *Plant cell* 2: 941-950.

SPRENGER, N., KELLER, F. 2000. Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: the roles of two distinct galactinol synthases. *The Plant Journal* 21: 249–258.

STROTHER, S. 1980. Homeostasis in germinating seeds. *Annals of Botany* 45: 217-218.

SWANSON, S.J., BETHKE, P.C., & JONES, R.L. 1998. Barley aleurone cells contain two types of vacuoles: characterization of lytic organelles by use of fluorescent probes. *Plant Cell* 10: 685-698.

TANAKA, K., OGAWA, M., & KASAI, J. 1976. The rice scutellum: studies by scanning electron microscopy and electron microprobe x-ray analysis. *Cereal Chemistry* 53: 643-649.

VERNON, D.M ., & BOHNERT, H.J. 1992. Increased Expression of an Inositol Methyl Transferase in *M. crystallinum* is Part of a Stress Response Distinct from CAM Induction. *Plant Physiology* 99: 1695-1698.

CHAPTER 2 MATERIALS AND METHODS

2.1. Seed quality assessment

Ten white normal maize cultivars designated A, B, C, D, E, F, G, I, J, N and two white quality protein (QPM) cultivars designated Q1 and Q2 were obtained, respectively, from Pannar Seed Co. (Greytown, KwaZulu-Natal, South Africa) and Quality Seed cc (Pietermaritzburg, KwaZulu-Natal, South Africa). The cultivars were screened to determine their quality and phytate levels as explained under phytate determination (Paragraph 2.3). Conductivity test, Cold germination test and electron microscopy (ESEM-EDAX and TEM) were used to determine seed quality.

2.1.1. Conductivity test

Three replications of 50 seeds per cultivar placed in a 500ml conical flask were imbibed in 250ml of aerated distilled water for 24 hours at room temperature (ISTA, 1995). The contents of each flask were gently stirred and electrical conductivity was determined using the YSI 32200 (Yellow Springs, Ohio, USA) conductivity metre.

2.1.2. Cold germination test

The paper towel germination test was used (ISTA, 1995). One hundred (100) seeds were placed equidistantly on moistened paper towel. A non-sterile soil was placed on the seeds; the soil was moistened with tap water to meet the germination requirement. After covering the paper were placed in trays in a cold room at 4°C for five days. At the end of five days they were placed in a germination chamber at 25°C in the presence of light as prescribed by ISTA (1995). Germination was recorded at the end of 7 days.

2.2. Electron microscopy

2.2.1. Transmission electron microscopic (TEM)

Primary fixation: The starchy endosperm and embryo were dissected from the maize seeds. The collected tissues were cut into $1mm^3$ pieces. The tissue pieces were fixed in glutaraldehyde for 24 h at 1°C. Half the tissue pieces were rinsed in 2% osmium tetroxide with pH 7.2 for 30 minutes and the other pieces were used as controls. The tissues placed in osmium tetroxide were then rinsed several times in 0.05M Na-cacodylate buffer.

Dehydration: The specimens were washed twice for 30 min each time with buffer and block-stained with freshly prepared 2% (m/v) uranyl acetate in 2% (v/v) ethanol for 45 min. The specimens were washed with double distilled water twice for 10 min and dehydrated as 10, 20, 30, 40, 50, 60, 70% (v/v) ethanol, for 10 min at each level. The samples were left overnight at 70% (v/v). The following day, the samples were dehydrated through 80, 90, 100% (v/v) ethanol at 10 min intervals between each step. The dehydration series was completed with two rinses of 15 minutes each in absolute alcohol. The Epon Araldite mixture is not miscible with alcohol and, therefore, from the 100 % alcohol one proceeds to the solvent propylene oxide in two 30 min changes.

Embedding and polymerization: Two washes in propylene oxide each of 30 minutes were performed. In order to ensure adequate infiltration of the material with the embedding mixture the tissues were placed into:

(a) 3 parts propylene oxide: 1 part Epon-Araldite for 2 hour.
(b) 2 parts propylene oxide: 2 parts Epon-Araldite for 2 hour.
(c) 1 part propylene oxide: 3 parts Epon-Araldite for 8 hours, or overnight caps off.
(d) Pure Epon-Araldite for 24 hours caps on.

The tissues were finally placed in appropriately labelled moulds, and polymerized for 48 hours at 70°C. The oven had a desiccant. After removal of the tissues from the oven they were allowed to cool at room temperature before attempting to trim or section them. Selected tissue regions were sectioned in the 150-170nm range. Trimming was done using the LKB ULTRAMICROTOME III (Stockholm, Sweden) with a knife clearance angle of 5° to produce gold sections (60-80nm thick) using a diamond knife (6° Micro Star Diamond Knife) and collected on formvar coated 200 mesh copper grids.

Staining: Sections were stained in 2% (m/v) uranyl acetate for 10 minutes rinsed in distilled water and placed in 2% 1 (m/v) lead citrate for 10 minutes with sodium hydroxide pellets in staining dish to absorb carbon dioxide. The grids were then rinsed in distilled water. The tissues were viewed on CM 120 Biotwin (Phillips, Eindhoven, Holland) transmission electron microscope with an accelerating voltage of 80 KV.

2.2.2. Environmental scanning electron microscopy (ESEM)

Energy-dispersive x-ray analysis using environmental scanning electron microscopy operating at 25kV with a pump detection of 500µm, pressure of 1 to 2torr, wet mode, purge custom, spot size 6, room temperature (approximately 20°C), working distance of 50µm and pressure of 10mm. A gaseous secondary detector was used to determine mineral element storage within the embryo and endosperm in maize caryopses (seeds). The Virtis bench top freeze drier (Benchtop 2k, 4k, 6k, Gardiner, New York) was used to freeze-dry the material. For the three caropses from each cultivar, three ESEM EDAX observations were done on randomly selected areas, which were kept constant for each treatment. Peak to background (P/B) ratios for Ca, K, Mg, and P were calculated for the spectra of each type, using the window width. Each specimen was mounted on a carbon planchette using carbon tape and viewed on a XL30 ESEM (Phillips, Eindhoven, Holland) (LIN *et al.*, 2005). Means and standard deviations were calculated. To determine whether element means differed, analysis of variance test was used at 5% level of significance (GenStat 7th edition).

2.3. Phytate analysis

For each tissue (embryo and endosperm) dry, a ground sample (0.5g) was extracted in 20ml of 0.4 M HCl (10% Sodium sulphate) with stir bars; the tube was covered with parafilm and set in a cooler to be stirred overnight (CHEN *et al.*, 1956).

Ferric Precipitations: The tubes were placed in centrifuge at 10000rpm for 20 minutes. The supernatants were filtered through filter paper and funneled into labeled tubes. From each sample, 10ml were pipetted into labeled glass corex tubes; 10ml of distilled water was added to each tube; 5ml of 15 mM Fec_3 (0.2 M HCl 5% sodium sulphate) was added to each tube. The tubes were placed in 90°C steam water bath for 30 minutes and placed later on ice water bath for 30 minutes. The tubes were placed for centrifuge at 7000rpm for 15 minutes. The liquid was poured off and 10 ml of 0.2 M HCl were added to each pellet sample tube. The tubes were vortexed until the pellets were suspended in liquid. The tubes were centrifuged at 7000rpm for 15 minutes. The liquid was poured off and the pellets were allowed to drain and covered with parafilm until time to do digestions.
Digests: Sulphuric acid (2ml) was added to each pellet sample and the tubes were placed on the 250°C heated block for 30 minutes. Four to 6 drops of 30% Hydrogen peroxide was added to each tube. This was repeated every 30 minutes until the samples were clear. The tubes were heated for one hour and then they were left under the hood to cool.
Colorimetric assay: Distilled water was added to each digested sample to bring the level up to 12.5ml. One tube was vortexed and then 100μl was pipetted from that tube three times. The samples were placed in three smaller glass tubes. Distilled water (3.9ml) was added to each triplicate sample to bring the level up to 4ml. The same was done for each tube until all the samples were placed into triplicate tubes. Five standards 1.39, 0.93, 0.465 and $0.155 \mu g.p^{-1}$ using 100μl of a blank digest as the sample was made and then 3.9 ml of distilled water were added to bring the level up to 4 ml. Each sample was vortexed and 4ml of Chen's reagent (APPENDIX 2.1) was added to each triplicate sample and the standard. The spectrophotometer (Anthelie Domont Cedex, France) at 820nm was used to read the standards and the samples.

2.4. Determination of effects of phosphorus and temperature on the seed quality

Following ESEM-EDAX and phytate analysis, two cultivars (Q1 and N), designated, respectively, as low and high phytate, were selected from the 12 cultivars were planted in a tunnel according to soil analysis, the soil pH was 5.13 and phosphorus content was $10 mg.l^{-1}$. A total of 10 seeds were planted per pot and thinned to three after emergence. Phosphorus was applied at the following rates in 20 kg of soil per pot: Low (0 = 0g) medium (P = 0.12g) and high (PP = 1.2g). Growth parameters such as emergence rate, plant height and leaf emergence rate were determined in the tunnel. Ten days after emergence, seedlings were transferred to three separate glasshouses, with the following temperatures: Low (L = 22/16°C day/night), medium (M = 27/21°C) and high (H= 33/27°C). The experiment was replicated three times, so that there were 18 pots per glasshouse. Thus, the experimental design was a factorial with 27 pots per cultivar, three temperatures and three phosphorus levels.

The environment conditions in the glasshouses during plant growth are shown in (APPENDIX 2.2). Watering (1 litre per pot) was done once a week but it was increased to every third day after the first month. Plant height and leaf emergence were monitored daily for a week and then at 3 day intervals until flowering. Tasseling, silking and anthesis were monitored. Self-pollination of each plant was performed to ensure purity of the new generation of seeds. Seeds were harvested at different development stages starting with milk stage (15 days after pollination), dough stage (22 days after pollination) and harvest maturity (33 days after pollination).

A germination test was performed according to ISTA rules ((ISTA, 1995) on the harvested seeds to assess seed quality, with seed lots germinated on two filter papers placed on a Labcon germinator set at alternating temperatures of 20/30°C, 16/8 h. Seeds with radicle protrusion were counted daily for 10 days.

The harvested seeds were analysed for mineral elements using ESEM-EDAX. The concentration of galactose, glucose, fructose, myo-inositol, raffinose, pinitol, verbascose, stachyose and sucrose in harvested seed were determined using Gas Liquid

Chromatography (GC) as described under (Paragraph 2.5). All data were analysed using general analysis of variance (GenStat Release 7.0 Rothamsted Experimental Station, UK).

2.5. Sugar analysis using gas liquid chromatography

2.5.1. Sugar standards preparation

Glucose, galactose, fructose, myo-inositol, pinnitol, raffinose, stachyose, sucrose and verbascose were purchased from Sigma. Sugar standards (5 mg) were dissolved in 5 ml of ethanol. The standards were dried overnight in Savant Speed Vacuum SC 200. The pellets were dissolved in 6ml of 2:1 sterilized dd. H_2O: Chloroform mixture and samples allowed to clear at 4°C for at least 48 h (MODI *et al.*, 2000). Aliquots (200µl) from the clear aqueous layer were evaporated in silylation vials under vacuum. Then 125µl pure o pyridine and 125µl stox reagent, which contains 5mg phenyl beta-D glucopyranoside, 200µl hexamethyldizisane and 20µl of tetrafluoric acid were added to the dry tissue. The reaction proceeded at room temperature for 60 minutes. Then 1µl of analyte was injected in a gas chromatograph (Varian 3800). The column used was DB-5, with a capillary column 30m long and a diameter of 0.25mm; detection was by flame ionization. The column was operated with a programmed initial temperature of 100°C with one minute hold time and increased at five degrees per minutes up to 220°C. From 220°C to 350°C an increase at $10°C.min^{-1}$ was allowed. The run time was 48 minutes in split mode. The injector port and the detector (flame ionization) were respectively at 310°C and 330°C with oven maximum temperature of 350°C (STREETER & STRIMBU, 1998). The carrier gas was N_2 with the flow rate of $1ml.min^{-1}$.

2.5.2. Gas chromatography sample preparation for sugar quantification

Soluble sugars were extracted according to BLACK *et al.* (1996) where 50mg of samples were ground at room temperature in a mortar and dissolved in 5ml of 80% ethanol. The extract was heated for 15 minutes at 80°C, and then centrifuged for 15 minutes at

11500rpm at room temperature in a Sorvall RC 5C centrifuge and the removal of supernatant. The pellet was resuspended and re-extracted in 5ml of ethanol and centrifuge again. The supernatants were combined and reduced to dryness in a Savant speed Vacuum SC 200. The pellets were dissolved in 6ml of 2:1 sterilized dd. H_2O: Chloroform mixture and samples allowed to clear at 4^oC for at least 48 h (MODI *et al.*, 2000). Aliquots (200µl) from the clear aqueous layer were evaporated in silylation vials under vacuum. Then, 125µl pure of pyridine and 125µl stox reagents, which contain 5mg phenyl beta-D glucopyranoside in pyridine, 200µl hexamethyldizisane and 20µl of tetrafluoric acid were added to the dry tissue. The samples were capped and heated at 60^oC for 20 minutes. Then, 1µl of the sample was injected in the GC with same program as the standards (Paragraph 2.5.1). The individual sugars were identified by co-chromatography with known standard sugars.

2.5.3. Calibration curve

The calibration curve was done using the peak area ratio of the standard and the internal standard by using the following sugar concentrations: 2.5, 1.25, 0.625, 0.3125, 0.15625 and $0\mu g.\mu l^{-1}$ with three replications each. The standard curve of all the sugars was found to be almost identical (r =1), thus the values were averaged and used to construct a standard curve to quantify unidentified sugar peaks.

2.5.4. Sugar recovery efficiency

The samples were spiked with all the sugar standards and the extraction and derivatization were done as mentioned above. The recovery was calculated based on the peak area ratio (spiked sugar standard to the internal standard) per slope of the various sugars multiplied by $2.5\mu g.\mu l^{-1}$.

References

BLACK, M., CORBINEAU, F., GRZESIK, M., GUY, P., & COME, D. 1996. Carbohydrate metabolism in the developing and maturing wheat embryo in relation to its desiccation tolerance. *Journal of Experimental Botany* 47:161-169.

CHEN, P.S., TORIBARA, T.Y., & WARNER, H. 1956. Microdeterminations of phosphorus. *Analytical Chemistry* 28:1756-1758.

ISTA.3rd ed, 1995. International rules for seed testing. Handbook of vigour test methods. (Hampton, J.G., & TeKrony, D.M., eds). ISTA vigour test committee, Zurich. Switzerland.

LIN, L., OCKENDEN, I., & LOTT, JNA. 2005. The concentrations and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid 1-1 (lpa 1-1) corn (*Zea mays* L.) grains and grain parts. *Canadian Journal of Botany* 83: 131-141.

MODI, A.T., MCDONALD, M.B., & STREETER, J.G. 2000. Soluble carbohydrates in soybean seeds during development and imbibition. *Seed Science and Technology* 28: 115-127.

STREETER, J.G., & STRIMBU, C.E. 1998. Simultaneous extraction and derivatization of carbohydrate from green plant tissues for analysis by gas-liquid chromatography. *Analytical Biochemistry* 259: 253-257.

CHAPTER 3 RESULTS AND DISCUSSION

3.1. Preliminary screening of maize cultivars

Normal maize cultivars showed significantly high levels of phyate compared with QPM cultivars (Figure 3.1). When phyate was determine in the endosperm and embryos, normal maize showed significant amounts of it in the normal maize endosperm, but there were no detectable levels of phyate in the QPM maize endosperm (Figure 3.1). The differences between normal maize and QPM maize has not been found in research before, but it is well known that 88 % of the grain phytic acid phosphorus (PA-P) is found in the embryo, with a significant amount of the remainder in the aleurone layer (O'DELL *et al.*, 1972; LIN *et al.*, 2005).

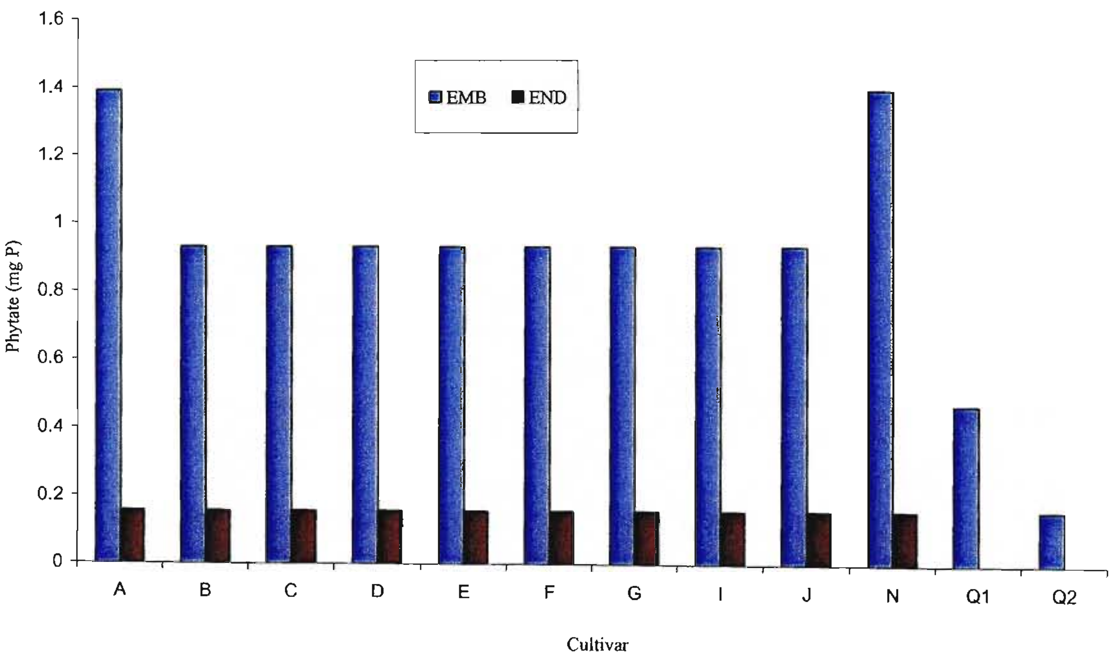


Figure 3.1. Phytate P content in embryo (emb) and endosperm (end) of normal maize cultivars (A, B, C, D, E, F, G, I, J, N) and quality protein cultivars (Q1 and Q2). Note: LSD ($P< 0.05$) = 0.04).

A further screening for mineral elements P, K and Mg, using ESEM-EDAX, showed that there were detectable levels of minerals in the endosperm, but there were significant amounts in the embryo (Figure 3.2), which concurs with previous studies (LIN *et al.*, 2005). Normal maize cultivars showed significantly higher (APPENDIX 3.1) levels of minerals than the quality protein maize (Figure 3.2). Phytic acid is the primary storage form of phosphorus and cations such as Mg and K in plant seeds and it represents 50% to over 80% of total P in mature seeds (LOTT *et al.*, 1984; RABOY, 1997). A low phytic content thus correlates with low phosphorus content in quality protein maize and is expected to be associated with low seed vigour.

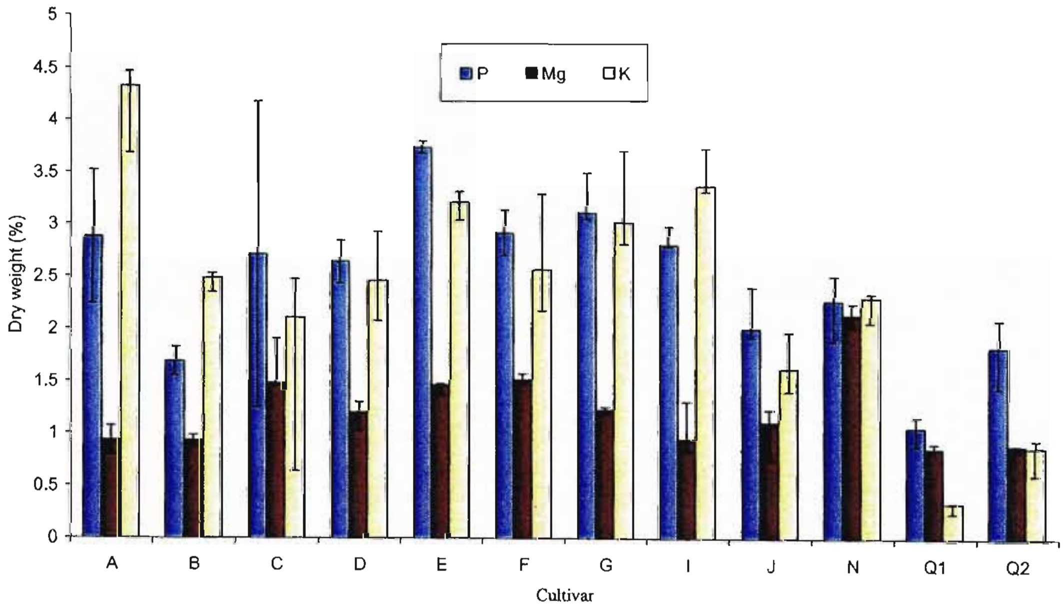


Figure 3.2. Mineral element content in embryo of normal maize (A, B, C, D, E, F, G, I, J, N) and quality protein maize (Q1 and Q2).

Indeed, a germination test on all the 12 cultivars showed that normal maize germinated significantly better (P< 0.001) than QPM maize (Figure 3.3). The poor germination was related to significant leakage of electrolytes from the QPM maize (Figure 3.4).

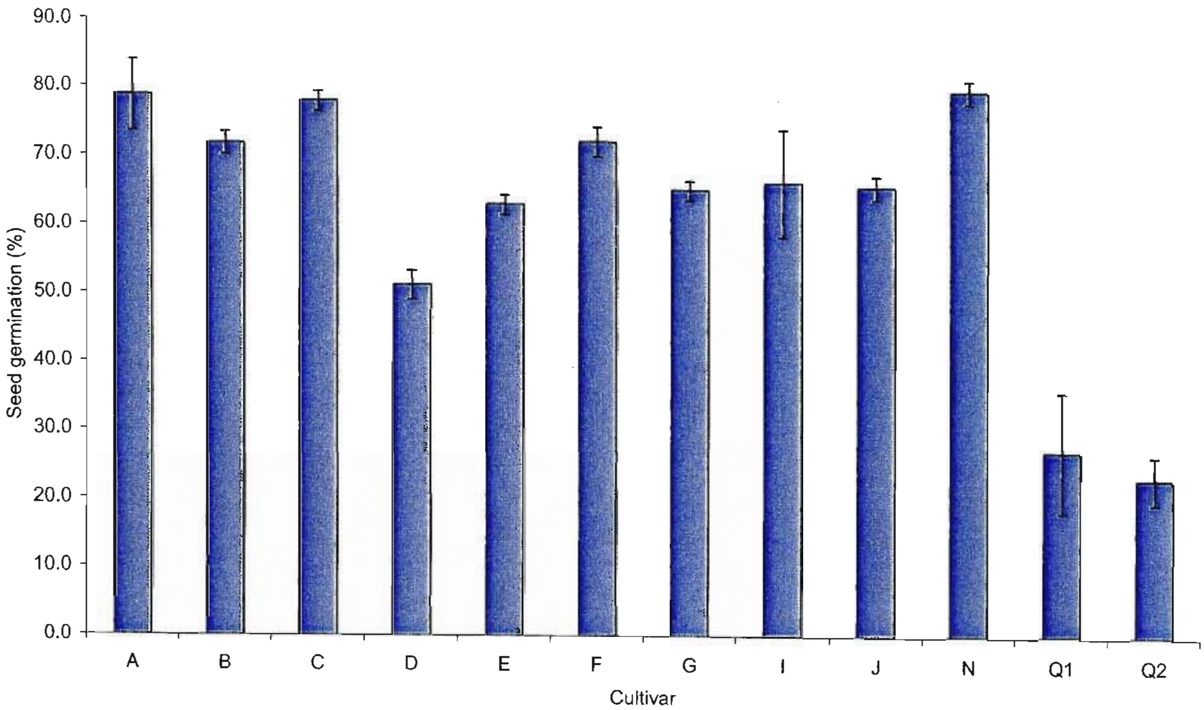


Figure 3.3. Seed germination of normal maize cultivars (A, B, C, D, E, F, G, I, J, N) cultivars and two quality protein maize (Q1 and Q2).

