DECLARATION

These studies represent original work done by me and have not been submitted in any form for any degree or diploma to any other university. Where use was made of the work of others, it has been duly acknowledged in the text.

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Alfred O. Odindo

December 2007

We certify that the above statement is correct.

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Professor Albert T. Modi, Supervisor

December 2007

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Dr. Colin Southway

Co-supervisor

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My family. Thank you Esther for your sacrifice, understanding and patience during my long student life. To Brian and Dan, thank you for having so much confidence in me.

DEDICATION

This thesis is dedicated to the memory of my beloved parents, Henry Opondo Odindo and Abigail Ajwang Opondo, who regretfully did not live to see this work… and for daring us to live our dreams....

Down how many roads among the stars

must man propel himself in search of the final secret?

The journey is difficult, immense,

at times impossible, yet that will not

deter some of us from attempting it….

We have joined the caravan, you might say,

at a certain point, we will travel as far

as we can, but we cannot in one lifetime

see all that we would like to see or to

learn all that we hunger to know

Loren Eiseley

The Immense Journey

ABSTRACT

Cowpea (*Vigna unguiculata*. L) is an important African crop. However, it is also an underutilized grain legume. Consequently, there is not enough research data on cowpea seed physiology. Whereas there is evidence of cowpea being a drought tolerant crop, there is no evidence to associate plant drought tolerance with seed quality in response to water stress. This study sought to understand the effect of production site and water stress on cowpea seed quality development with respect to germination capacity and vigour. Patterns of raffinose family of oligosaccharides (RFO) during seed development to mature dry stage were used to physiologically relate seed performance to water stress. The effect of water stress and exogenous ABA on the accumulation of stress LEA proteins (dehydrins) in relation to seed quality development and germination was investigated. RFOs are known for their roles in desiccation sensitivity but no studies have shown their significance in cowpeas.

Seeds of six cowpea cultivars were produced at two distinct growth sites characterised by irrigated and dry land conditions. The seeds were assessed during six developmental stages, for water content, dry matter accumulation, and performance. Harvested seeds were then planted in a pot experiment under controlled conditions to examine the effect of water stress on seed quality development and data collected during three developmental stages. Harvested seeds from the pot experiment were subsequently analyzed for changes in RFO accumulation during development using gas chromatography. The seeds were also used to investigate the effect of water stress and ABA on the accumulation of stress LEA proteins (dehydrins) in relation to seed quality development in cowpea.

In addition, this study evaluated the use of image analysis as a method that can be used to objectively determine seed coat colour variation in cowpea. Statistical variation in individual seed's solute leakage for cowpea cultivars differing in seed coat colour and produced under different environmental conditions was explored and correlations were done between seed conductivity test with other aspects of seed performance during germination. Furthermore the results of the conductivity test were compared with accelerated aging test, in relation to seed performance.

The study provided evidence that cowpea seed lots produced under different environmental, and possibly management conditions may not differ with respect to seed quality as determined by germination capacity and vigour. However, significant differences between sites with respect to seed maturation patterns determined by water content and dry matter accumulation were observed. Adverse maternal environmental effects on the subsequent performance of seeds in a drought tolerant crop may not necessarily lead to poor performance. Cultivar differences in response to simulated drought conditions at the whole plant and tissue level can be considerable and highly variable; however, these differences may not have adverse effects on the germination and vigour of the seeds. Drought avoidance mechanisms at the whole plant level in cowpea are quite efficient in allowing the species to adapt to simulated drought conditions. These mechanisms may allow the cowpea cultivars to maintain metabolism and restore conditions for their continued growth under water stress; and produce few seeds of high germination capacity and vigour.

Stachyose was found to be the predominant member of the raffinose family of oligosaccharides in cowpea. It is suggested that stachyose accumulation could be used as an indicator of stress tolerance in cowpea. However, the relationship between RFO concentration and the acquisition of desiccation remained as a matter of speculation in the present study and is still generally inconclusive. There was no evidence to suggest the acquisition of maximum desiccation tolerance is associated with maximum seed vigour. It is suggested in cowpea, which is drought tolerant, that maximum vigour does not necessarily imply the acquisition of maximum desiccation tolerance; rather there is a minimum level of desiccation tolerance that is required for the development of optimal seed vigour.

The use of an *in vivo* approach in the study of LEA function in cowpea enabled the accurate comparison of two different groups of LEA proteins in developing cowpea seeds under conditions of water stress and in relation to germination and vigour. Both group 1 LEA and group 2 LEA (dehydrin) were shown to increase in concentration in response to water stress. In addition group 1 LEA protein was observed to be relatively abundant in cowpea seeds. A maternal influence on LEA protein gene expression under conditions of water stress, which may induce dehydrin accumulation

during the earlier stages of seed development, was implied by the observation that dehydrin-like proteins were induced after two weeks of development in cowpea plants subjected to stress during the vegetative phase. In addition, the exogenous application of ABA delayed radicle protrusion; this was associated with a delay in the disappearance of LEA proteins and is suggestive of a relationship between LEA protein accumulation and the acquisition of desiccation tolerance.