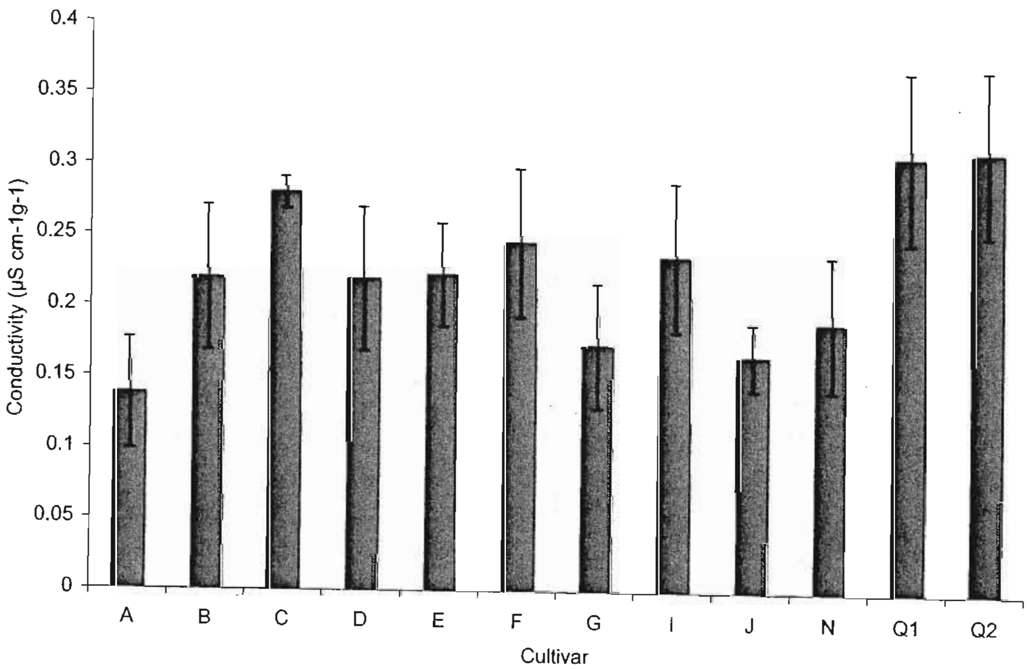


Figure 3.4. Leakage (EC) from normal maize (A, B, C, D, E, F, G, I, J, N) cultivars and two quality protein maize (Q1 and Q2).

That normal maize showed a significantly low level of leakage of substances compared with QPM maize (Q1 and Q2) (Figure 3.4). These results were also confirmed by the cold test results, showing normal maize is more vigorous than quality protein maize (Figure 3.5). Poor laboratory germination and poor seedling performance were previously shown to be correlated with high electrolyte leakage in seeds (MODI, 2005). Leakage of seeds can be associated with poor integrity of cell membranes as determined by deteriorative biochemical activity that impair the ability to re-organize and repair damage (MCDONALD, 1999). Low vigour seeds are those seeds with high electrolyte leakage during imbibition.

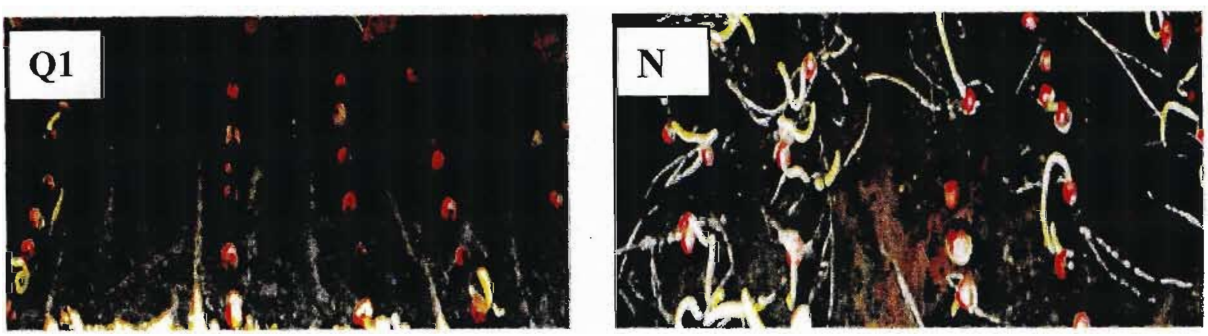


Figure 3.5. Comparison of QPM (cultivar Q1) maize with normal maize (cultivar N) for germination during a cold test.

Having examined all 12 cultivars for phytate and selected mineral content, it was decided that two cultivars (N and Q1) respectively high and low phytate content is studied further to determine the effect of temperature and P nutrition under glasshouse conditions. The premise for this study was that the next generation of seeds would maintain the characteristics of the present seeds, with respect to seed performance. Myo-inositol the precursor of phytic acid has been reported to maintain membrane integrity during cell biogenesis. This in return reduces the leaching of cations essential for germination (BOHNERT *et al.*, 1995). Hence, it was also decided to investigate the effects of growth temperature and P nutrition on soluble carbohydrates, including myo-inositol, in the selected maize cultivars.

3.2. Comparison of two selected cultivars

3.2.1 Seedling establishment

A comparison of the normal maize cultivar (N) with the QPM maize cultivar (Q1) for the amounts of P, Mg and K (Figure 3.2) suggested that cultivar N would show a better stand establishment that cultivar Q1 (BOHNERT *et al.*, 1995). Hence, prior to the start of the glasshouse experiment (in the tunnel), it was decided to determine the growth parameters of the two cultivars: emergence, plant height and leaf emergence rate. Results showed that there was no difference between cultivars with respect to seedling emergence rate (APPENDIX 3.2). Application of phosphorus did not have a significant effect on emergence rate either (Figure 3.6).

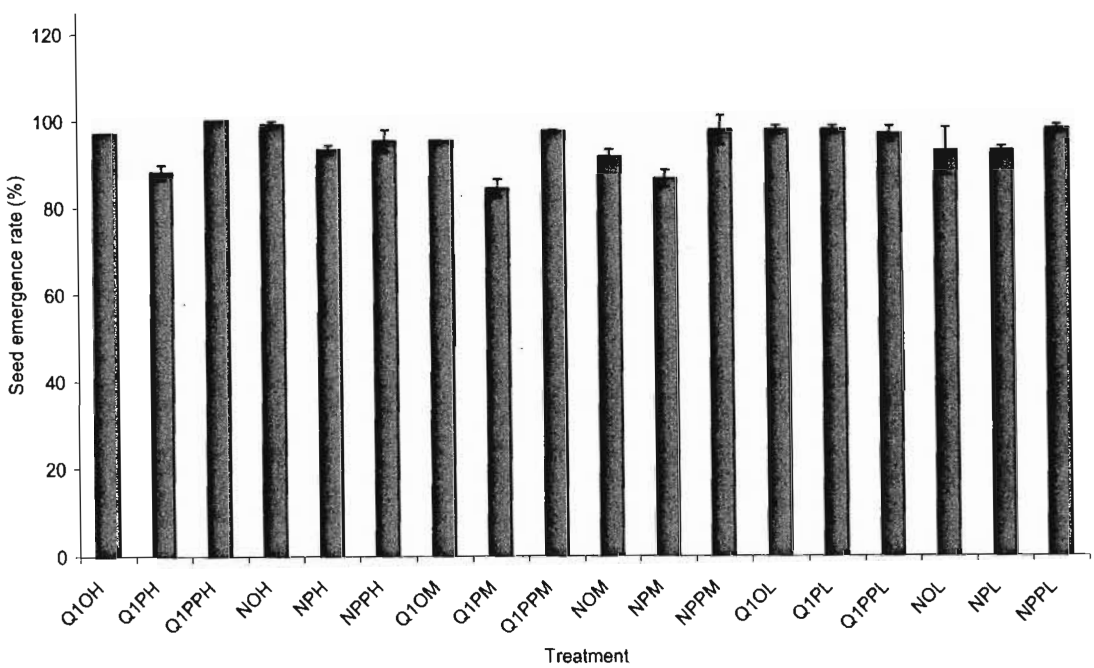


Figure 3.6. Seedling emergence rate of quality protein maize (Q1) and normal maize (N) at phosphorus levels (0, P and PP) prior to subjection to glasshouse temperature treatment.

A previous study by RABOY *et al.* (1985) showed that the ability of soybean seeds to germinate was not affected by phytic acid in the seeds. It is important to note that germination and seedling emergence are different stages of growth, although there maybe similarity of the fundamental factors of seed quality that affects them. Therefore, the fact that the results shown in Figures 3.3 and 3.5 do not exactly concur with Figure 3.6 should not be a surprise. The seedling performance shown in Figure 3.6, may be an indication of how poor quality seeds may improve their performance due to the availability of a favourable soil environment during plant growth.

3.2.2. Plant growth

Plant height: Normal maize had a higher plant height than quality protein maize at the three temperature regimes (Figures 3.7–3.9). The initial increase in temperature from 22/16°C to 27/21°C induced an expected increase in plant height, however, further increase in temperature to 33/27°C provided no further significant change in plant height (APPENDIX 3.3). Temperature is generally known for its correlation with plant development and its effect on accumulation of reserves in seed tissues (FAGERIA, 1992). Although, normal maize plant height was higher than quality protein maize at the three phosphorus levels (Figure 3.10-3.12) it was not significant (Appendix 3.3). Furthermore, plant height significantly ($P<0.01$) improved with phosphorus nutrition; the greatest plant height was recorded at temperatures treatments of 27/21°C and 33/27°C and the smallest at 22/16°C. The plant height increased significantly after week nine in the three phosphorus levels.

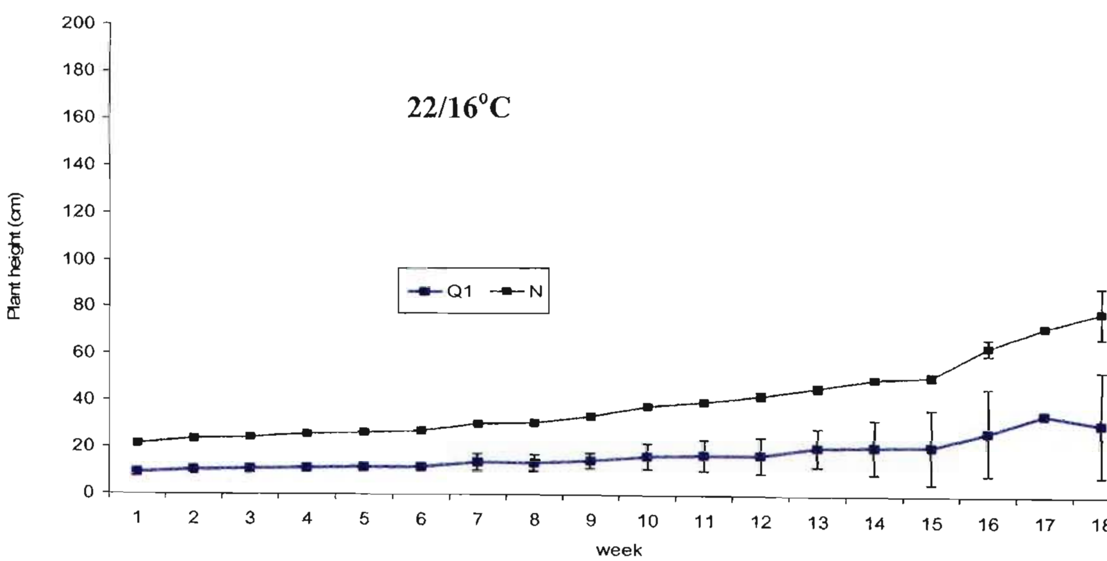


Figure 3.7. Mean plant height during plant development at the low temperature regime, for the QPM (Q1) and normal maize cultivars (N).

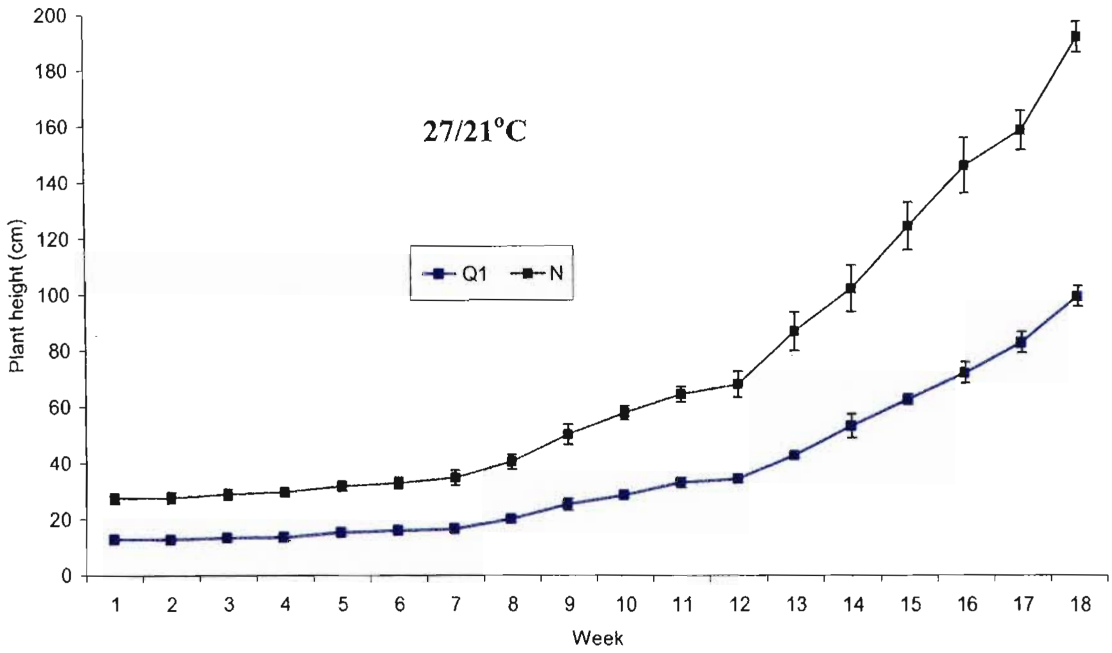


Figure 3.8. Mean plant height during plant development at the medium temperature regime, for the QPM (Q1) and normal maize cultivars (N).

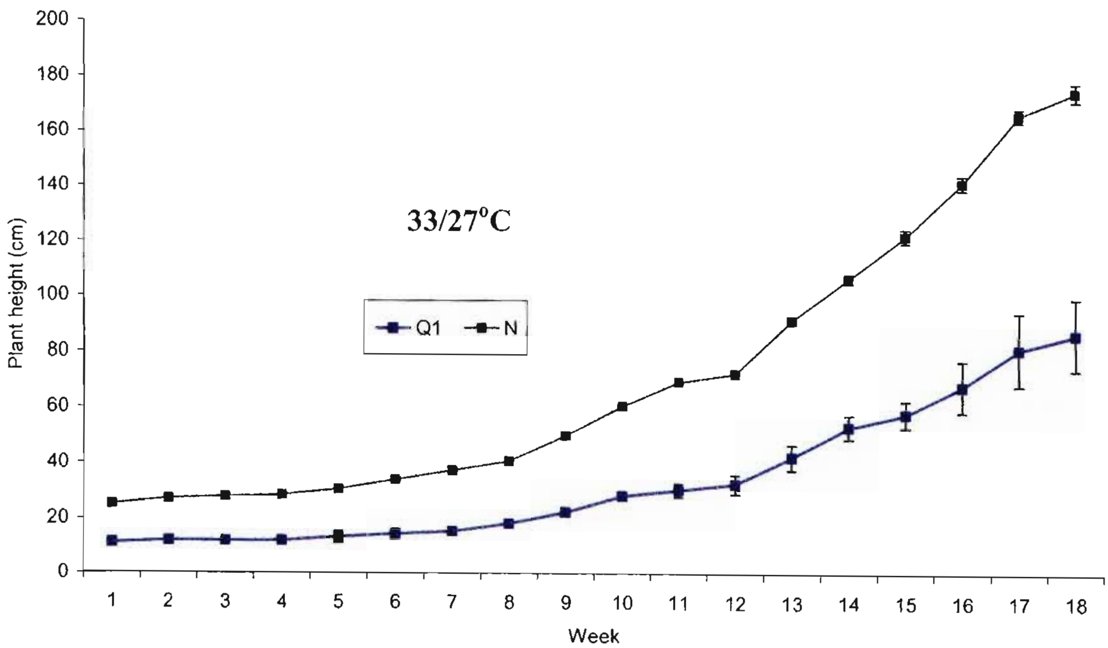


Figure 3.9. Mean plant height during plant development at the high temperature regimes, for the QPM (Q1) and normal maize cultivars (N).

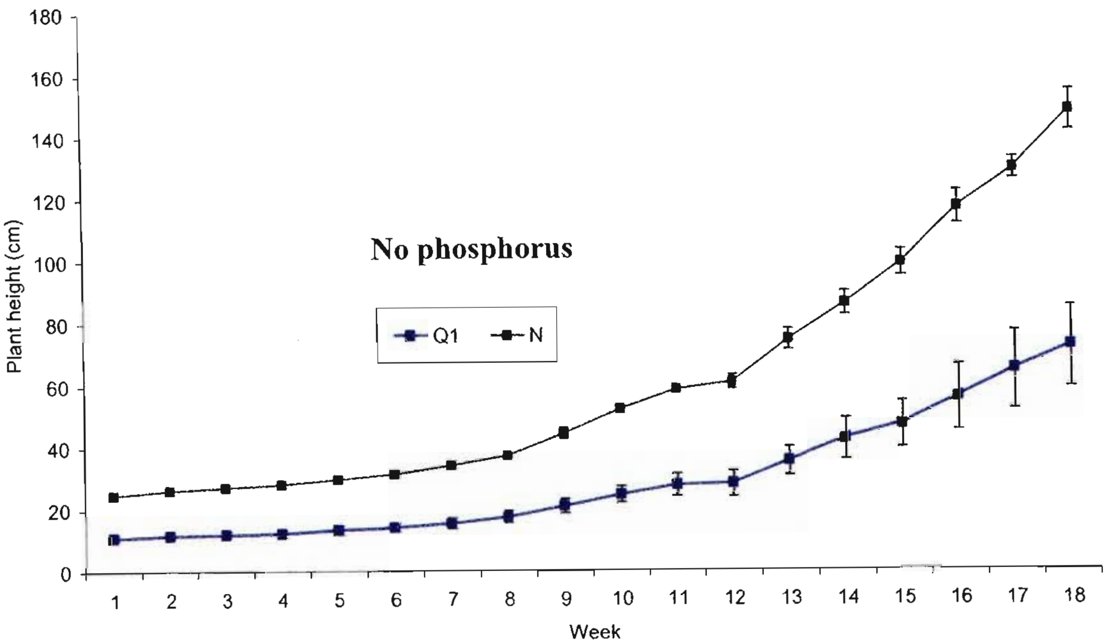


Figure 3.10. Mean plant height during plant development when no phosphorus was applied to the QPM (Q1) and normal maize cultivars (N).

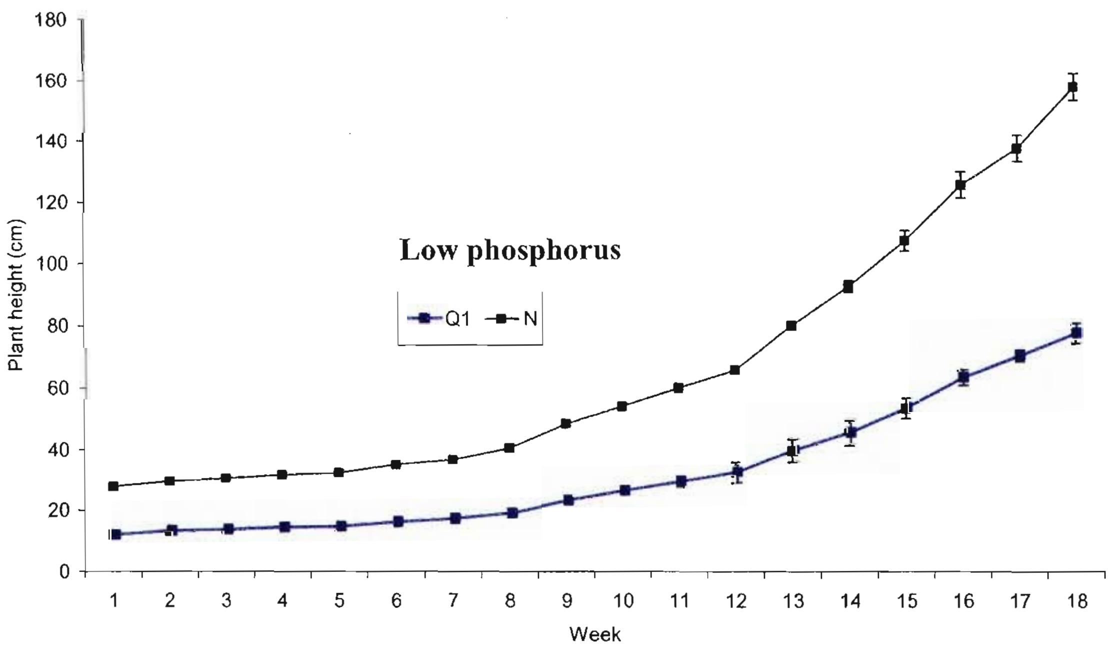


Figure 3.11. Mean plant height during plant development when low phosphorus was applied to the QPM (Q1) and normal maize cultivars (N).

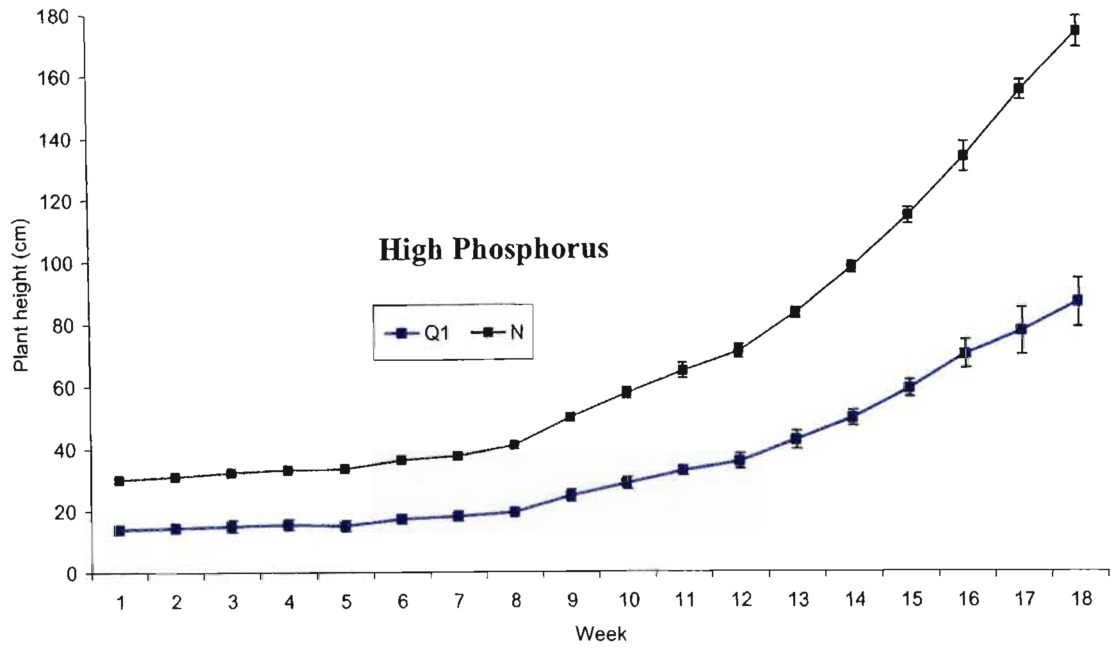


Figure 3.12. Mean plant height during plant development when high phosphorus was applied to the QPM (Q1) and normal maize cultivars (N).

Leaf number: Quality protein maize had low leaf emergence rate comparatively to the normal maize cultivar (Figures 3.13-3.15). Leaf emergence rate significantly increased with temperature; however, there was no significant difference between the temperature 27/21°C and 33/27°C (APPENDIX 3.3). These results concur with previous results reported by FAGERIA (1992) that within a favourable temperature range, plants grow faster as temperature increases, provided other environmental factors, such as nutrients, water and light are not limiting. Quality protein maize showed a low leaf emergence rate compared with the normal maize cultivar. Leaf emergence rate significantly ($P < 0.01$) improved with phosphorus nutrition (APPENDIX 3.3). Leaf emergence rate increased at a faster rate from the ninth week (Figures 3.16-3.18).

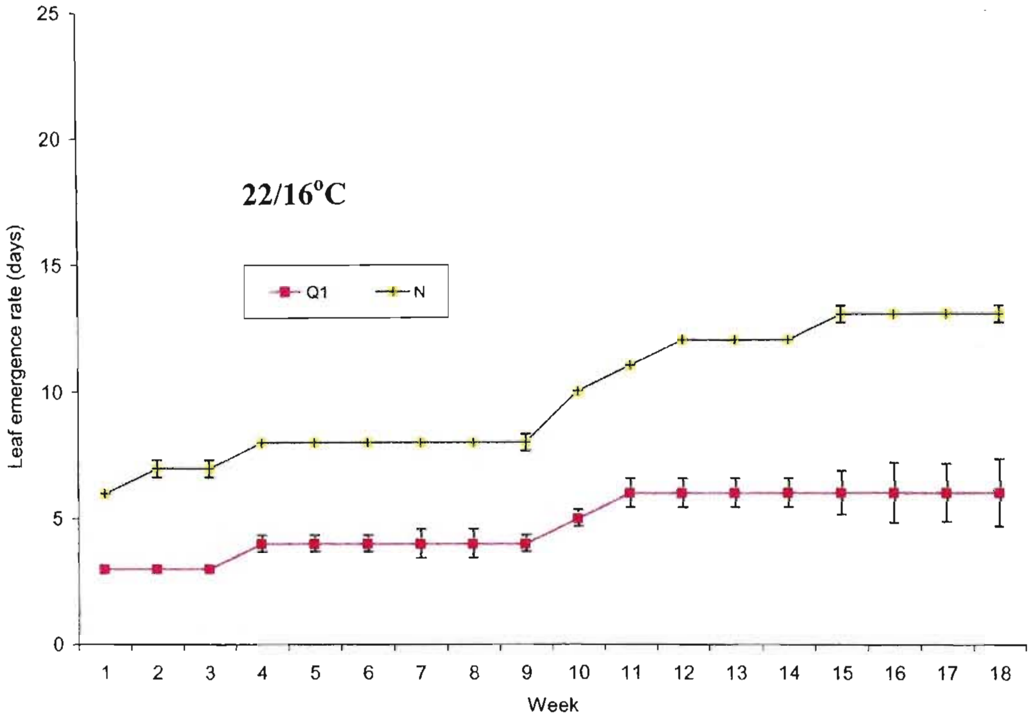


Figure 3.13. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a low growth temperature regime.

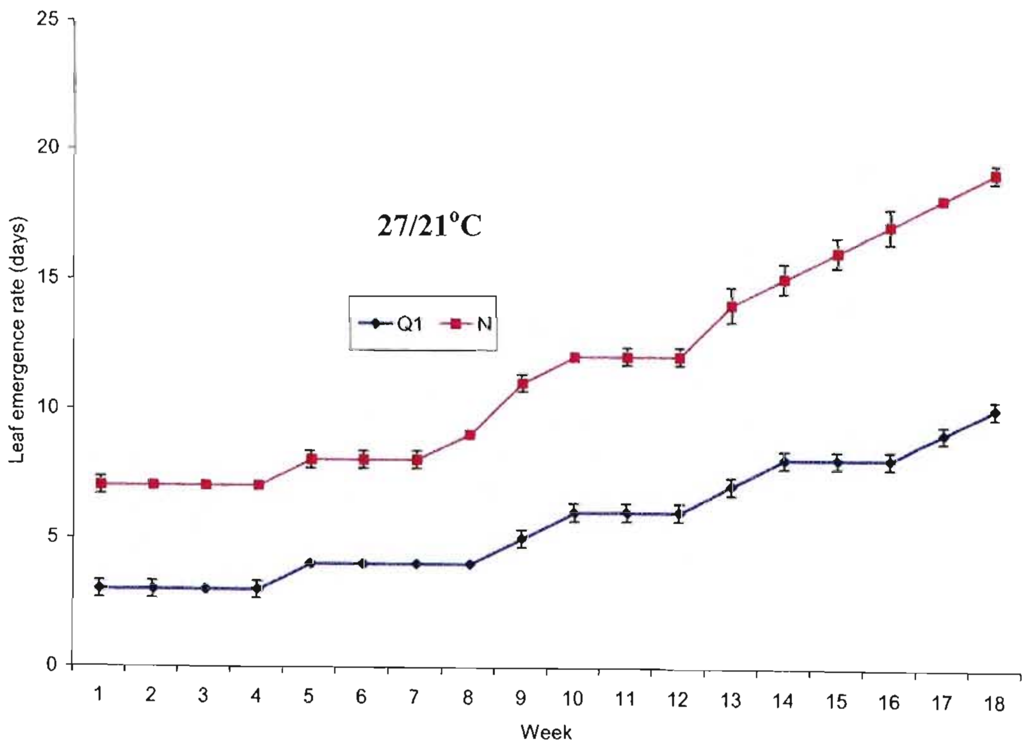


Figure 3.14. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a medium growth temperature regime.

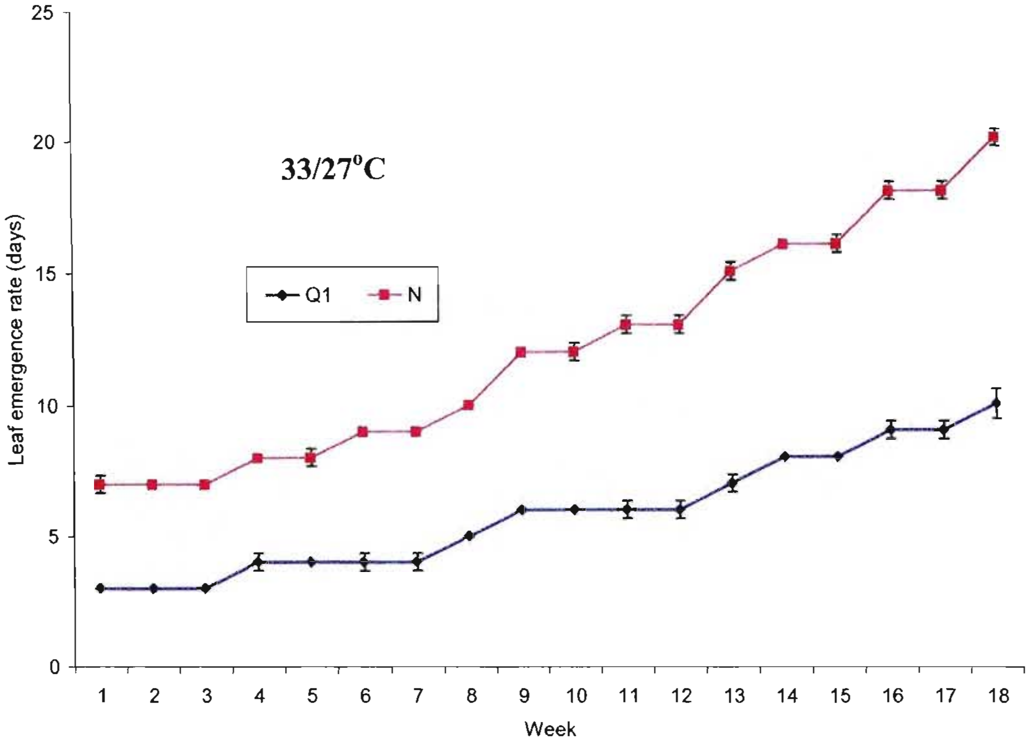


Figure 3.15. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a high growth temperature regime.

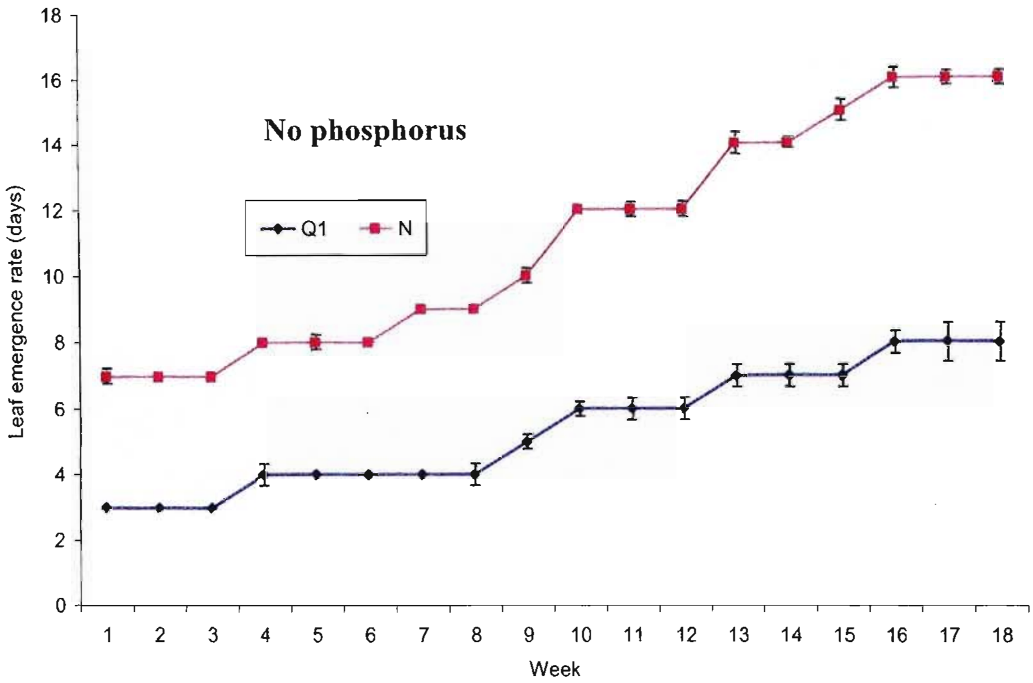


Figure 3.16. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a no phosphorus treatment.

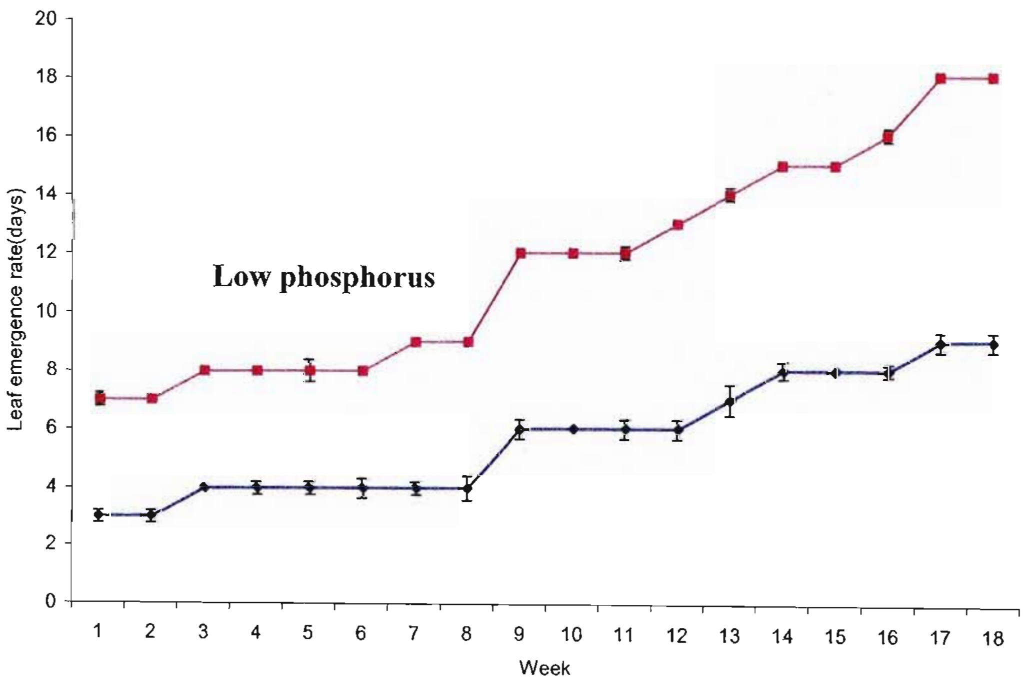


Figure 3.17. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a low phosphorus treatment.

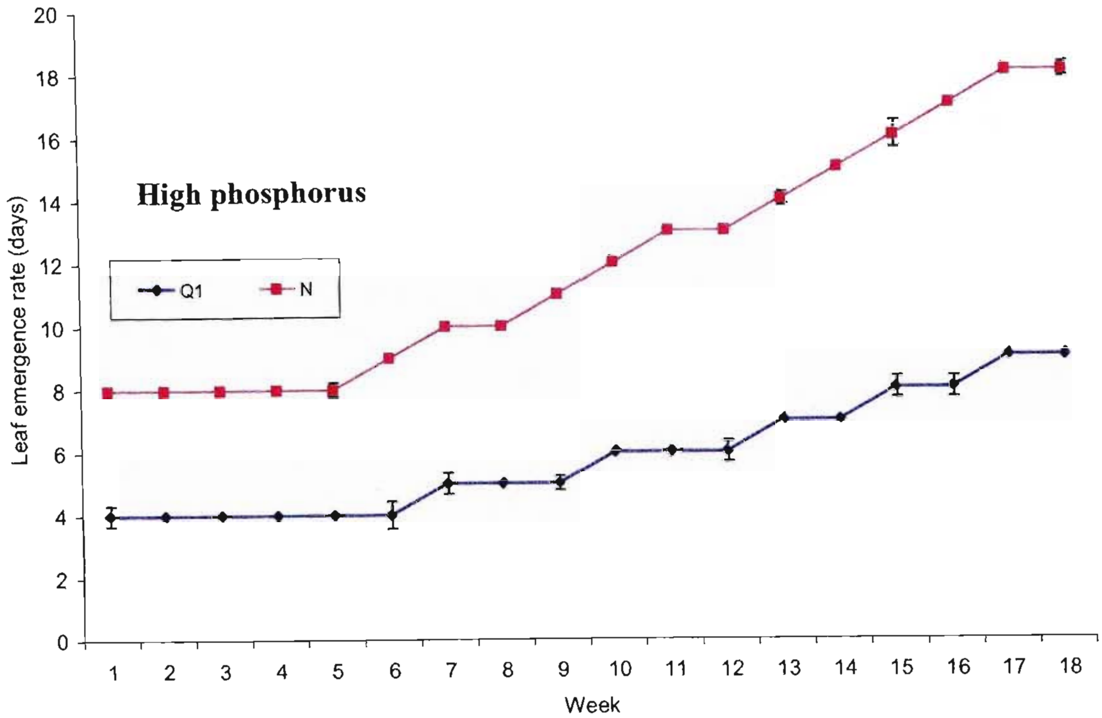


Figure 3.18. Mean leaf number for QPM (Q1) and normal maize cultivars (N) during plant development in response to a high phosphorus treatment.

3.2.3. Seed germination

There was a significant difference ($P< 0.01$) in seed germination capacity during seed development (APPENDIX 3.4). At 15 days after flowering, there was a significant ($P< 0.01$) low germination % at temperatures 33/27°C and 27/21°C and no germination at 22/16°C (Figure 3.19). At 22 days after flowering, the dough stage of seed development, there was a significant increase in germination at temperatures 33/27°C and 27/21°C and no germination at 22/16°C, with the exception of the normal maize with the highest phosphorus nutrition. This finding is in agreement with a previous study by BAILLY *et al.* (2004), who found that seed vigour increased during development and the maturation drying phase. Furthermore, a study by BEWLEY & BLACK (1985) showed a correlation between abscisic acid (ABA) content and germinability and ABA levels are higher in young, non-germinable seeds than in older ones, e.g., in peas, soybean and wheat. ABA is implicated in phytate synthesis (Figure 1.2). The quality protein cultivar had a significantly low ($P< 0.01$) germination % than normal maize at different harvesting stages of seed development and this correlates with the vigour test (Figure 3.5). Furthermore, the seed of quality protein maize at temperature 22/16°C did not germinate during milk and dough stage. MERTZ (1992) and MUNCK (1992) have reported the negative agronomic characteristics of the quality protein maize. Germination % of both cultivars' was significantly influenced by phosphorus nutrition in temperatures 33/27°C and 27/21°C, however, the temperature 22/16°C showed no significant effect ($P< 0.01$) (APPENDIX 3.4). The interaction between temperature and P nutrition is shown in Table 3.1.

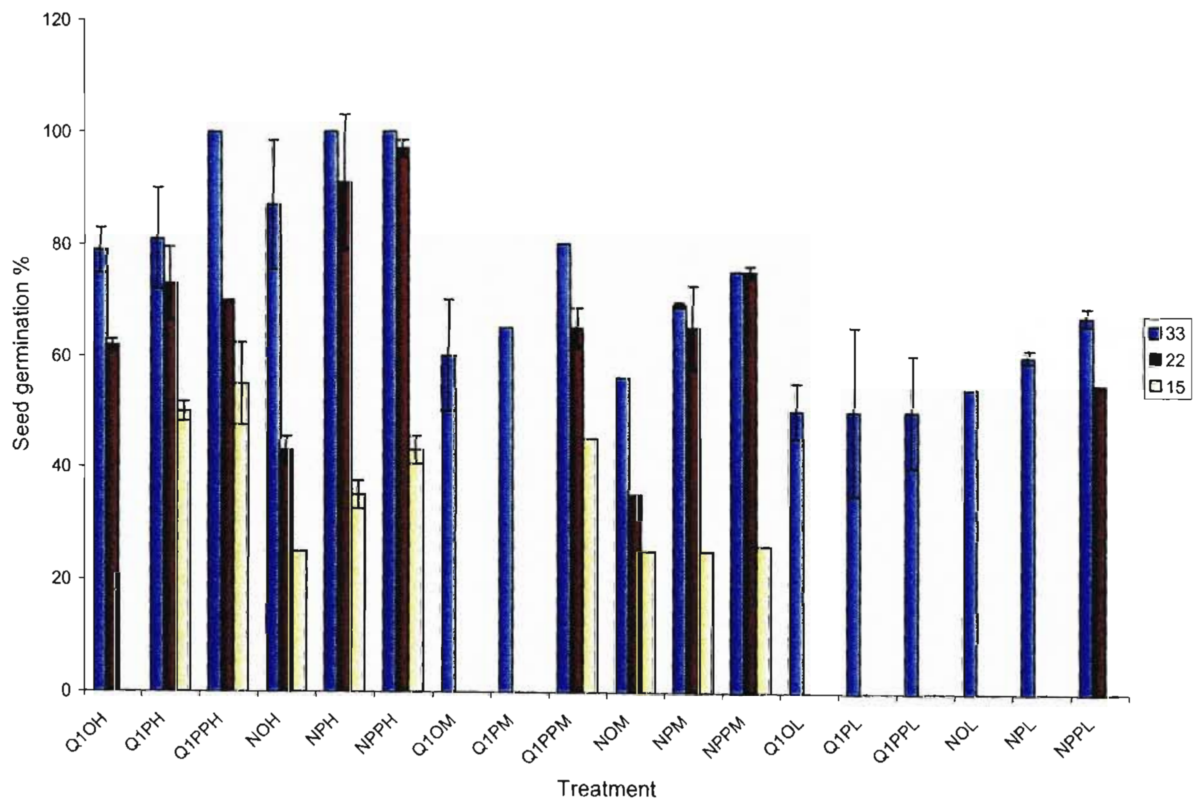


Figure 3.19. Seed germination of quality protein maize (Q1) and normal maize (N) at phosphorus levels (0, P and PP) and temperature levels (L, M, and H) during three stages of seed development milk stage (15 DAP), dough stage (22 DAP) and harvest maturity (33 DAP).

Table 3.1. The interaction of temperature and phosphorus nutrition on seed germination % of normal and quality protein maize during seed development. Note: Values sharing the same letter were not significantly different (P= 0.05).

Fertilizer	22/16°C	27/21°C	33/27°C	Mean
0	25^{c}	27.7^{c}	53.3^{b}	35.3^{A}
P	25^{c}	28.3^{c}	82.5^{a}	45.3^{A}
PP	25.8^{c}	53.3^{b}	25.8^{c}	35^{A}
Mean	25.3^{B}	36.4^{C}	54^{A}	38.5^{A}

There was a significant interaction between phosphorus nutrition and temperature (Table 3.1 and APPENDIX 3.4). At 0 phosphorus, there was no change in germination with an increase in temperature from 22/16°C to 27/21°C. However, when the temperature increased to 33/27°C, there was a significant increase in seed germination % (Table 3.1). One might except from a temperature increase from 22/16°C to 27/21°C in a crop such as maize that seed germination % capacity would increase with temperature until it reached a supra-optimal level. However, it is important to note that nutrients are also important in improving germination capacity. At low phosphorus level, there was a non-statistical increase in germination % from temperature 22/16°C to 27/21°C. There was a significant increase in seed germination at temperature 33/27°C. Furthermore, at high phosphorus level there was a significant increase in seed germination capacity from temperatures 22/16°C to 27/21°C whilst, there was a significant reduction in germination capacity from temperature 27/21°C to 33/27°C. Temperatures above 32°C are considered as unfavourable for maize (THOMPSON, 1975). Therefore, this could explain the reduction in seed germination capacity.

3.2.4. Electron microscopy studies

Using an environmental scanning electron microscope and transmission electron microscope. The globoids, the site of phytate synthesis and storage in seeds was identified, it was established that regardless of maize type, the embryo of maize caryopses is rich globoids, whereas the endosperm has no globoids, but it contains starch grains (Figure 3.20).

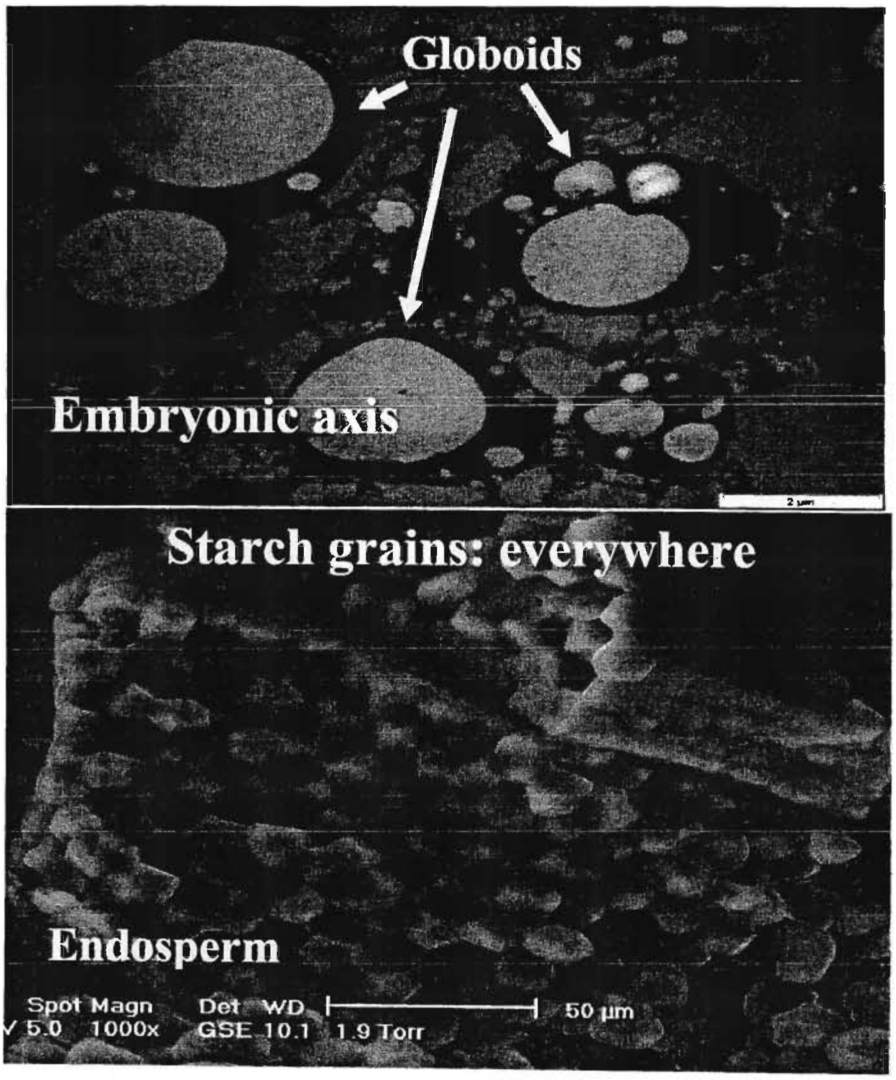


Figure 3.20. Electron micrographs of maize seed tissues showing the presence of globoids in the embryonic axis, and their absence in the starch-rich endosperm.

Determination of mineral elements (P, K and Mg) in maize seed tissues was performed in embryo and endosperm, separately. Results showed that there were no significant differences in potassium with respect to temperature, phosphorus, seed parts, cultivar, stages of development and their interactions (APPENDIX 3.5). Magnesium content was significantly ($P<0.05$) affected by temperature, phosphorus, seed parts, cultivar and stages of seed development (APPENDIX 3.5) (Figures 3.21-3.23).

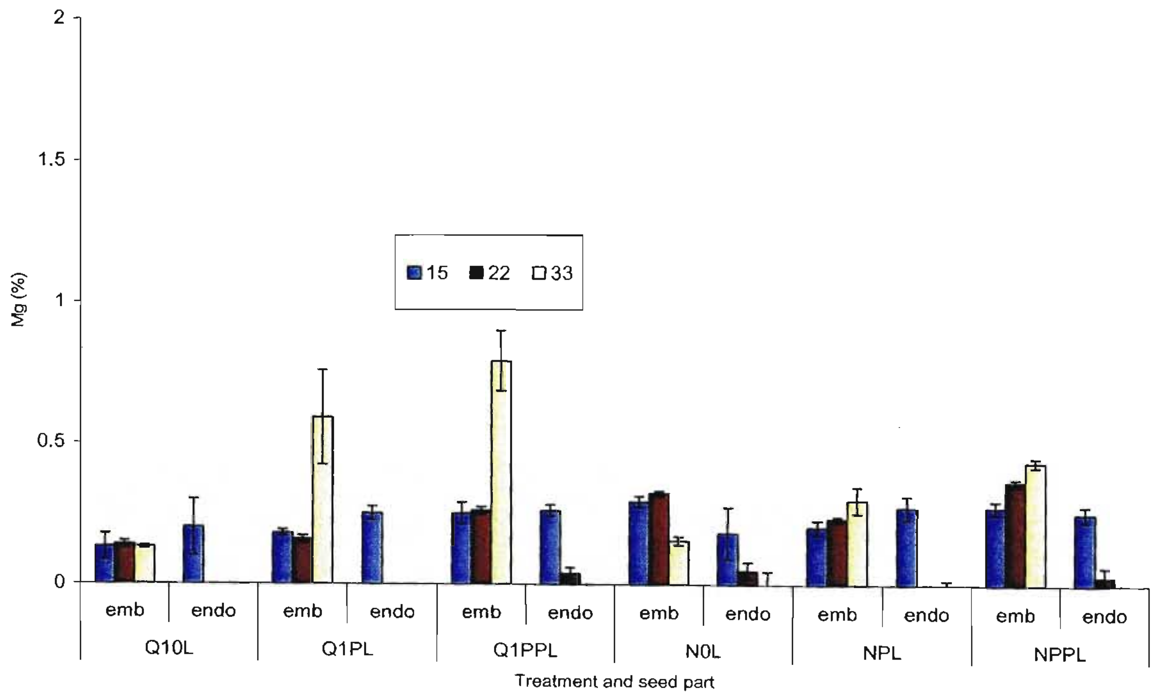


Figure 3.21. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for Mg content (%) in response to phosphorus (0, P, and PP) levels at the low temperature regime (L). Seeds were examined at 15, 22 and 33) days after pollination (inset).

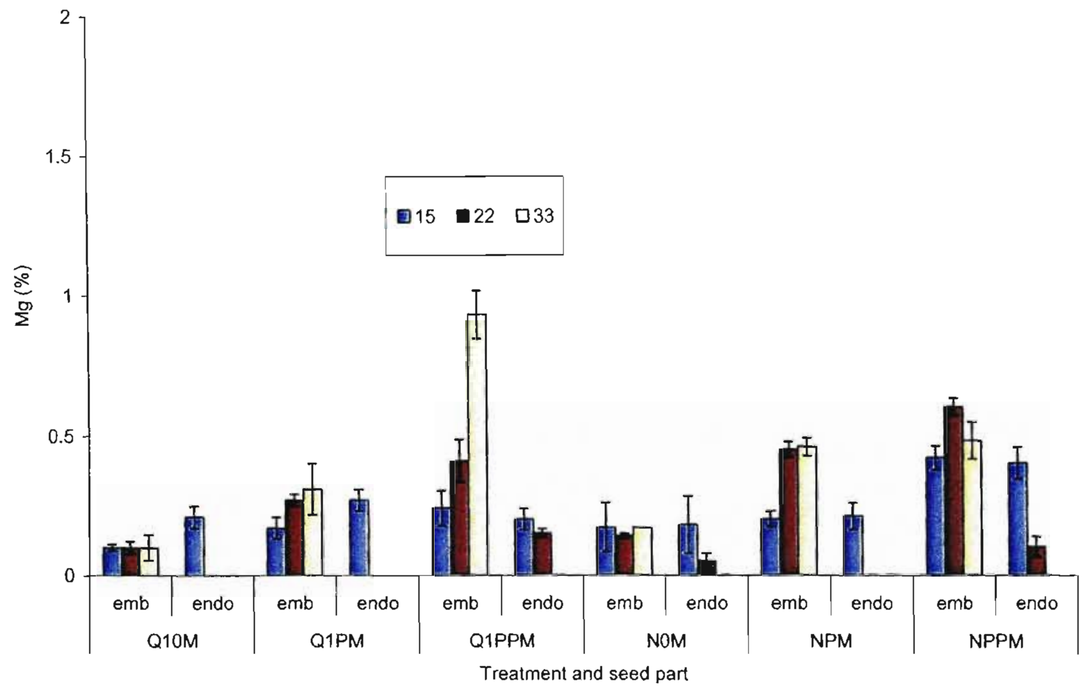


Figure 3.22. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for Mg content (%) in response to phosphorus (0, P, and PP) levels at the medium temperature regime (M). Seeds were examined at 15, 22 and 33 (days after pollination (inset).

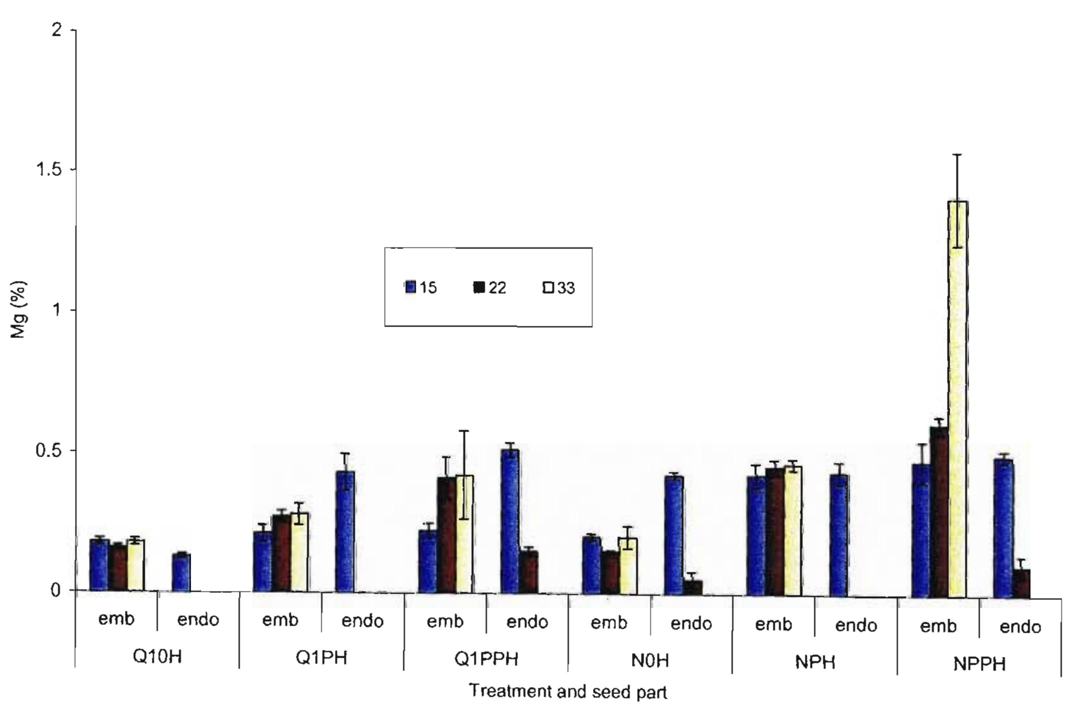


Figure 3.23. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for Mg content (%) in response to phosphorus (0, P, and PP) levels at the high temperature regime (last H). Seeds were examined at 15, 22 and 33 (days after pollination (inset).

An increase in temperature from 22/16°C to 27/21°C resulted in a reduction in magnesium content. However, there was a significant increase in magnesium from 27/21°C to 33/27°C. Furthermore, magnesium was significantly influenced by phosphorus nutrition (APPENDIX 3.5; Figures 3.21-3.23). There was a significant increase in magnesium from 0 phosphorus level to low (L) phosphorus level and a non-statistical increase from low phosphorus level to high (H) phosphorus level. The seed of normal maize had a significant higher magnesium content than the QPM maize, except at temperature 22/16°C where, the magnesium content was the highest for quality protein at 33 days after flowering. In the embryonic axis, Mg content increased with maturity (Figures 3.20-3.22), possibly because the mineral was accumulated in phytate. In the endosperm, there was a decrease in Mg content as the seed matured (Figure 3.22) (LIN *et al.*, 2005).

Changes in P content in maize seed tissues were generally similar to those found for Mg (Figures 3.24-3.26). There was an increase in phosphorus content with temperature and stage of seed development which was influenced by an interaction between temperature and phosphorus nutrition (Table 3.2) At both low and high phosphorus nutrition, the phosphorus content decreased significantly from temperature 22 /16°C to 27/21°C and increased significantly at temperature 33/27°C. One might except, the temperature 27/21°C to be ideal for maize. However, other physiological factors may have counteracted the effect of phosphorus nutrition. Under the temperature 22/16°C there was no significant differences in respect to phosphorus nutrition, cultivars and seed development stages whilst, there was significant difference at temperature 27/21°C and 33/27°C. This leads us to conclude that, the temperature 22/16°C is sub-optimal for phosphorus mineral content.

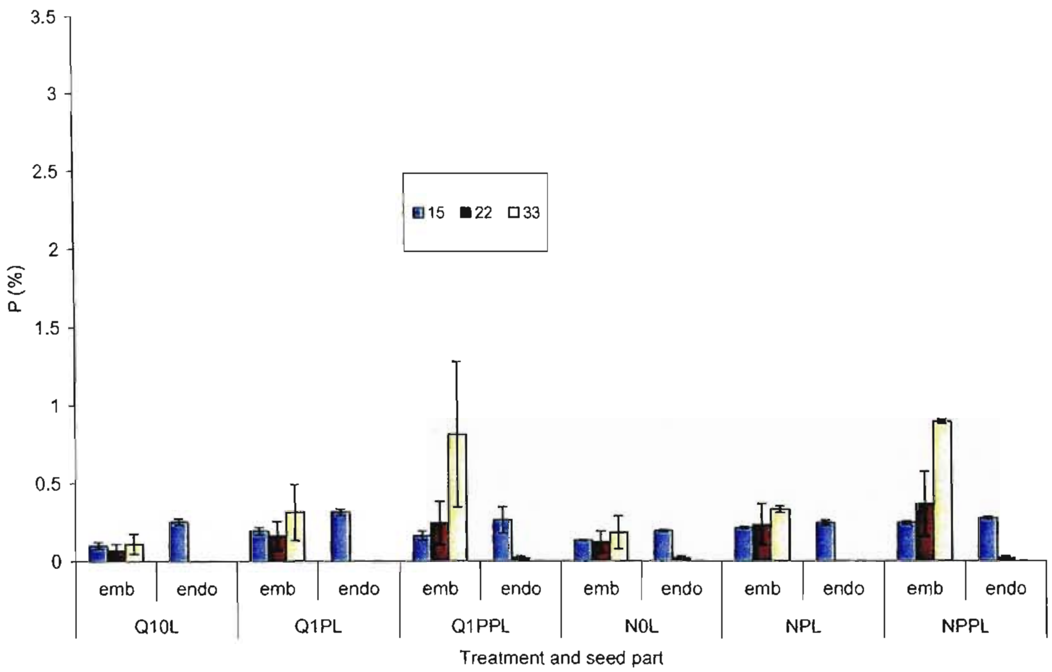


Figure 3.24. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for P content (%) in response to phosphorus (0 P, and PP) levels at the low temperature regime (L). Seeds were examined at 15, 22 and 33 days after pollination (inset).

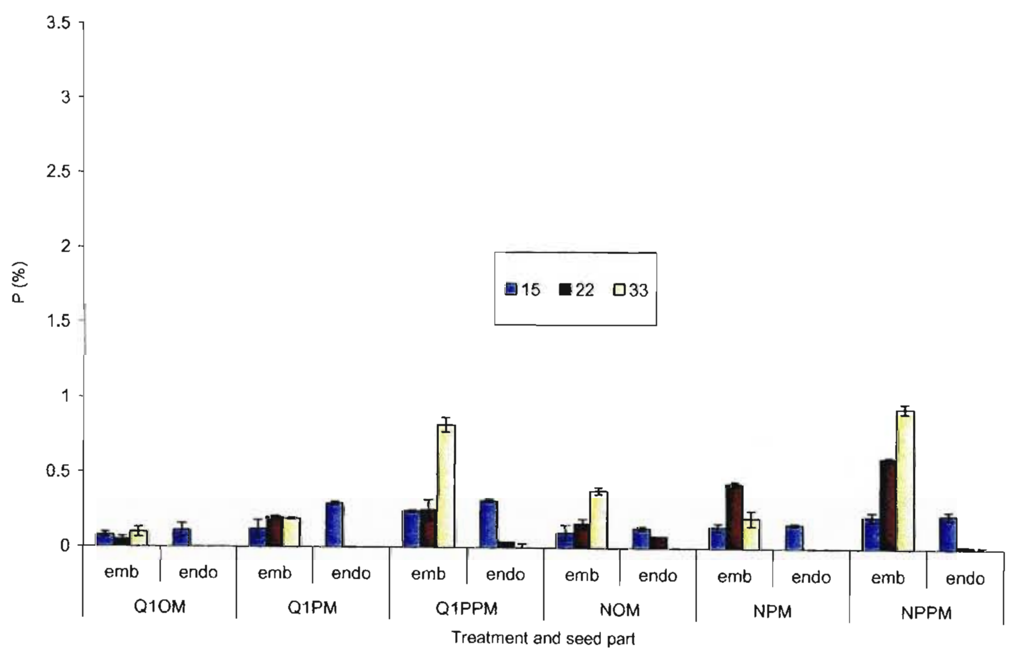


Figure 3.25. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for P content (%) in response to phosphorus (0, P, and PP) levels at the medium temperature regime (M). Seeds were examined at 15, 22 and 33 days after pollination (inset).

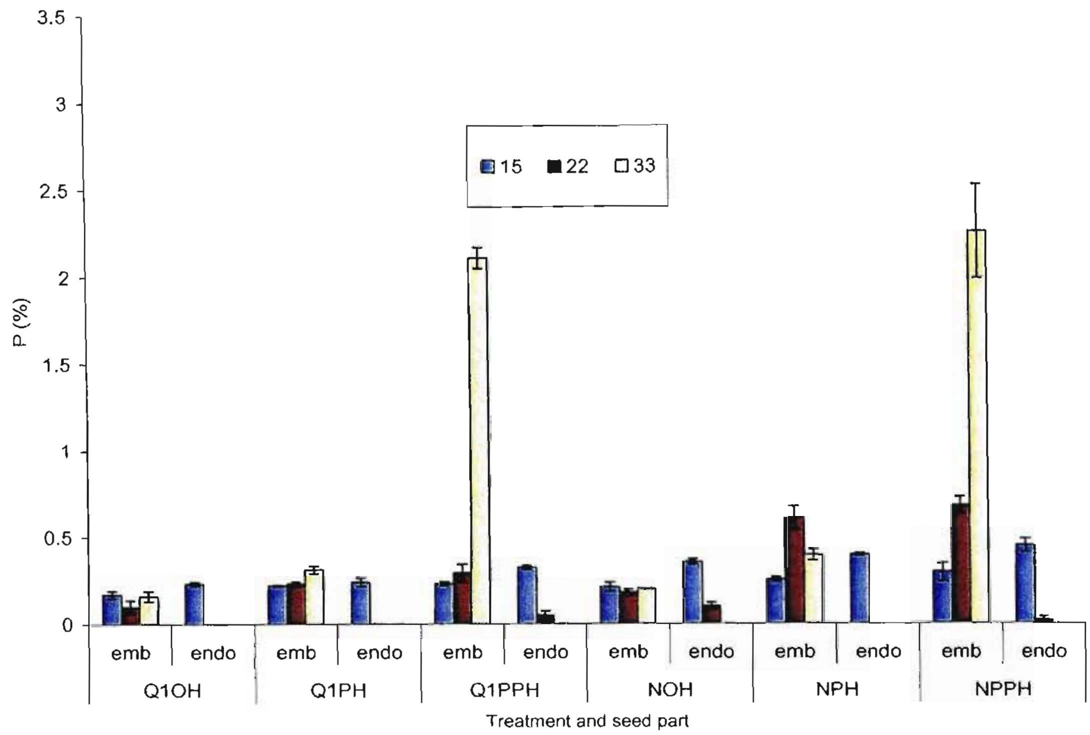


Figure 3.26. Comparison of maize cultivars (Q1 and N) and seed tissues embryo (emb) and endosperm (endo) for Mg content (%) in response to phosphorus (0, P, and PP) levels at the high temperature regime (last H). Seeds were examined at 15, 22 and 33 days after flowering (inset).

Table 3.2. The interaction of temperature and phosphorus nutrition on phosphorus % of seed from normal and quality protein maize. Values sharing the same letters are not significantly different ($P < 0.05$).

Fertilizer	22/16°C	27/21°C	33/27°C	Mean
0	0.0967^{f}	0.1122^{f}	0.1475^{ef}	0.1188^{C}
P	0.2286^{cd}	0.175^{e}	0.2358^{c}	0.213^{B}
PP	0.3544^{b}	0.2358^{c}	0.46^{a}	0.35^{A}
Mean	0.226^{A}	0.174^{A}	0.281^{A}	0.227^{B}

3.2.5. Phytate content

Normal maize phytic acid content was significantly higher than quality protein maize and there was a general tendency for phytate to increase with seed maturity stage (Figure 3.27). There was also a significant interaction between phosphorus, cultivar and stage of seed development (APPENDIX 3.6). At the temperature of 33/27°C, phytic acid increased with seed maturity, with the highest concentration at harvest maturity. This agrees with findings by RABOY & DICKINSON (1987) which showed a linear increase of phytic acid with maturity. Furthermore, there was a significant linear increase in phytic acid content with phosphorus nutrition in quality protein maize. The normal maize showed a significant increase in phytic acid from the control to the lowest phosphorus level and no differences from the lowest to the highest concentration at stages 22 and 33 days after pollination of seed development. At 15 days after flowering there was a significantly lower phytic acid content under high phosphorus nutrition. This suggests that the medium 90.12 $g.pot^{-1}$) phosphorus level was optimal for phytic acid content in normal maize.

At the temperature of 27/21°C, there was an increase in phytic acid content with phosphorus nutrition (Figure 3.27). It has been shown that in general, phytate content in grains is higher with increases in supply of P (RABOY & DICKINSON, 1993).

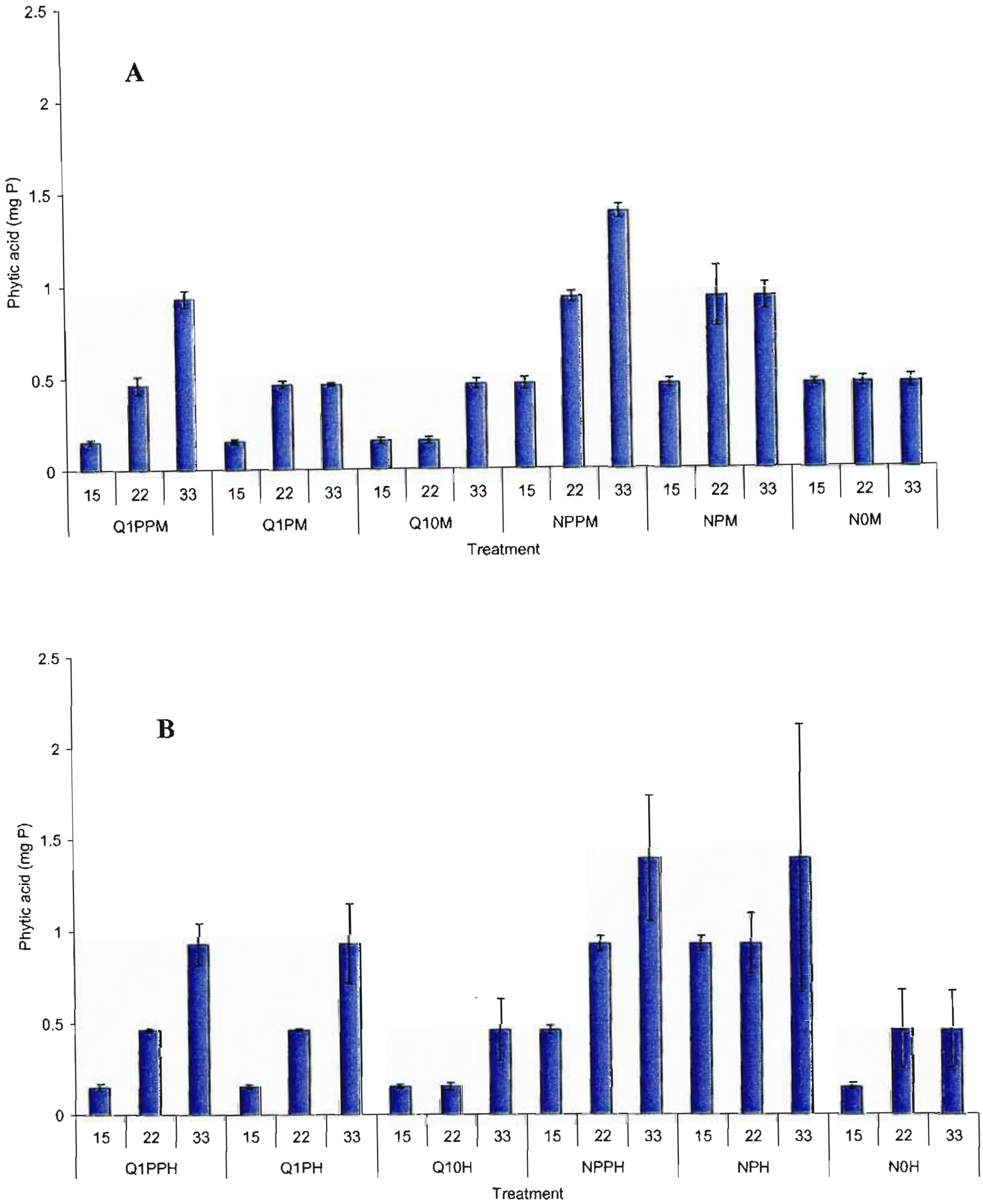


Figure 3.27. Changes in phytate content in quality protein (Q1) and normal (N) maize seeds during development (15, 22 and 33 days after flowering) at different levels of phosphorus (PP = high, P = medium and 0 = low) and growth temperatures (M = 27/21; A= top figure and H = 33/27; B= bottom figure).

Temperatures 33/27°C and 27/21°C for both cultivars at the milk stage (15 days) showed no difference in phytic acid content with respect to phosphorus nutrition. This is due to the fact that, the mineral element is utilized for seed germination and very little is stored as phytic acid at this stage of development (HEGEMAN *et al.*, 2001). There were no data collected at 22/16°C due to seed limitation. However, it would be expected that a lesser amount of phytate will be available at this temperature because temperature 33/27°C produced the highest phytate content followed by temperature 27/21°C (APPENDIX 3.6).

3.2.6. Soluble carbohydrate determination

Observation of the occurrence of all soluble carbohydrates determined in this study showed that in both cultivars sucrose, glucose and fructose (in the order mentioned) were predominant sugars at most stages of seed development, regardless of the growth temperature and phosphorus application (Figure 3.28). The pattern was the same at all temperatures, but sugars responded to temperature in the order: high > medium > low. Hence, only data from the high temperature are shown in Figure 3.28. However, the oligosaccharides, raffinose, stachyose and verbascose, were not detectable in this study. Figure 3.28 gives a general impression that the amount of sugars decreased as the phosphorus level decreases, but this pattern is not clear. Hence, it was necessary to examine individual sugars to determine the response of cultivars to temperature and phosphorus treatments. APPENDIX 3.7 shows that there were significant effects of temperature and phosphorus on soluble carbohydrate occurrence in both cultivars. However, cultivar differences were only found for galactose (APPENDIX 3.7 D). Glucose, fructose and galactose showed a significant effect of both temperature and phosphorus treatments (APPENDICES 3.7 C, D and E). Sucrose showed a significant effect of temperature (APPENDIX 3.7 B). Myo-inositol showed no significant main effect (APPENDIX 3.7 A). However, there were significant interactions of temperature, cultivar and stage in their effects on almost all the soluble carbohydrates determined except for sucrose (APPENDIX 3.7).

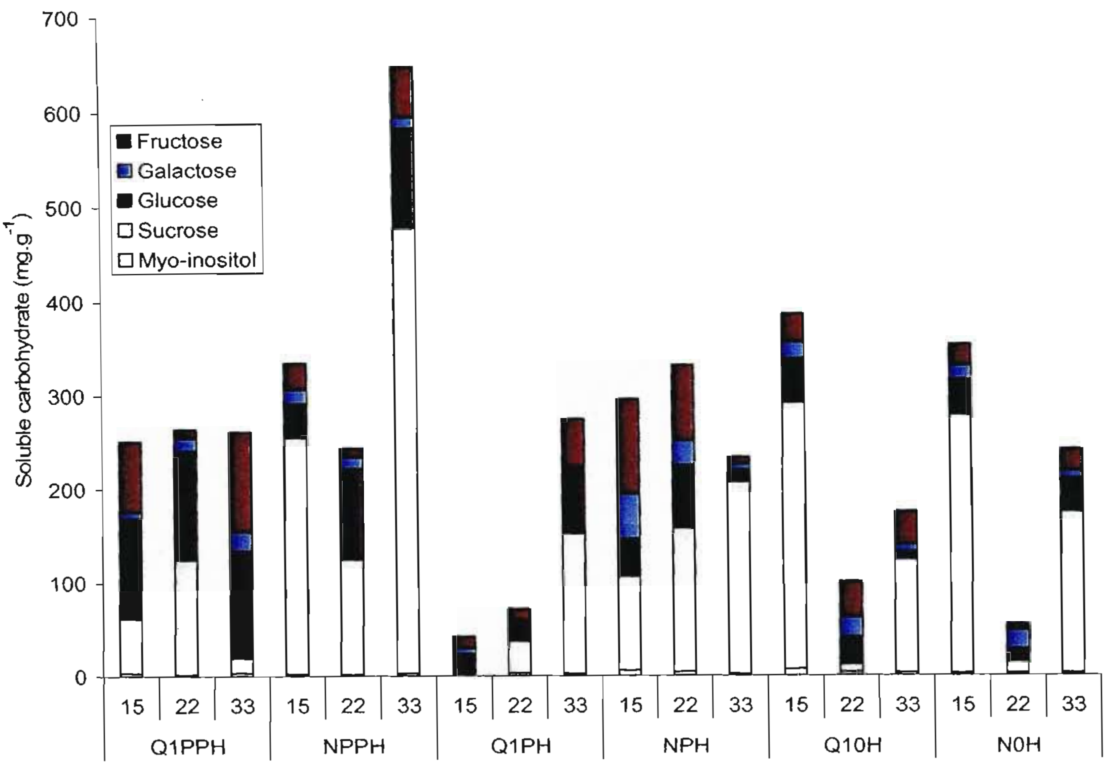


Figure 3.28. General pattern of soluble carbohydrate occurrence in seeds of two maize cultivar (Q1 and N) in response to phosphorus treatments (PP, P and 0) at high temperature regime (33/27°C).

The report by SUN *et al.* (1994) that sucrose is the prominent sugar accumulated in maize embryos has been confirmed in this study (Figure 3.29). Sucrose showed significant response to low temperature and high temperature stress, the former causing higher concentrations, but its occurrence was moderate at medium temperatures (Figure 3.29).

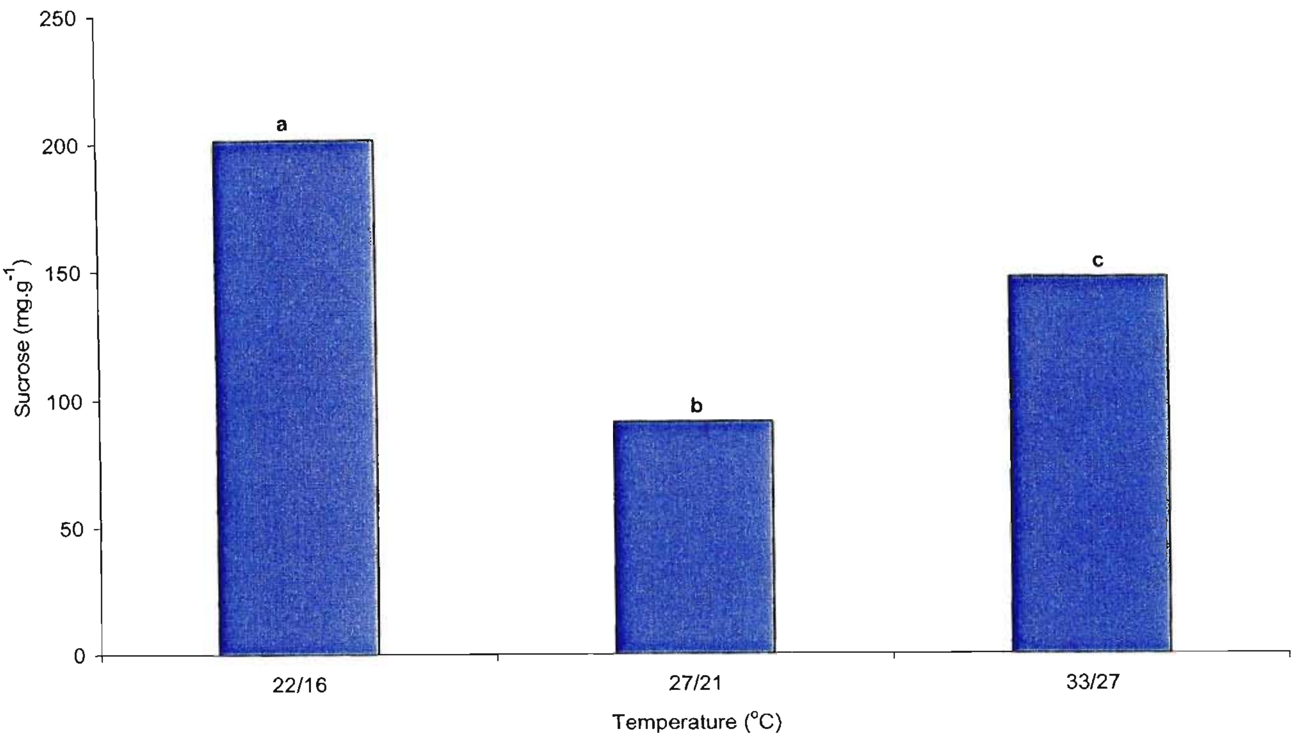


Figure 3.29. Mean effect of temperature on sucrose across cultivars and phosphorus levels.

The pattern of other monosaccharide occurrence in maize seeds clearly showed a decrease as the temperature increased (Figures 3.30-3.32). However, their occurrence in response to phosphorus application varied, with fructose and glucose showing an increase with an increase in P only at low and high, but not medium temperatures. No previously published studies explaining the relationship between soluble carbohydrates and P and temperature effects on maize were found. Therefore, it is difficult to compare the data on sucrose, glucose, fructose and galactose with previous findings. Soluble carbohydrates, have however, been implicated in seed quality (PETERBAUER & RITCHER, 2001).

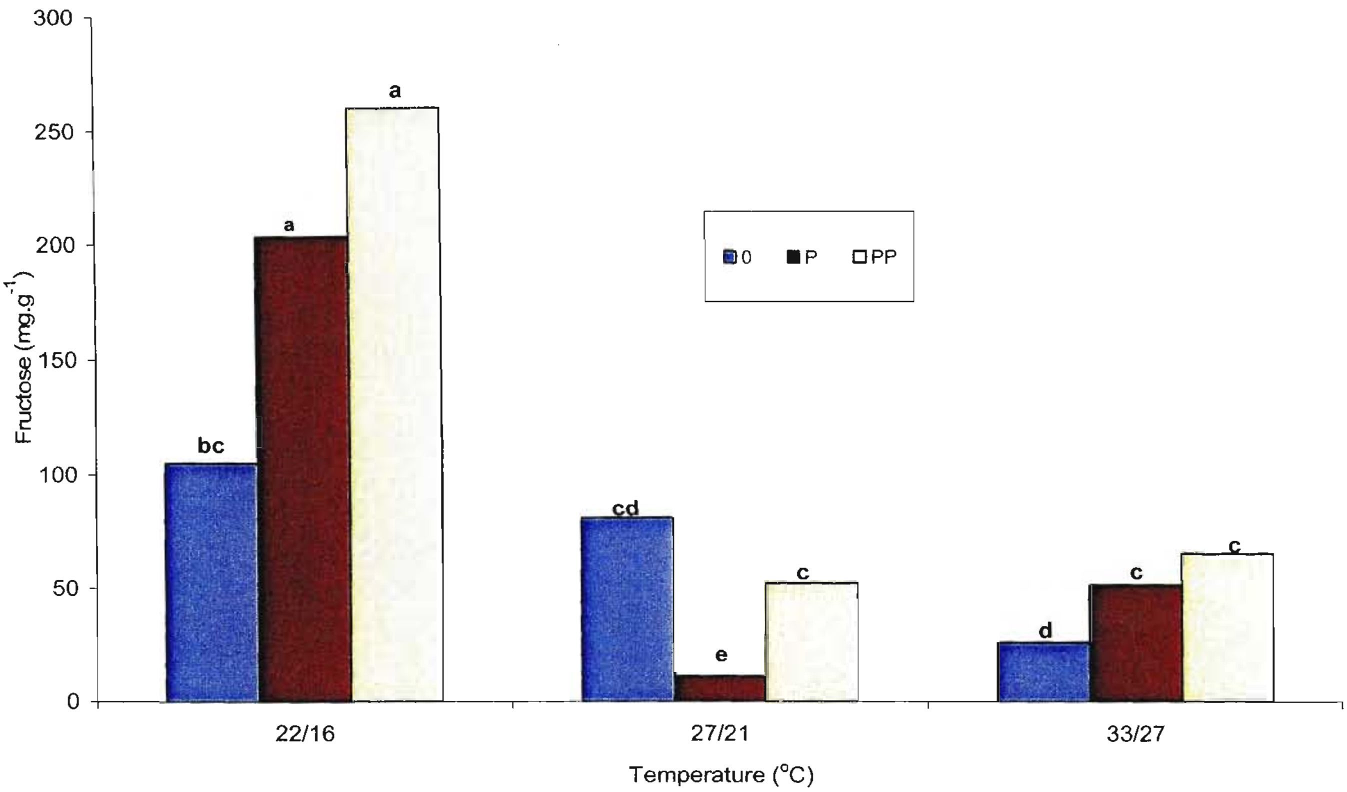


Figure 3.30. Mean effect of temperature on fructose across cultivars and phosphorus levels.

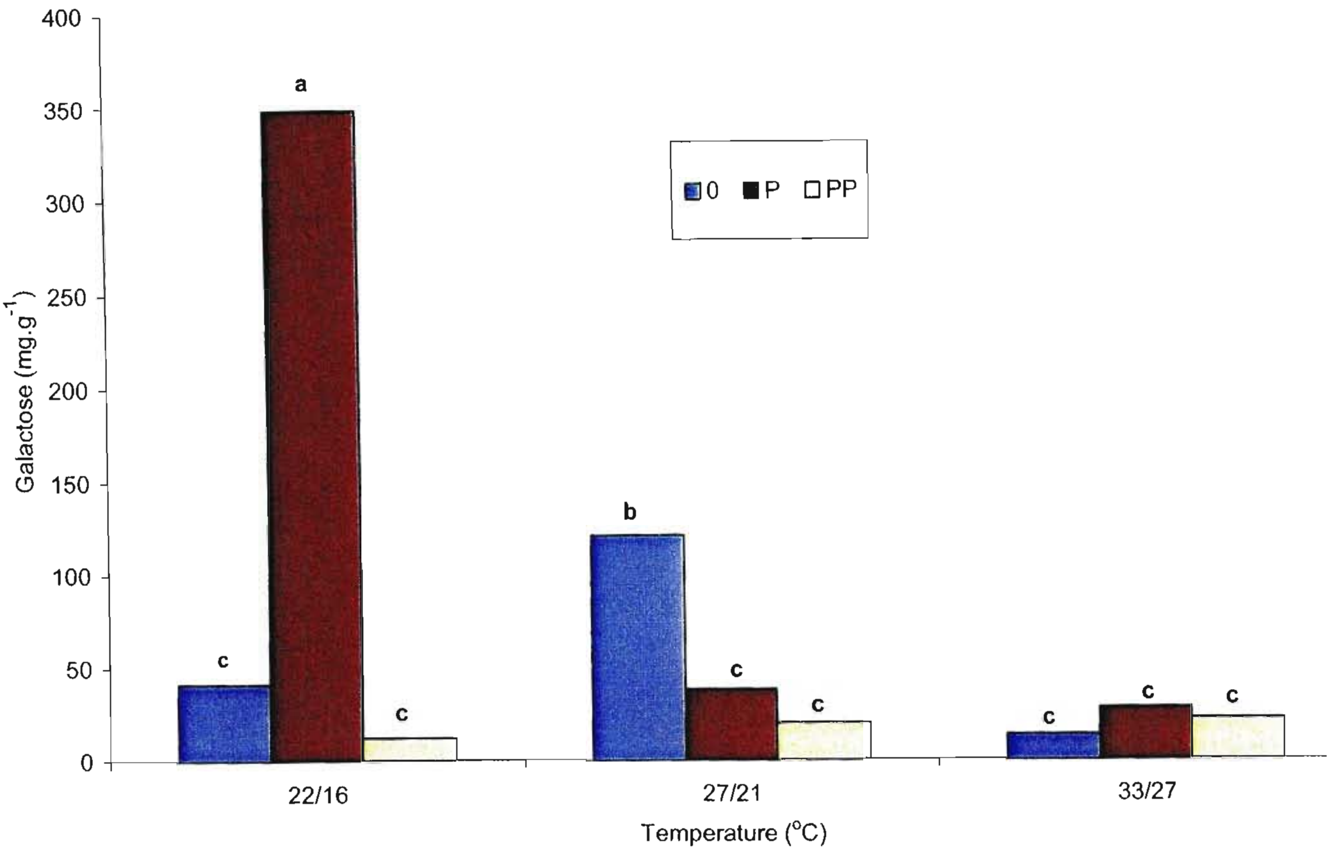


Figure 3.31. Mean effect of temperature on galactose across cultivars and phosphorus levels.

Quality protein maize had a significant ($P<0.01$) lower galactose content relatively to the normal maize (APPENDIX 3.7). Galactose content decrease with maturity of seed which of evident of galactose role in seed germination. This agrees with a previous study by BLACKMAN *et al.* (1992) that changes in carbohydrates occur during seed development and germination. Generally, as seeds mature the mono-saccharide content decreases and the oligosaccharide content increases. Galactose content was significantly affected by temperature and phosphorus nutrition (APPENDIX 3.7). The control phosphorus level resulted in a significant increase in galactose at the temperature of 22/16°C whilst, there was no response in the other two temperatures (Figure 3.31). The lowest phosphorus level resulted in significantly high galactose content at the temperature of 22/16°C while temperatures 27/21°C and 33/27°C had a significantly lower galactose content (Figure 3.31). The highest phosphorus level showed no response across temperatures.

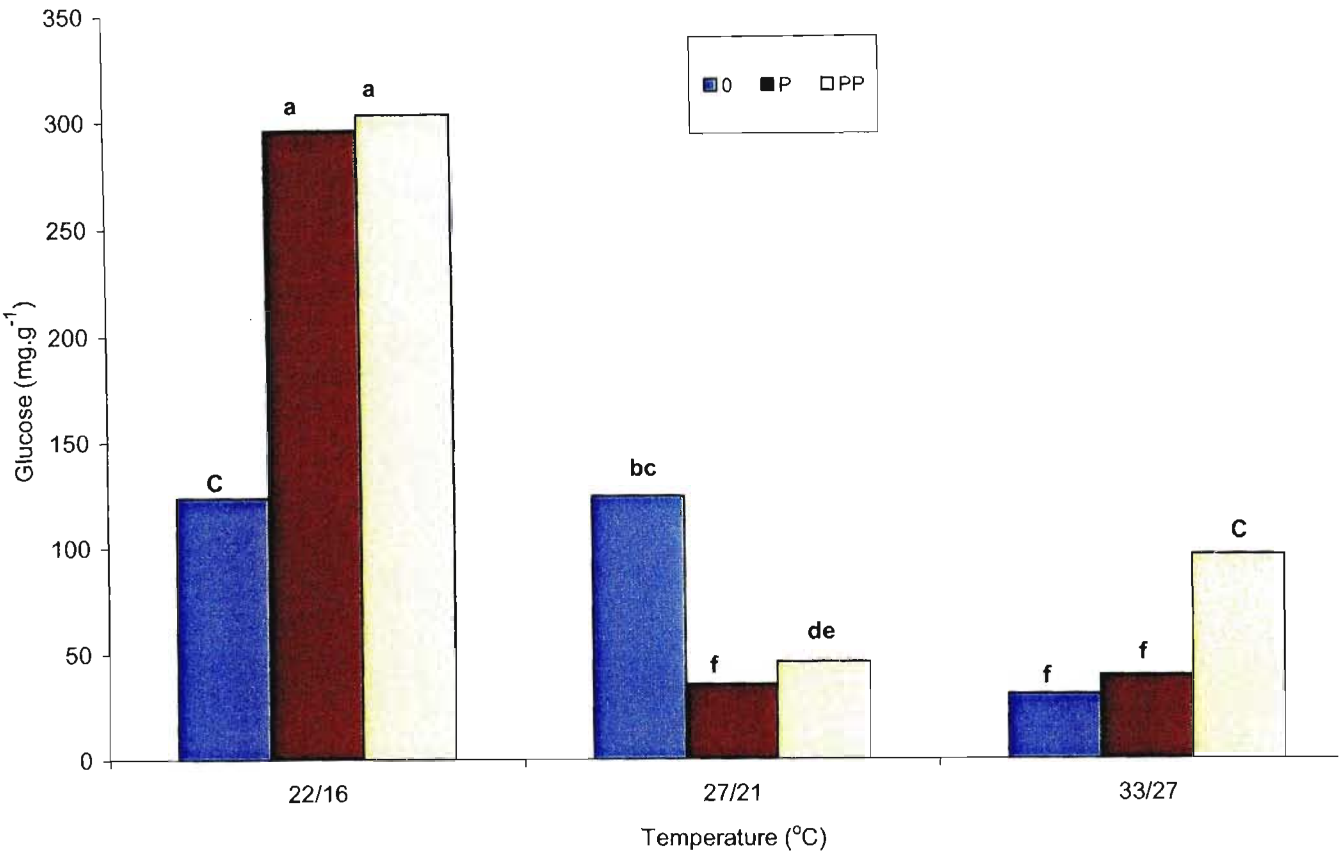


Figure 3.32. Mean effect of temperature on glucose across cultivars and phosphorus levels.

Glucose content was the highest at 15 days after flowering and there was significant decrease at later stages of seed development (APPENDIX 3.7). With respect to the phosphorus nutrition, in the control, there were no differences in glucose content in temperatures 22/16°C and 27/21°C, however, there was a significant decrease in glucose content at the temperature 33/27°C. This could be implying that, the temperature of 33/27°C is supra-optimal for glucose accumulation in maize seeds. At high phosphorus nutrition there was a significant decrease in glucose content from temperature 22/16°C to 27/21°C. From temperature 27/21°C to 33/27°C there was a significant increase in glucose content at high P levels.

Of particular interest for the purposes of this study was how myo-inositol would occur in seeds in response to phosphorus nutrition. This was of interest because myo-inostol forms the basic structure of phytate (Figure 1.1). This study showed that myo-inostol occurred in very low concentrations in maize seeds, compared with other detectable soluble carbohydrates (Figure 2.27). The reason for this low occurrence may be that myo-inositol is a very reactive cyclitol (sugar alcohol), easily used in the metabolic activities for the formation of higher level cyclitols (PETERBAUER & RITCHER, 2001) and phytate (HEGEMAN *et al.* 2001).

In the present study, there were significant interactions between temperature and cultivar ($P = 0.002$), temperature and stage ($P = 0.009$), phosphorus and stage ($P = 0.04$), cultivar and stage (0.008) and a temperature x phosphorus x cultivar x stage interaction ($P = 0.0035$), with respect to the occurrence of myo-inositol in maize seeds (APPENDIX 3.7 A). These interactions show that myo-inositol occurrence was influenced by the treatments (P and temperature) in this study, and that the quality protein maize and normal maize cultivars' response was dependent upon conditions of temperature and phosphorus content in seeds. Previous research reported that myo-inositol should be significantly high during early stages of seed development and decline as it is metabolised for the synthesis of high molecular weight oligosaccharides (MODI *et al.* 2000). The cultivar x stage interaction in this study confirmed that relationship. Of significant importance for the purposes of this study was the increase in myo-inostol in response to an increase in phosphorus application and growth temperature (Figure 3.32). Note also that the quality protein maize (Q1) showed lower myo-inostol levels compared with the normal maize cultivar (Figure 3.32).

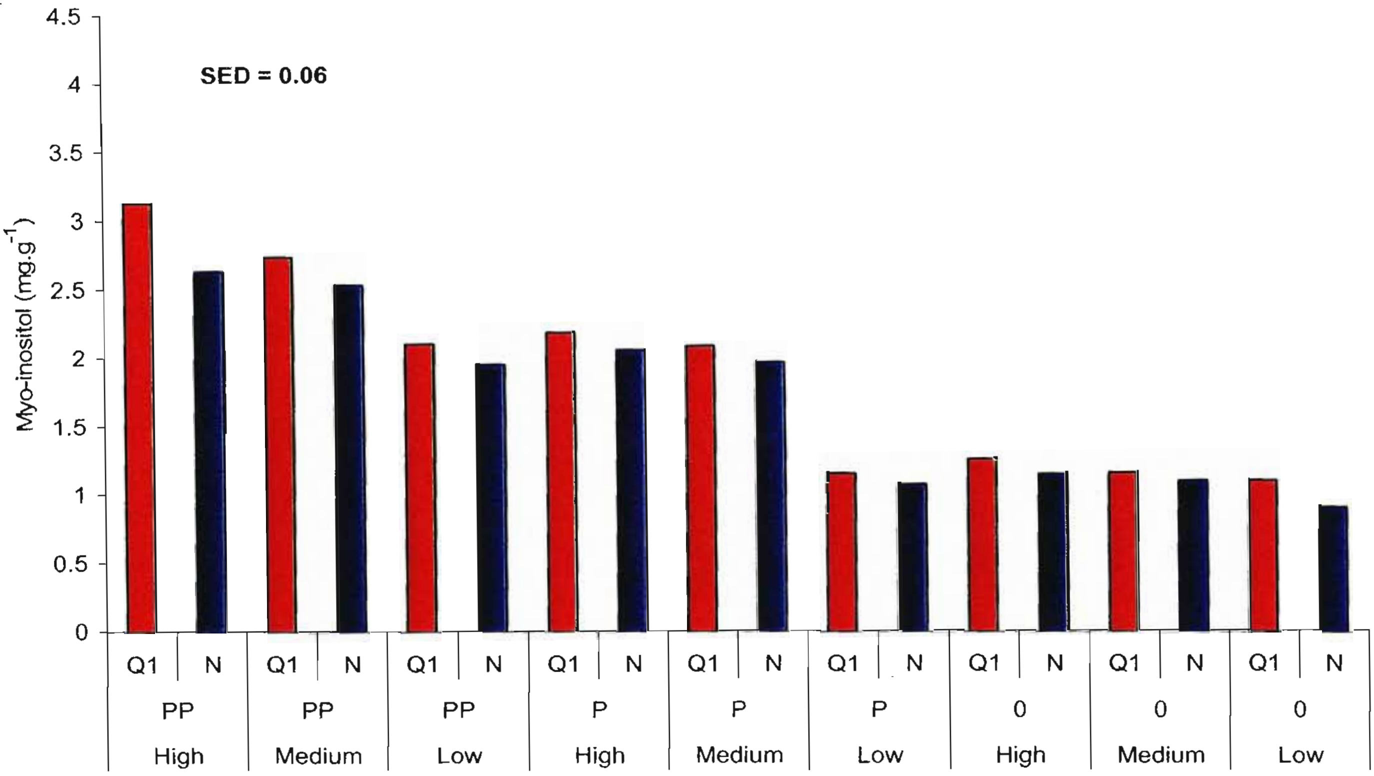


Figure 3.32. Myo-inositol concentrations in quality protein maize (Q1) and normal maize (N) cultivars at different levels of phosphorus (PP = 1.2 g, P = 0.12 g and 0 = 0 g/20 kg soil) under glasshouse conditions of high (33/27°C day/night), medium (27/21°C) and low (21/16°C).

References

BAILLY, C., LEYMARIE, J., LEHNER, A., ROUSSEAU, S., COME, D. & CORBINEAU, F. 2004. Catalase activity and expression in developing sunflower seeds as related to drying. *Journal of Experimental Botany* 55: 475-483.

BEWLEY, J.D., & BLACK, M. 1985. Seeds. Physiology of development and germination. New York, Plenum Press.

BLACKMAN, S.A., OBENDORF, R.L., & LEOPOLD, A.C. 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. *Plant Physiology* 100: 225-230.

BOHNERT, J.H., NELSON, D.E., & JENSEN, R.G. 1995. Adaptations to environmental stresses. *Plant Cell* 7: 1099-1111.

FAGERIA, N.B. 1992. Maximizing crop yields. New York, Marcel Dekker, Inc.

HEGEMAN, C.E., GOOD, L.L., & GRABAU, E.A. 2001. Expression of D-myo-inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. *Plant Physiology* 125: 1941-1948.

LIN, L., OCKENDEN, I., & LOTT, J.N.A. 2005. The concentration and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid 1-1 (lpa 1-1) corn (*Zea mays* L.) grain and grain parts. *Canadian Journal of Botany* 83: 131-141.

LOTT, J.N.A., GOODCHILD, D.J., & CRAIG, S. 1984. Studies of mineral reserves in pea (*Pisum sativum*) cotyledons using low-water-content procedures. *Australian Journal of Plant Physiology* 69: 829-833.

MCDONALD, M.B. 1999. Seed deterioration: physiology repair and assessment. *Seed Science and Technology*, 27: 177-237.

MERTZ, E.T. 1992. Discovery of high lysine, high tryptophan cereals. In: quality protein maize. (E.T. MERTZ, ed), pp 1-8. American Association of Cereal Chemists, St. Paul, MN.

MODI, A.T., MCDONALD, M.B., & STREETER, J.G. 2000. Soluble carbohydrates in soybean seeds during development and imbibition. *Seed Science and Technology* 28: 115-127.

MODI, A.T. 2005. Assessment of pepper seed performance using desiccation sensitivity. *Seed Science and Technology* 33: 19-30.

MUNCK, L. 1992. The case of high-lysine barley breeding. In: barley: genetics, biochemistry, molecular biology and biotechnology. (P.R. SHEWRY, ed), pp 573-601. CAB International, Wallingford, U.K.

O'DELL, B.L., DE BOLAND, A.R., & KOIRTYOHANN, S.R. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. *Journal of Agricultural and Food Chemistry* 20: 718-721.

PETERBAUER, T., & RICHTER, A. 2001. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Science Research* 11: 185-197.

PETERBAUER, T., LAHUTA, L.B., BLÖCHL, A., MUCHA, J., JONES, D.A., HEDLEY, C.L., GÒRECKI, R.J., RICHTER, A. 2001. Analysis of the raffinose family oligosaccharide pathway in pea seeds with contrasting carbohydrate composition. *Plant Physiology* 127: 1764–1772.

RABOY, V., HUDSON, S.J., & DICKINSON, D.B. 1985. Reduced phytic acid content does not affect on germination of soybean seeds. *Plant Physiology* 79: 323-325.

RABOY, V., & DICKINSON, D.B. 1987. The timing and rate of phytic acid accumulation in developing soybean seeds. *Plant Physiology* 85: 841-844.

RABOY, V., & DICKINSON, D.B.1993. Phytic acid levels in seeds of *Gycine max* and *Glycime Soja* as influenced by phosphorus status. *Crop Science* 33: 1300-1305.

RABOY, V. 1997. Accumulation and storage of phosphate and minerals. *In*: cellular and molecular biology of plant seed development. (Larkins, B.A & Vasil, I.K, eds); pp. 441-477. Kluwer Academic, Netherlands.

SUN, W.Q., IRVING, T.C., & LEOPOLD, A.C. 1994. The role of sugar vitrification and membrane phase transition in seed desiccation tolerance. *Physiology Plant* 90: 621-628.

THOMPSON, L.M. 1975. Weather variability climatic change and grain. *Production Science*188: 535-541.

CHAPTER 4 GENERAL CONCLUSIONS

This study determined the seed quality responses of two maize types (quality protein and normal) to phosphorus nutrition under three temperature conditions, designated as low, medium and high. A preliminary study to screen seed of 10 cultivars of normal maize and two cultivars of quality protein maize (QPM) showed that normal maize seeds contained significantly more phytate levels than QPM. Further, it was decided that one cultivar of each maize type, showing the highest levels of phytate in each group, would be studied to investigate the effects of P nutrition and growth temperature under controlled environment conditions. ESEM-EDAX is used for determination of the sites of phytate synthesis and storage (globoids) as well as mineral content in seed tissues. Calorimetry is used to determine phytate concentration in seeds. Gas chromatography is used for the determination of soluble carbohydrates in seeds; and international rules for testing seeds (ISTA rules) were used to determine seed quality with respect to germinability and vigour. Seed quality parameters such as conductivity test, germination test and growth parameters (Plant height and leaf emergence rate) assisted to correlate phytate content with seed vigour.

Preliminary screening studies showed that quality protein maize has lower phytate content than normal maize, in both the endosperm and embryonic axis tissues. The germination capacity of quality protein maize was found to be significantly less than that of normal maize, but this difference was not found in seedling emergence. Further studies, on the cultivars that were selected for high phytate, showed that the QPM cultivar consistently displayed a less vigorous seedling than the normal maize, with respect to seedling size. This difference between cultivars was shown at three day/night temperature regimes (33/27°C, 27/21°C and 21/16°C) and three seed P nutrition regimes (0, 0.12 and 1.2 g / 20 kg soil) in pot trials occurring under natural light conditions. Plants were grown under the aforementioned temperature and P nutrition regimes until flowering, when self pollination was undertaken. Seeds were sampled at 15, 22 and 33 days after pollination to determine germination capacity, phytate content, selected mineral (P, K and Mg) and soluble carbohydrate content. The glasshouse study confirmed the findings of the preliminary study, that normal maize had higher phytate content than QPM maize. It was further found that high phytate levels were associated with high mineral (P and Mg) content levels in maize seeds. The levels of phytate and P were positively influenced by P nutrition. This finding agrees with the findings of RABOY & DICKINSON (1984). It was also established that high phytate seeds displayed high vigour, as determined by a cold test. Phosphorus nutrition was found to be related to increased levels of some soluble carbohydrates, notable glucose and fructose. Myo-inositol occurred at significantly lower concentrations than the other soluble carbohydrates. P nutrition however, improves myo-inositol concentrations in correlation with increasing temperature conditions of seed development, in agreement with previous studies (HEGEMAN *et al.,* 2001; (PETERBAUER & RITCHER, 2001). The increase in seed germination capacity, mineral element content and phytate content increases with maturity confirmed that seed vigour is increases during seed development as previously shown by BAILLY *et al.* (2004).

This study confirmed that temperature is the main factor controlling the rate of seed development (FAGERIA, 1992), since leaf emergence rate and plant height were significantly influenced by temperature. Furthermore, there was a positive correlation between seed germination capacity and temperature. Mineral element Mg and P, phytate content were influenced by temperature. The temperature of 33/27°C was optimal, whereas soluble carbohydrates such as glucose, fructose and galactose formation were enhanced at low temperature stress of 22/16°C.

The findings of this study are significant for an understanding of the differences between QPM and normal maize, with respect to seed quality. In addition, the findings about the positive effect of P nutrition on seed quality, which apparently influenced increased phytate concentrations, showed that phytate has a positive role in seed quality. The role of phytate is in storage of mineral elements that may be essential during germination for metabolic processes such as osmotic potential regulation (e.g. K^+), enzyme activity (e.g. MgATPase) and ATP synthesis (P is an important element of ATP). The limitation of this study was that the cultivars were not tested under field conditions to determine their performance under conditions similar to those for large scale crop production. On the laboratory studies: there are a number of proteins that are triggered during temperature and nutrient stress, these proteins could be determined and correlated with P nutrition and seed performance (BOHNERT *et al.*, 1995).

In conclusion, the study provided evidence to confirm previous findings that phytic acid content increase linear during seed development (RABOY & DICKINSON, 1987). Whereas, previous studies have shown that low temperature stress during seed development enhances the formation of phytic acid (HORVATIC & BALINT, 1996), the current study reported phytic acid formation to be enhanced during high temperature stress. This study provided evidence that phytic acid content correlates with seed vigour which was evident in the normal and quality protein maize. In addition, this study confirmed previous findings that, phytate and mineral element content were significantly low in endosperm due to the fact that cells of the starchy endosperm of cereals die during the maturation phase of seed development (LIN *et al.*, 2005). This study also showed a relation between phytate content and mineral element in seeds. However, phosphorus nutrition and temperature stress did not impact on myo-inositol.

References

BAILLY, C., LEYMARIE, J., LEHNER, A., ROUSSEAU, S., COME, D. & CORBINEAU, F. 2004. Catalase activity and expression in developing sunflower seeds as related to drying. *Journal of Experimental Botany* 55: 475-483.

BOHNERT, J.H., NELSON, D.E., & JENSEN, R.G. 1995. Adaptations to environmental stresses. *Plant Cell* 7: 1099-1111.

FAGERIA, N.B. 1992. Maximizing crop yields. New York, Marcel Dekker, Inc.

HEGEMAN, C.E., GOOD, L.L., & GRABAU, E.A. 2001. Expression of D-myo-inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. *Plant Physiology* 125: 1941-1948.

HORVATIC, M., & BALINT, L. 1996. Relationship among the phytic acid and protein content during maize grain maturation. *Journal of Agronomy and Crop Science* 176: 73-77.

LIN, L., OCKENDEN, I., & LOTT, J.N.A. 2005. The concentration and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid 1-1 (lpa 1-1) corn (*Zea mays* L.) grain and grain parts. *Canadian Journal of Botany* 83: 131-141.

PETERBAUER, T., & RICHTER, A. 2001. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Science Research* 11: 185-197.

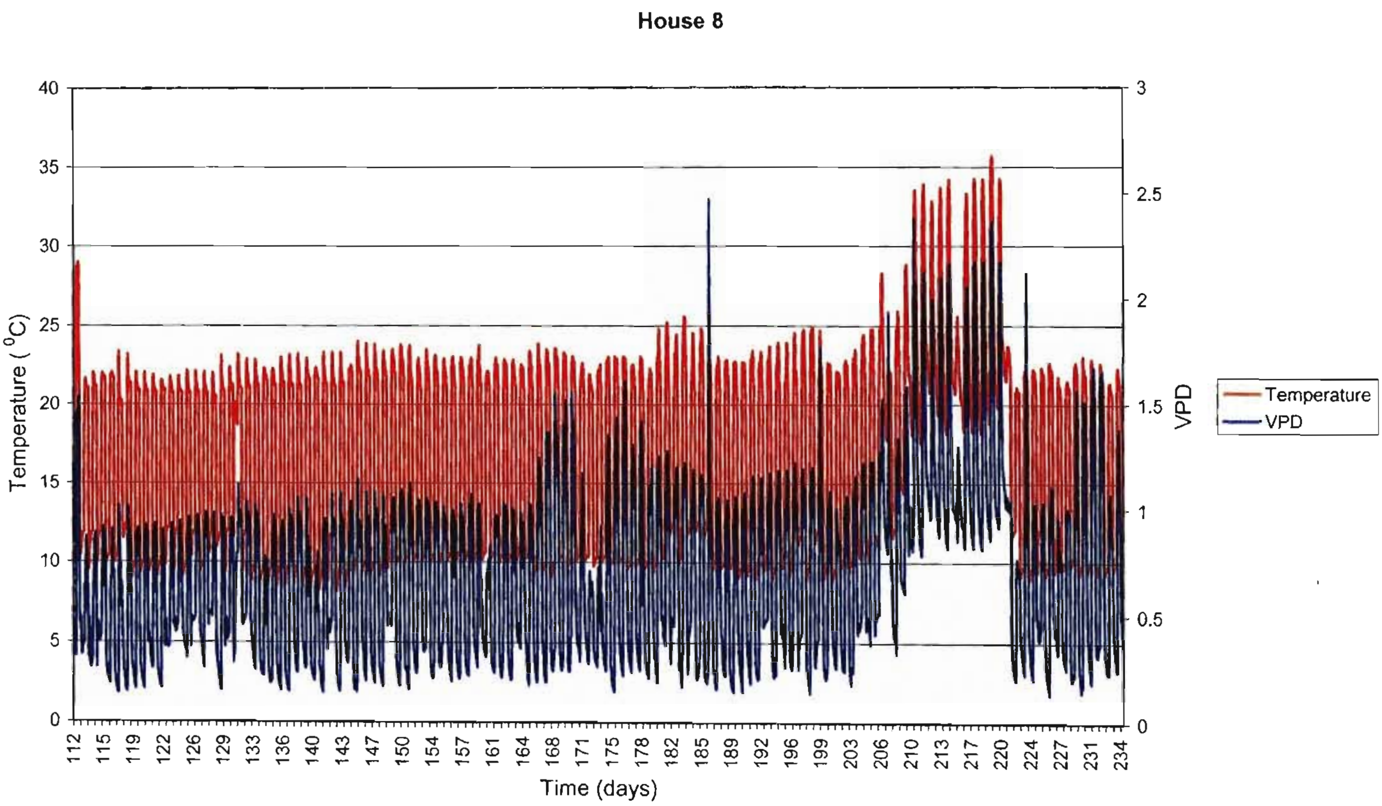
RABOY, V., & DICKINSON, D.B. 1984. Effect of phosphorus and zinc nutrition on soybean seed phytic acid and zinc. *Plant Physiology* 75: 1094-1098.

RABOY, V., & DICKINSON, D.B. 1987. The timing and rate of phytic acid accumulation in developing soybean seeds. *Plant Physiology* 85: 841-844.

APPENDIX 2.1. Chen's reagent

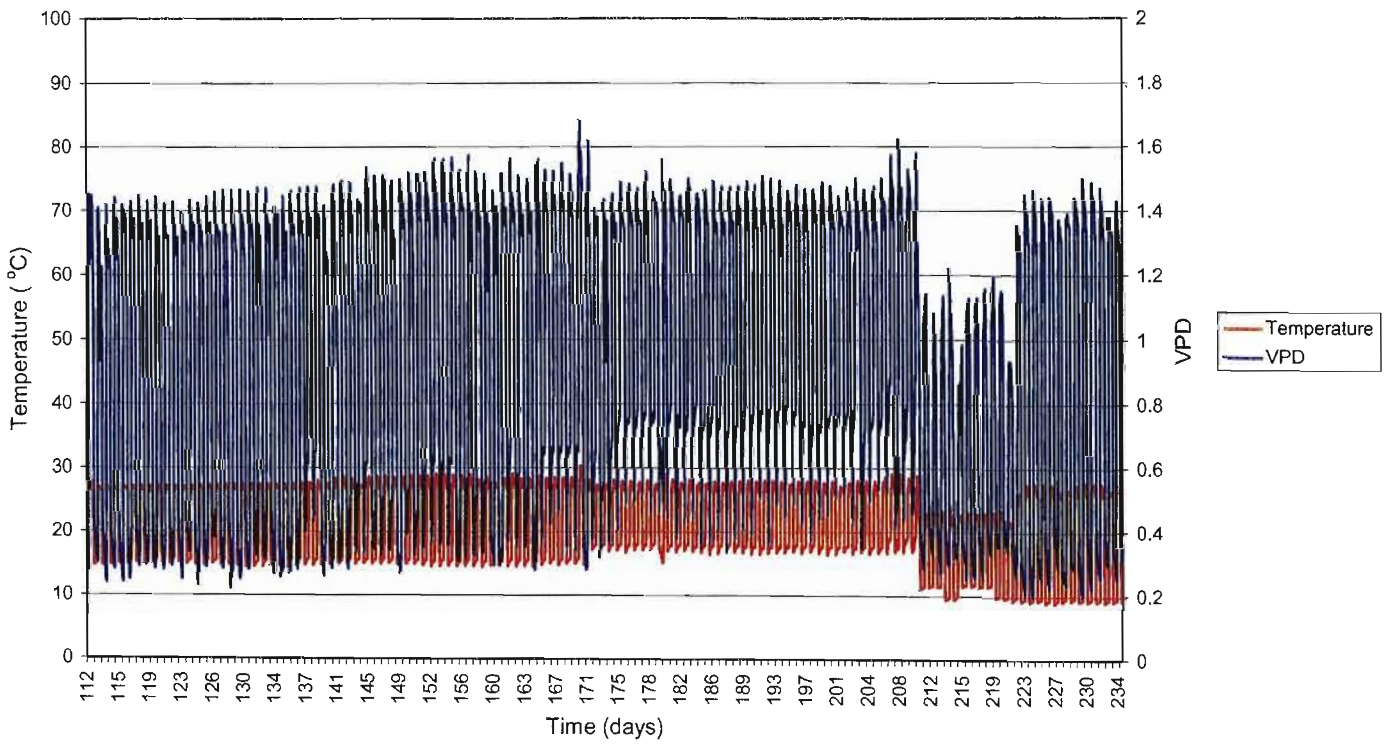
1 volume 6N H_2SO4
1 volume 2.5% Ammonium Molybdate
1 volume 10% Ascorbic Acid (store stock solution at 4°C)
2 volumes dd H_2O

APPENDIX 2.2. Climatic conditions in the glasshouse where maize were grown house 10 (22/16°C), house 9 (27/21°C) and house 8 (33/27°C) at actual days of the year in 2005.

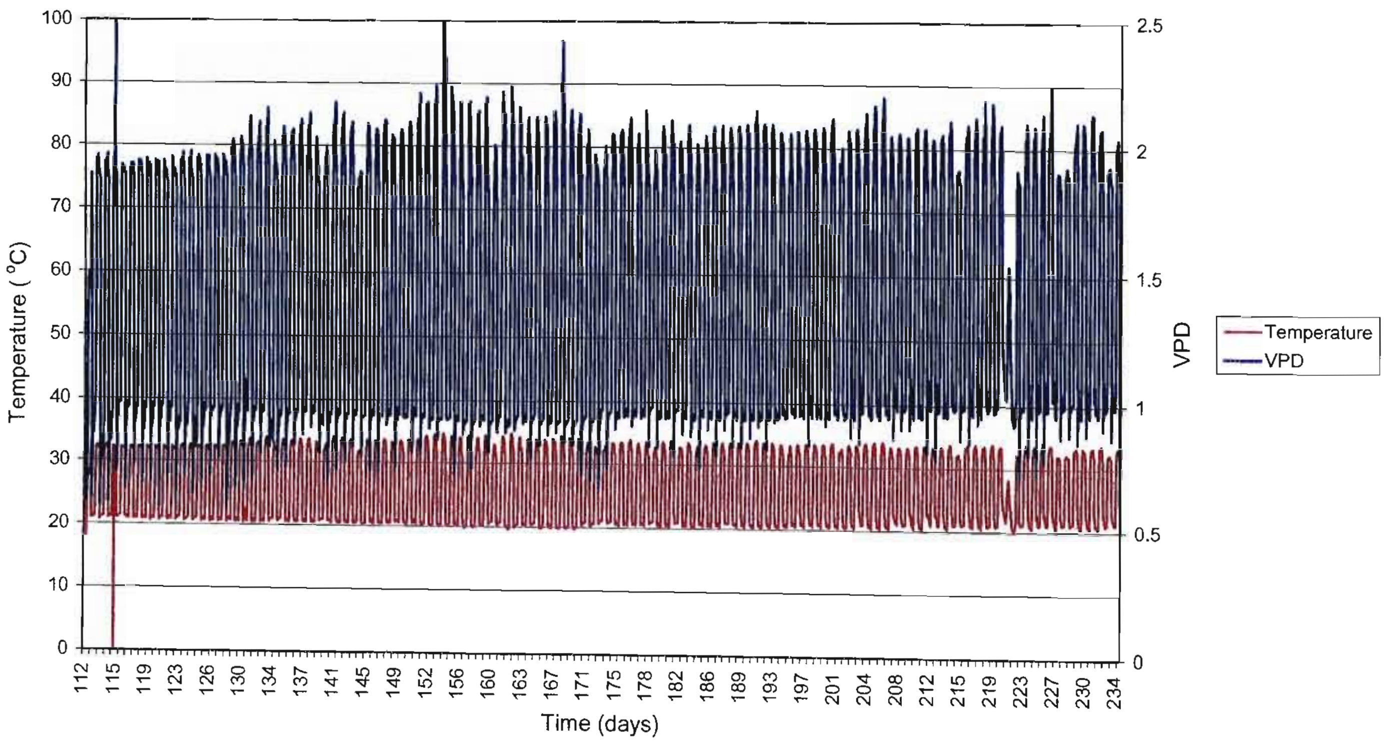


Appendix 2.2 (continued)

House 9



House 10



APPENDIX 3.1. Analysis of variance for maize seed mineral element content from EDAX.

Analysis of variance

A. Variate: Phosphorus

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.2752	0.1376	0.33	
Rep*Units* stratum					
Variety	11	25.4320	2.3120	5.62	<.001
Residual	22	9.0543	0.4116		
Total	35	34.7614			

Analysis of variance

B. Variate: Magnesium

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.00245	0.00122	0.02	
Rep*Units* stratum					
Variety	11	2.98734	0.27158	4.99	<.001
Residual	22	1.19628	0.05438		
Total	35	4.18607			

Analysis of variance

C. Variate: Potassium

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.6768	0.3384	0.74	
Rep*Units* stratum					
Variety	11	55.4774	5.0434	11.07	<.001
Residual	22	10.0196	0.4554		
Total	35	66.1738			

APPENDIX 3.2. Analysis of variance of seed emergence

Analysis of variance

Variate: Seed emergence rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	159.15	79.57	1.97	
Rep*Units* stratum					
cult	1	0.30	0.30	0.01	0.932
fert	2	216.70	108.35	2.68	0.083
temp	2	160.26	80.13	1.98	0.154
cult.fert	2	42.26	21.13	0.52	0.598
cult.temp	2	62.48	31.24	0.77	0.470
fert.temp	4	219.63	54.91	1.36	0.269
cult.fert.temp	4	29.63	7.41	0.18	0.946
Residual	34	1376.19	40.48		
Total	53	2266.59			

APPENDIX 3.3. Analysis of variance of plant height and leaf emergence rate during seed development in response to temperature and phosphorus nutrition.

Analysis of variance

A. Variate: Plant_height

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	205.8	102.9	0.21	
Rep*Units* stratum					
cult	1	788.3	788.3	1.61	0.205
temp	2	35765.4	17882.7	36.42	<.001
fert	2	3456.5	1728.2	3.52	0.030
cult.temp	2	1123.5	561.7	1.14	0.319
cult.fert	2	326.8	163.4	0.33	0.717
temp.fert	4	1260.6	315.2	0.64	0.633
cult.temp.fert	4	1541.2	385.3	0.78	0.535
Residual	952	467463.1	491.0		

Analysis of variance

B. Variate: Leaf_emergence rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.000	0.000	0.00	
Rep*Units* stratum					
cult	1	9.091	9.091	2.63	0.105
temp	2	236.858	118.429	34.22	<.001
fert	2	33.463	16.731	4.83	0.008
cult.temp	2	2.681	1.341	0.39	0.679
cult.fert	2	4.335	2.168	0.63	0.535
temp.fert	4	6.420	1.605	0.46	0.762
cult.temp.fert	4	4.115	1.029	0.30	0.880
Residual	952	3295.037	3.461		
Total	971	3592.000			

APPENDIX 3.4. Analysis of variance of seed emergence rate of glasshouse maize during three stages of development (15, 22 and 33 days after pollination).

Analysis of variance

Variate: Seed germination %

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	1	778.7	778.7	4.65	
Rep*Units* stratum					
temp	2	40090.7	20045.4	119.65	<.001
fert	2	4860.4	2430.2	14.51	<.001
cult	1	2523.0	2523.0	15.06	<.001
stage	2	41224.0	20612.0	123.03	<.001
temp.fert	4	5870.1	1467.5	8.76	<.001
temp.cult	2	808.2	404.1	2.41	0.099
fert.cult	2	5570.2	2785.1	16.62	<.001
temp.stage	4	5644.5	1411.1	8.42	<.001
fert.stage	4	3132.7	783.2	4.67	0.003
cult.stage	2	7.4	3.7	0.02	0.978
temp.fert.cult	4	3178.4	794.6	4.74	0.002
temp.fert.stage	8	6457.0	807.1	4.82	<.001
temp.cult.stage	4	3565.6	891.4	5.32	0.001
fert.cult.stage	4	4896.4	1224.1	7.31	<.001
temp.fert.cult.stage	8	7904.8	988.1	5.90	<.001
Residual	53	8879.3	167.5		
Total	107	145391.4			

APPENDIX 3.5. Analysis of variance of mineral element content in seeds of two maize cultivars subjected to temperature and phosphorus treatments at three stages of seed development.

Analysis of variance

A. Variate: Potassium

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	1.0679	0.5339	1.05	
Rep*Units* stratumt					
temp	2	0.6709	0.3354	0.66	0.517
fert	2	0.9295	0.4647	0.92	0.401
cult	1	2.7501	2.7501	5.42	0.021
stage	2	4.4572	2.2286	4.39	0.013
parts	1	1.6943	1.6943	3.34	0.069
temp.fert	4	4.7024	1.1756	2.32	0.058
temp.cult	2	1.3146	0.6573	1.30	0.276
fert.cult	2	0.7560	0.3780	0.75	0.476
temp.stage	4	3.7519	0.9380	1.85	0.120
fert.Stage	4	5.5706	1.3927	2.75	0.029
cult.stage	2	0.8212	0.4106	0.81	0.446
temp.parts	2	1.4320	0.7160	1.41	0.246
fert.Parts	2	5.2538	2.6269	5.18	0.006
cult.Parts	1	0.0063	0.0063	0.01	0.911
stage.Parts	2	14.4103	7.2052	14.21	<.001
temp.fert.cult	4	4.1483	1.0371	2.05	0.089
temp.fert.stage	8	6.6170	0.8271	1.63	0.117
temp.cult.stage	4	3.9168	0.9792	1.93	0.106
fert.cult.Stage	4	4.8537	1.2134	2.39	0.052
temp.fert.parts	4	2.7462	0.6866	1.35	0.251
temp.cult.parts	2	2.9747	1.4874	2.93	0.055
fert.cult.parts	2	3.3350	1.6675	3.29	0.039
temp.stage.parts	4	3.4842	0.8710	1.72	0.147
fert.stage.parts	4	1.6193	0.4048	0.80	0.527
cult.stage.parts	2	3.8161	1.9081	3.76	0.025
temp.fert.cult.stage	8	5.9649	0.7456	1.47	0.169
temp.fert.cult.parts	4	2.4753	0.6188	1.22	0.303
temp.fert.stage.parts	8	7.5468	0.9433	1.86	0.068
temp.cult.stage.parts	4	3.3751	0.8438	1.66	0.160
fert.cult.stage.parts	4	2.5353	0.6338	1.25	0.291
temp.fert.cult.stage.parts	8	7.8716	0.9839	1.94	0.055
Residual	214	108.5189	0.5071		
Total	323	225.3880			

B. Variate: Magnesium

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.00278	0.00139	0.04	
Rep*Units* stratum					
temp	2	0.29990	0.14995	3.79	0.024
fert	2	2.63157	1.31579	33.28	<.001
cult	1	0.14230	0.14230	3.60	0.059
stage	2	2.69061	1.34530	34.03	<.001
parts	1	3.59945	3.59945	91.04	<.001
temp.fert	4	0.24345	0.06086	1.54	0.192
temp.cult	2	0.02855	0.01427	0.36	0.697
fert.cult	2	0.00142	0.00071	0.02	0.982
temp.stage	4	0.84164	0.21041	5.32	<.001
fert.stage	4	0.55098	0.13774	3.48	0.009
cult.stage	2	0.04159	0.02079	0.53	0.592
temp.parts	2	0.56387	0.28194	7.13	0.001
fert.parts	2	1.13780	0.56890	14.39	<.001
cult.parts	1	0.43927	0.43927	11.11	0.001
stage.parts	2	2.89755	1.44878	36.65	<.001
temp.fert.cult	4	1.02755	0.25689	6.50	<.001
temp.fert.stage	8	0.61262	0.07658	1.94	0.056
temp.cult.stage	4	0.04622	0.01155	0.29	0.883
fert.cult.stage	4	0.05980	0.01495	0.38	0.824
temp.fert.parts	4	0.02238	0.00559	0.14	0.967
temp.cult.parts	2	0.29930	0.14965	3.79	0.024
fert.cult.parts	2	0.03335	0.01668	0.42	0.656
temp.stage.parts	4	1.19945	0.29986	7.58	<.001
fert.stage.parts	4	1.43804	0.35951	9.09	<.001
cult.stage.parts	2	0.09662	0.04831	1.22	0.297
temp.fert.cult.stage	8	1.41390	0.17674	4.47	<.001
temp.fert.cult.parts	4	0.32111	0.08028	2.03	0.091
temp.fert.stage.parts	8	0.30501	0.03813	0.96	0.465
temp.cult.stage.parts	4	0.33845	0.08461	2.14	0.077
fert.cult.stage.parts	4	0.14355	0.03589	0.91	0.460
temp.fert.cult.stage.parts	8	0.34754	0.04344	1.10	0.365
Residual	214	8.46055	0.03954		
Total	216	50.3250			

C.Variate: P

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.00115	0.00058	0.04	
Rep*Units* stratum					
Temp	2	0.43647	0.21824	14.02	<.001
fert	2	3.41027	1.70513	109.57	<.001
cult	1	1.01114	1.01114	64.98	<.001
stage	2	1.84580	0.92290	59.31	<.001
parts	1	3.26003	3.26003	209.49	<.001
temp.fert	4	0.23010	0.05753	3.70	0.006
temp.cult	2	0.11386	0.05693	3.66	0.027
fert.cult	2	0.25395	0.12698	8.16	<.001
temp.stage	4	0.26852	0.06713	4.31	0.002
fert.stage	4	2.11279	0.52820	33.94	<.001
cult.stage	2	0.48649	0.24324	15.63	<.001
temp.parts	2	0.30869	0.15435	9.92	<.001
fert.parts	2	2.20261	1.10131	70.77	<.001
cult.parts	1	1.17602	1.17602	75.57	<.001
stage.parts	2	3.59470	1.79735	115.50	<.001
temp.fert.cult	4	0.41747	0.10437	6.71	<.001
temp.fert.stage	8	0.68049	0.08506	5.47	<.001
temp.cult.stage	4	0.24871	0.06218	4.00	0.004
fert.cult.stage	4	0.35138	0.08785	5.65	<.001
temp.fert.parts	4	0.08236	0.02059	1.32	0.262
temp.cult.parts	2	0.52656	0.26328	16.92	<.001
fert.cult.parts	2	0.84664	0.42332	27.20	<.001
temp.stage.parts	4	1.15660	0.28915	18.58	<.001
fert.stage.parts	4	2.18209	0.54552	35.06	<.001
cult.stage.parts	2	0.87195	0.43597	28.02	<.001
temp.fert.cult.stage	8	0.75258	0.09407	6.05	<.001
temp.fert.cult.parts	4	0.53717	0.13429	8.63	<.001
temp.fert.stage.parts	8	0.33305	0.04163	2.68	0.008
temp.cult.stage.parts	4	1.41830	0.35458	22.79	<.001
fert.cult.stage.parts	4	0.86753	0.21688	13.94	<.001
temp.fert.cult.stage.parts	8	1.03858	0.12982	8.34	<.001
Residual	214	3.33018	0.01556		
Total	323	36.35426			

APPENDIX 3.6. Analysis of variance of phytate content in seeds of two maize cultivars subjected to temperature and phosphorus treatments at three stages of seed development.

Variate: Phytate_content

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	5	0.84460	0.16892	2.38	
Rep*Units* stratum					
fert	2	3.69896	1.84948	26.05	<.001
cult	1	2.93750	2.93750	41.38	<.001
stage	2	7.06780	3.53390	49.78	<.001
temp	1	0.23583	0.23583	3.32	0.070
fert.cult	2	0.13166	0.06583	0.93	0.398
fert.stage	4	0.31723	0.07931	1.12	0.350
cult.stage	2	0.89349	0.44674	6.29	0.002
fert.temp	2	0.25552	0.12776	1.80	0.168
cult.temp	1	0.00972	0.00972	0.14	0.712
stage.temp	2	0.42718	0.21359	3.01	0.052
fert.cult.stage	4	0.70259	0.17565	2.47	0.046
fert.cult.temp	2	0.03403	0.01702	0.24	0.787
fert.stage.temp	4	0.22056	0.05514	0.78	0.542
cult.stage.temp	2	0.09649	0.04825	0.68	0.508
fert.cult.stage.temp	4	0.20142	0.05036	0.71	0.587
Residual	175	12.42364	0.07099		
Total	215	30.49822			

APPENDIX 3.7. Analysis of variance of soluble carbohydrate in seeds of two maize cultivars subjected to temperature and phosphorus treatments at three stages of seed development.

A. Variate: Myo-inositol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	30.16	15.08	0.90	
Rep*Units* stratum					
temp	2	75.36	37.68	2.24	0.112
fert	2	25.17	12.59	0.75	0.476
cult	1	44.53	44.53	2.65	0.107
stages	2	34.24	17.12	1.02	0.365
temp.fert	4	122.06	30.52	1.81	0.132
temp.cult	2	219.29	109.65	6.52	0.002
fert.cult	2	74.13	37.07	2.20	0.116
temp.stages	4	242.44	60.61	3.60	0.009
fert.stages	4	175.07	43.77	2.60	0.040
cult.stages	2	168.27	84.13	5.00	0.008
temp.fert.cult	4	159.33	39.83	2.37	0.057
temp.fert.stages	8	228.27	28.53	1.70	0.108
temp.cult.stages	4	146.54	36.64	2.18	0.077
fert.cult.stages	4	152.95	38.24	2.27	0.066
temp.fert.cult.stages	8	292.98	36.62	2.18	0.035
Residual	106	1783.89	16.83		
Total	161	3974.69			

B. Variate: Sucrose

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	12221.	6110.	0.96	
Rep*Units* stratum					
temp	2	329647	164824	25.81	<.001
fert	2	23960	11980	1.88	0.158
cult	1	3110	3110	0.49	0.487
stages	2	28877	14438	2.26	0.109
temp.fert	4	48962	12240	1.92	0.113
temp.cult	2	348033	174016	27.24	<.001
fert.cult	2	124125	62062	9.72	<.001
temp.stages	4	477976	119494	18.71	<.001
fert.stages	4	232433	58108	9.10	<.001
cult.stages	2	98940	49470	7.75	<.001
temp.fert.cult	4	131728	32932	5.16	<.001
temp.fert.stages	8	281095	35137	5.50	<.001
temp.cult.stages	4	32041	8010	1.25	0.293
fert.cult.stages	4	61026	15257	2.39	0.056
temp.fert.cult.stages	8	90219	11277	1.77	0.092
Residual	106	677042	6387		
Total	161	3001435			

C. Variate: Fructose

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	1891	946	0.21	
Rep*Units* stratum					
temp	2	724318	362159	79.37	<.001
fert	2	84666	42333	9.28	<.001
cult	1	5458	5458	1.20	0.277
stages	2	751564	375782	82.35	<.001
temp.fert	4	194175	48544	10.64	<.001
temp.cult	2	162438	81219	17.80	<.001
fert.cult	2	5707	2853	0.63	0.537
temp.stages	4	829878	207469	45.47	<.001
fert.stages	4	78608	19652	4.31	0.003
cult.stages	2	34239	17120	3.75	0.027
temp.fert.cult	4	57665	14416	3.16	0.017
temp.fert.stages	8	437223	54653	11.98	<.001
temp.cult.stages	4	142662	35665	7.82	<.001
fert.cult.stages	4	16405	4101	0.90	0.468
temp.fert.cult.stages	8	108282	13535	2.97	0.005
Residual	106	483678	456		
Total	161	4118857			

D. Variate: Galactose

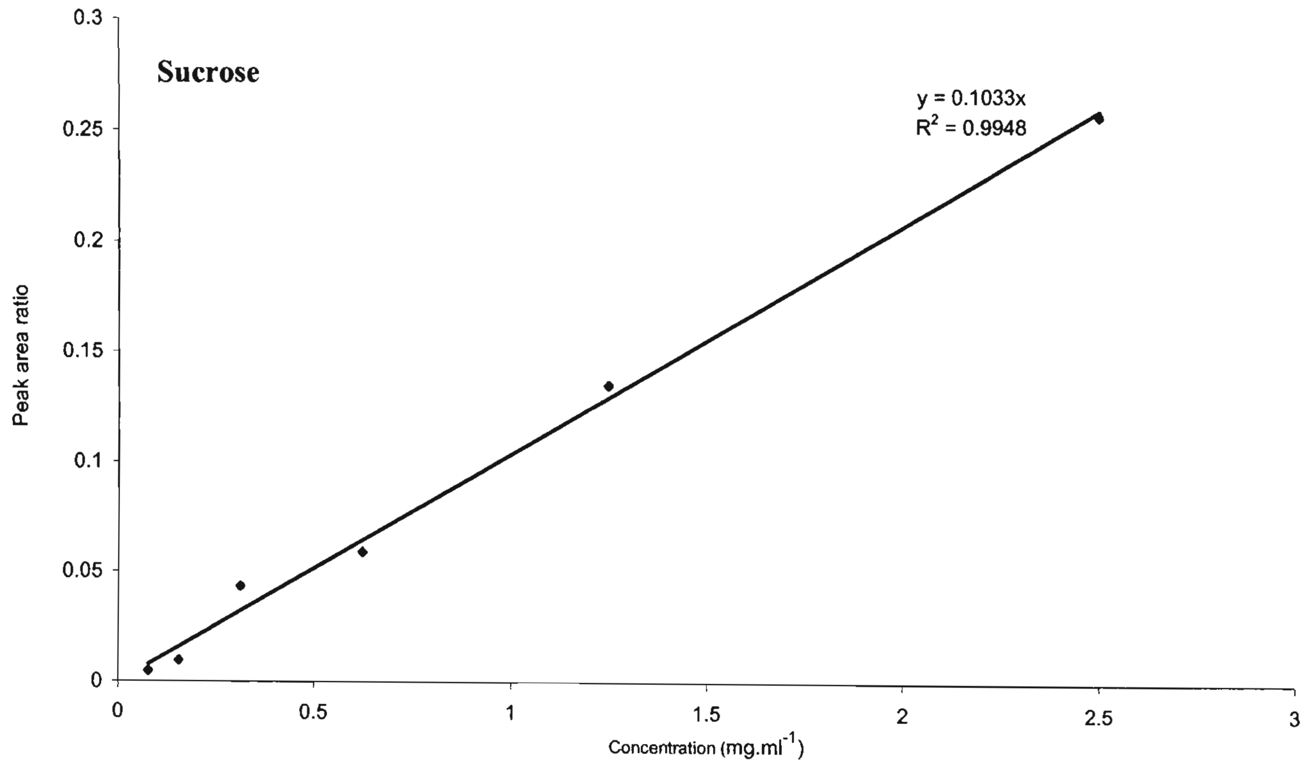
Variate: Gal

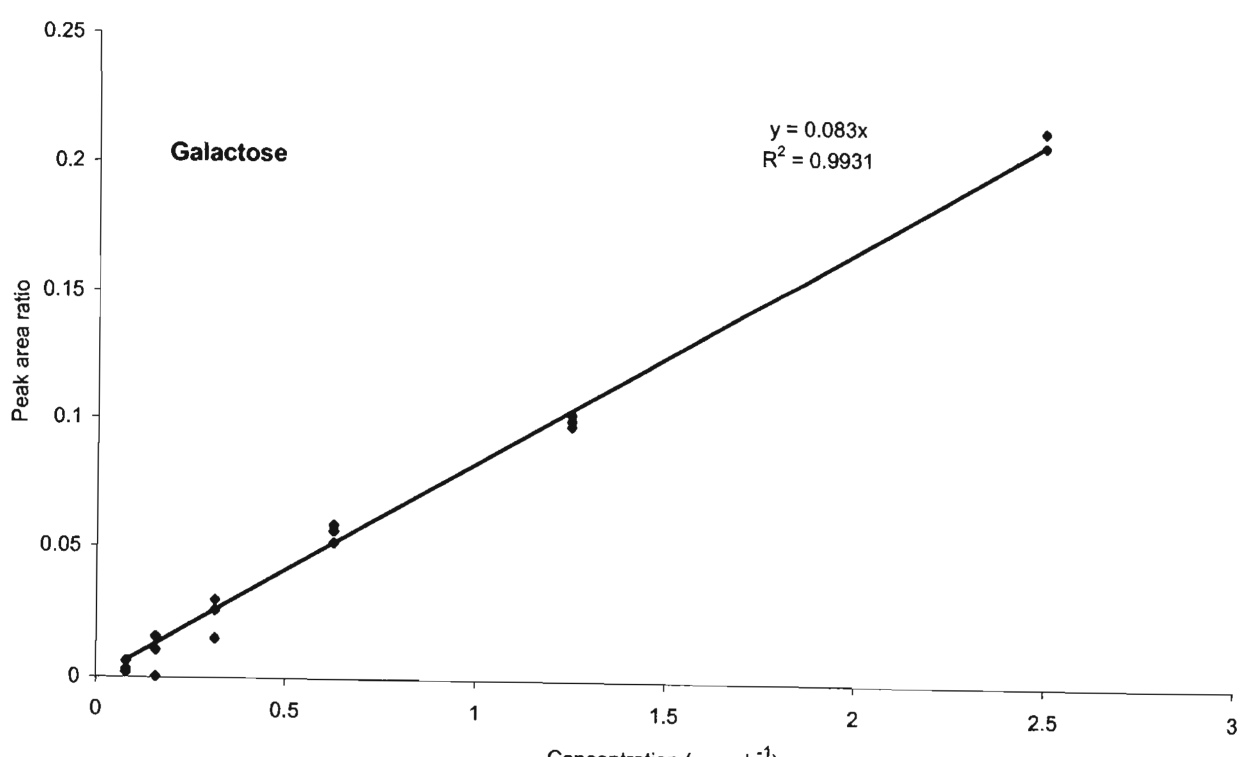
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	4303	2151.	0.88	
Rep*Units* stratum					
temp	2	353493	176747	72.35	<.001
fert	2	402896	201448	82.46	<.001
cult	1	29611	29611	12.12	<.001
stages	2	209219	104610	42.82	<.001
temp.fert	4	946681	236670	96.88	<.001
temp.cult	2	6923	3461	1.42	0.247
fert.cult	2	11443	5722	2.34	0.101
temp.stages	4	238551	59638	24.41	<.001
fert.stages	4	164058	41014	16.79	<.001
cult.stages	2	8565	4282	1.75	0.178
temp.fert.cult	4	67580	16895	6.92	<.001
temp.fert.stages	8	867292	108411	44.38	<.001
temp.cult.stages	4	110774	27693	11.34	<.001
fert.cult.stages	4	100147	25037	10.25	<.001
temp.fert.cult.stages	8	114311	14289	5.85	<.001
Residual	106	258961	2443		
Total	161	3894808			

E. Variate: Glucose

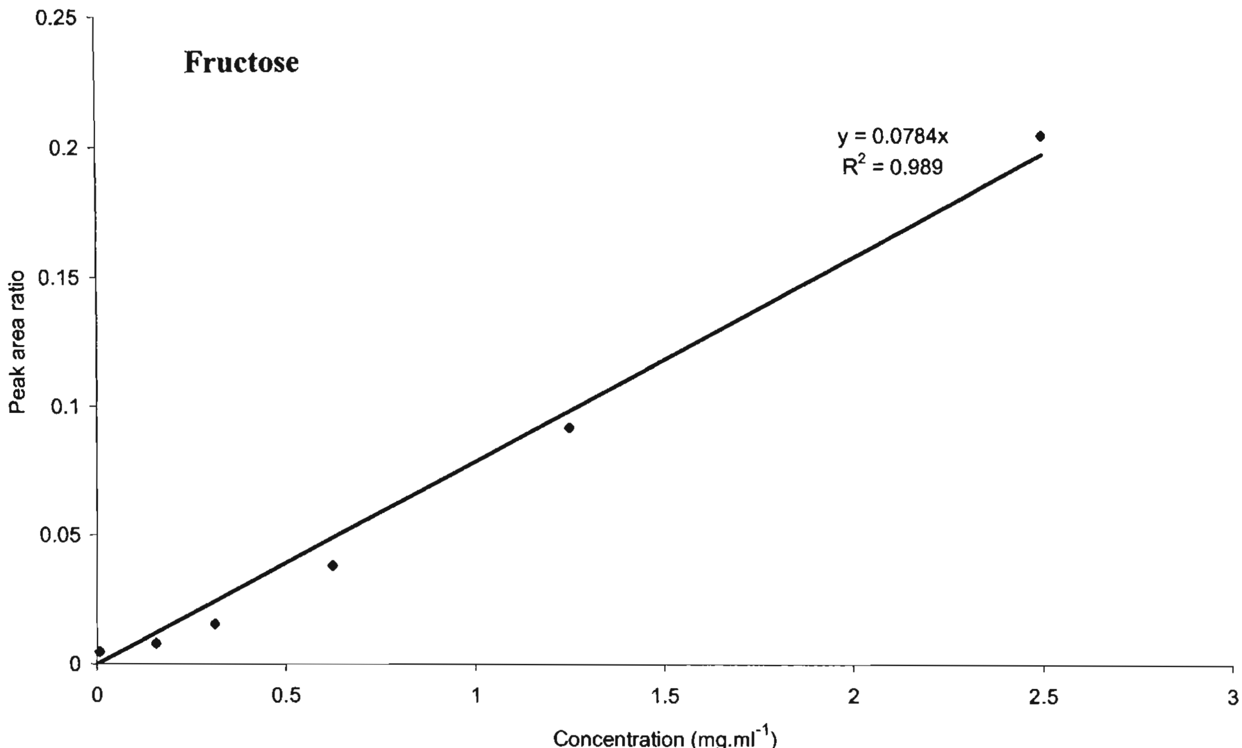
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	5245	2622	0.45	
Rep.*Units* stratum					
temp	2	1156168	578084	99.62	<.001
fert	2	83911	41956	7.23	0.001
cult	1	6762	6762	1.17	0.283
stages	2	1028853	514426	88.65	<.001
temp.fert	4	416201	104050	17.93	<.001
temp.cult	2	214689	107345	18.50	<.001
fert.cult	2	9611	4806	0.83	0.440
temp.stages	4	1444350	361088	62.22	<.001
fert.stages	4	63651	15913	2.74	0.032
cult.stages	2	43499	21750	3.75	0.027
temp.fert.cult	4	46685	11671	2.01	0.098
temp.fert.stages	8	787522	98440	16.96	<.001
temp.cult.stages	4	136847	34212	5.90	<.001
fert.cult.stages	4	14659	3665	0.63	0.641
temp.fert.cult.stages	8	311305	38913	6.71	<.001
Residual	106	615125	5803		
Total	161	6385083			

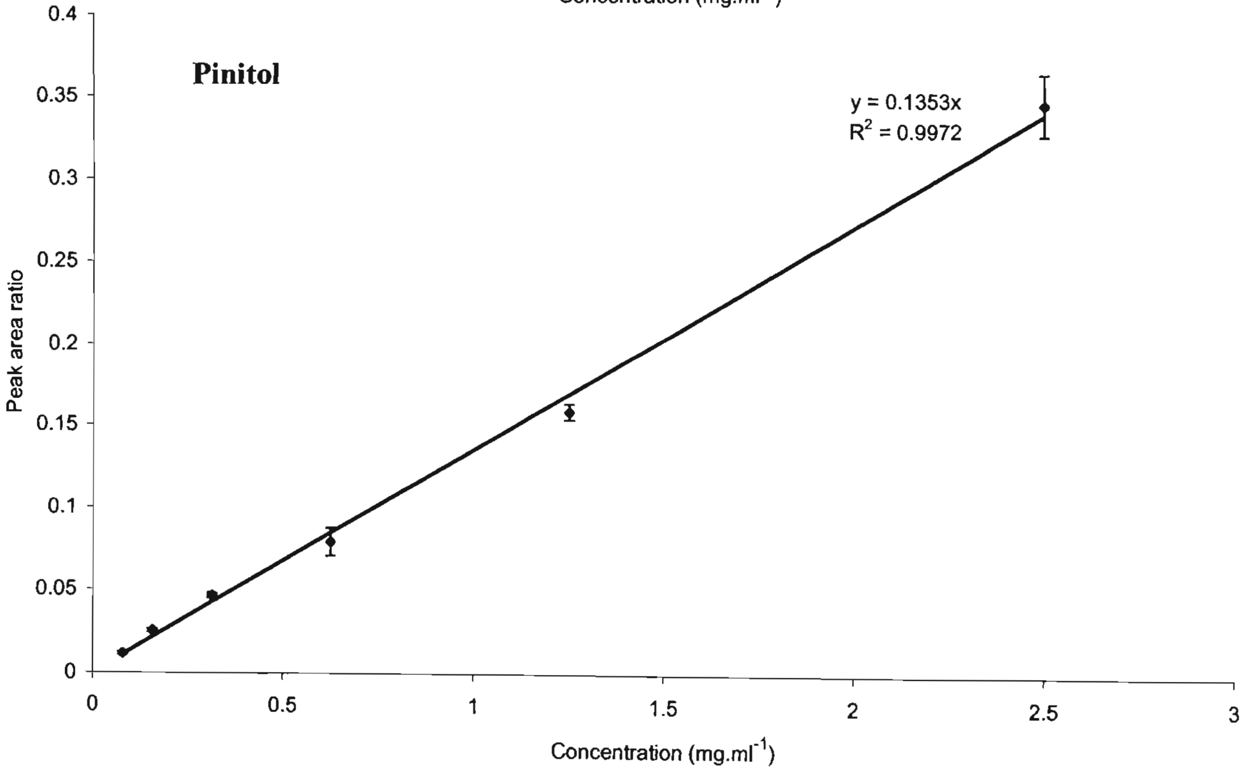
APPENDIX 3.8. Soluble carbohydrate calibration curves.



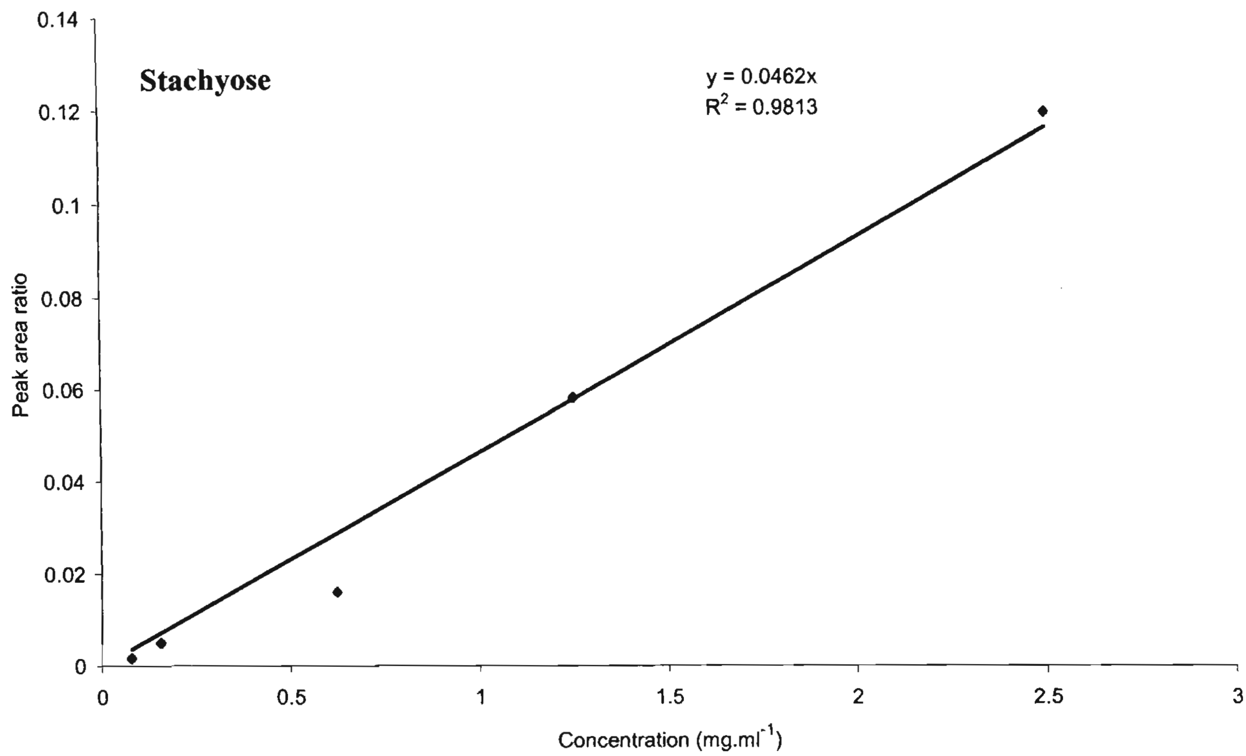


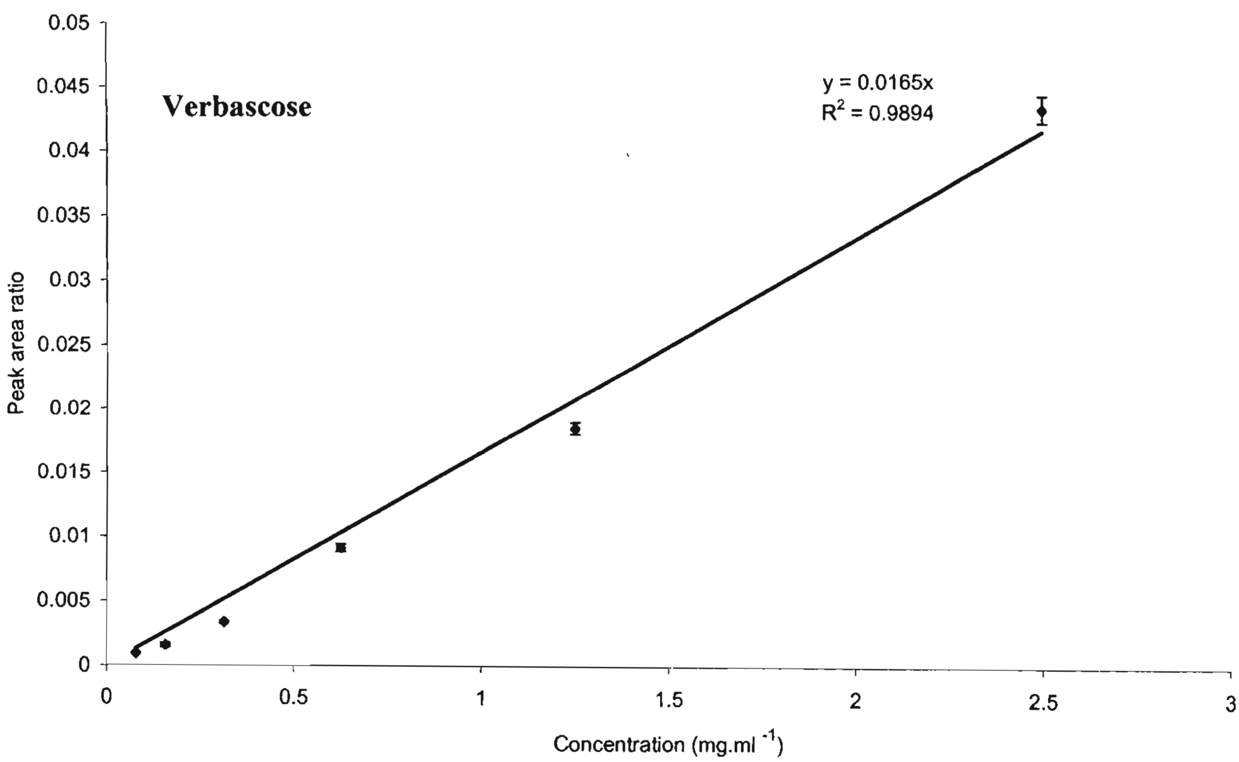
Appendix 3.8. (Continued…)



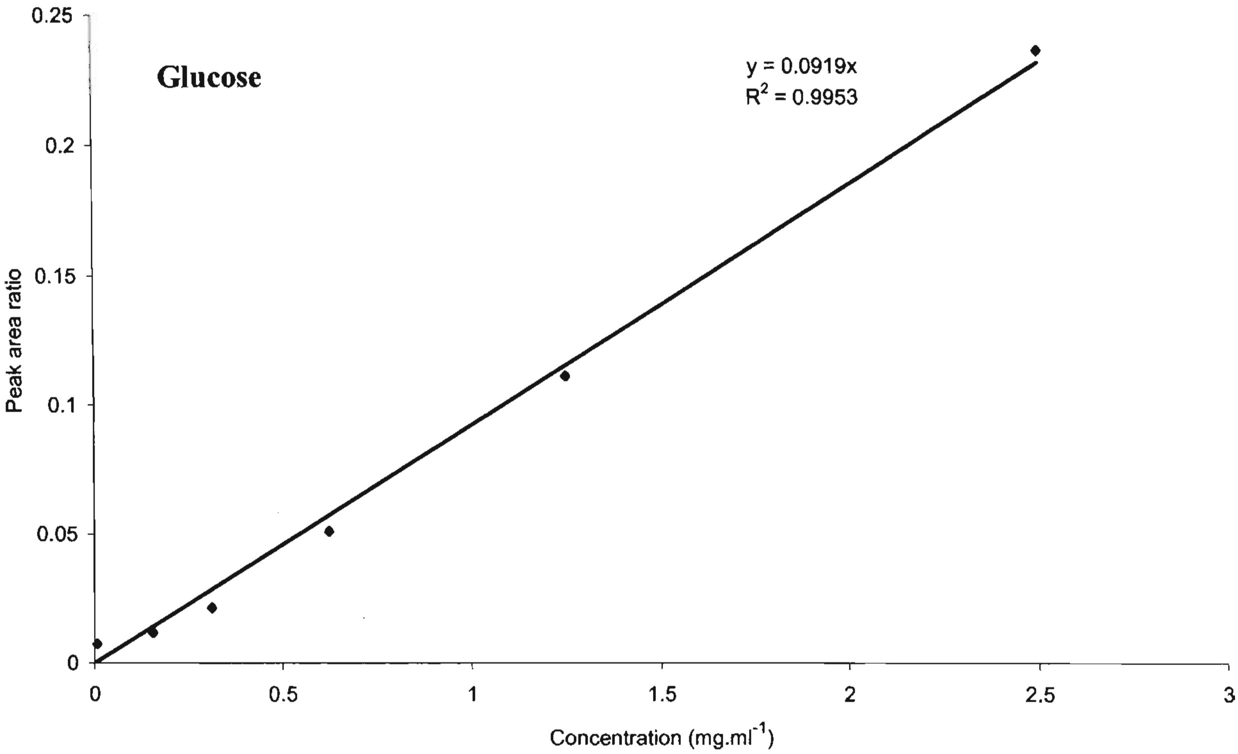


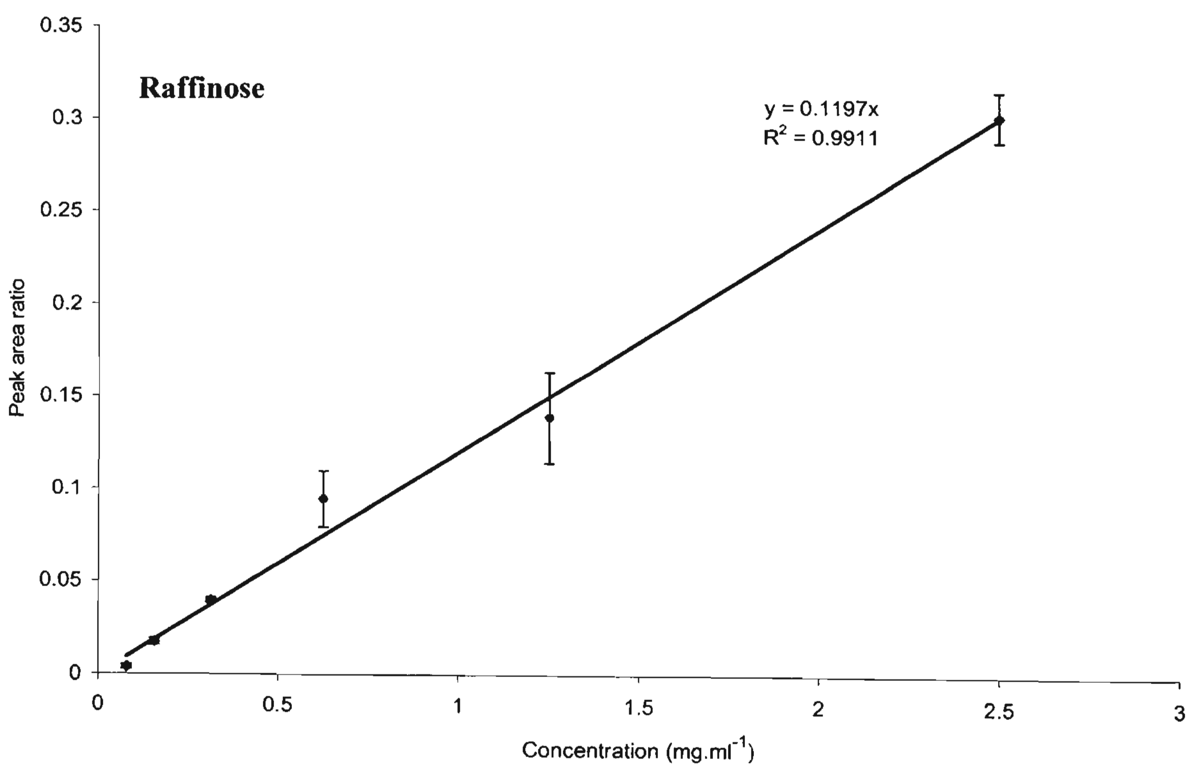
Appendix 3.8. (Continued…)



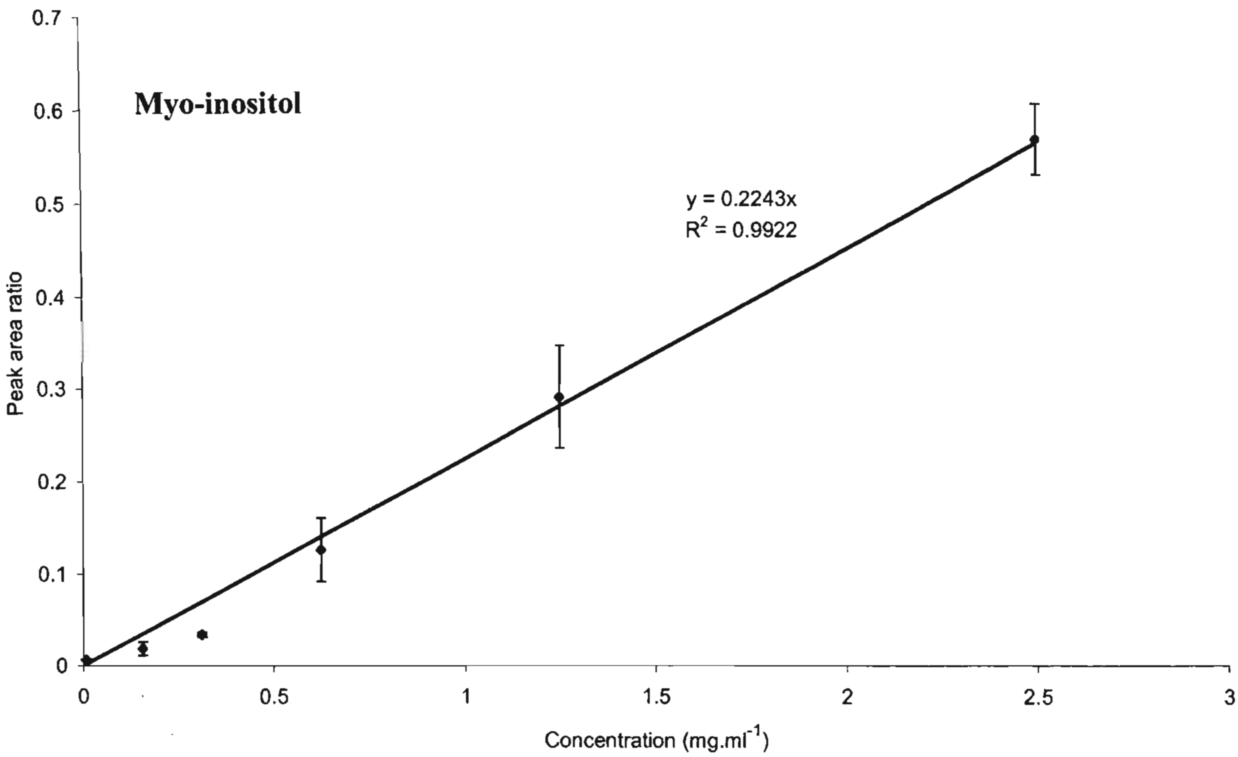


Appendix 3.8. (Continued...)





Appendix 3.8. (Continued…)



Appendix 3.9. Recovery efficiencies of soluble carbohydrates.

Recoveries %	Soluble carbohydrate
91	Glucose
65	Myo-inositol
93.4	Raffinose
82	Fructose
95	Sucrose

Appendix 3.10. Gas chromatogram of soluble carbohydrate retention times.