The study has demonstrated that image analysis can objectively discriminate seed coat colour variation in cowpea. Dark coloured seeds in general performed better than light coloured seeds; however seed coat colour was not always associated with better performance. A newly developed Aging Stress Differential Index (ASDI) has been used in this study to demonstrate a link between seed coat colour and sensitivity to water stress. The ASDI correlated well with the observations relating stress tolerance to stachyose accumulation.

The skewed distribution patterns in individual electrical conductivity and the presence of extreme values may have implications with respect to the suitability of using standard statistical analyses which compare mean values to evaluate such data. In addition variation in individual electrical conductivity may also be influenced by cultivar differences and the chemical composition of the seed coat. Therefore associations between seed coat colour and electrical conductivity as a measure of performance should be treated with caution. The AA test does reflect changes in seed vigour, however ranked electrical conductivity values after AA did not consistently reflect differences in seed performance between cultivars and sites, and they did not correlate well with other aspects of performance.

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1 LITERATURE REVIEW

1.1 Introduction

Cowpea (*Vigna unguiculata* (L) Walp) is one of the most ancient crops known to man (Martin *et al.*, 1967) with its centre of origin in West Africa (Ng and Padulosi, 1988). It is an important food legume and a valuable component of the traditional cropping systems in the semi-arid tropics covering Asia, Africa, Central and South America (Mortimore *et al.*, 1997; Singh and Tawarali, 1997). Four cultigroups of cowpea are recognized (Baudoin and Marechal, 1985): (1) *unguiculata*, which is the most common form; (2) *biflora* or catjang which is characterized by small erect pods and found mostly in Asia; (3) *sesquipedalis* or yard-long bean also mostly found in Asia and characterized by its very long pods which are consumed as a green 'snap bean'; and (4) *textiles*, which is found in West Africa and used for fibers obtained from its long peduncles. Cowpea exhibits a wide range of growing habits (Figure1.1) (Ehlers and Hall, 1997). It is a short-day plant with many accessions that are photoperiod sensitive (Ehlers and Hall, 1997). Its seeds are characterised by a wide variation in seed coat colour (Figure1.2).

Figure 1-1 Different cowpea plant types. (A) Erect and spreading, (B) late spreading, (C) spreading and semi-erect and (D) semi-spreading (Singh *et al.*, 2003).

Figure 1-2 Cowpea seeds are characterized by a wide variation in seed coat colour (Author's collection from various sources in Kenya and South Africa, 2003).

Cowpea is of major importance to the nutrition and livelihoods of millions of people in less-developed countries of the tropics (Singh *et al.*, 2003). The species can play a significant role in food security initiatives aimed at addressing problems of food production in these regions. The legume is consumed in several ways. The dried seeds are an important protein source (22 -23 % protein content) (Bressani, 1985), and can be ground into a meal which is used in a number of ways (Nout, 1996; Nielsen *et al.*, 1997). Fresh seeds and immature pods are frozen or canned and consumed as 'green

beans' in developed countries. The young shoots and leaves are eaten as a leafy vegetable and provide one the most widely used pot herbs in tropical Africa; they are often dried and can be stored for dry season use. Cowpea is equally important as a nutritious fodder for livestock (Singh and Tawarali, 1997). In West Africa, mature cowpea pods are harvested and the haulms are cut whilst still green; these are stored for use and for sale as a livestock feed supplement in the dry season (Singh and Tawarali, 1997). The species can also be used as a green manure or cover crop. The seeds are sometimes used as coffee substitutes.

Although cowpea is an important crop, not many countries have initiated cowpea improvement programmes (Singh *et al.*, 2003). No recent and/or reliable data on global cowpea production can be found since FAO stopped publishing cowpea statistics (FAO, 2006). The production data for cowpea is pooled with that of common bean (*Phaseolus vulgaris*) (FAO, 2006). However, it is estimated that the worldwide area under cowpea is about 14 million ha with over 4.5 million tons of annual production (Singh *et al.*, 2003). More than 60% of the production and 75% of the area is spread over the arid and semi -arid regions of sub-Saharan Africa (Ehlers and Hall, 1997).

Being a drought tolerant and hot weather crop, cowpea is well-adapted to the semi-arid regions of the tropics where other food legumes do not perform well (Singh *et al.*, 2003). (van Rij, 1997) observed that the rainfall requirement for cowpea in Southern Africa can be as low as 300 mm, spread over the growing season. In the Sahel region, yields of up to 1000 kg ha^{-1} have been recorded under conditions of limited moisture (181 mm per year) and high temperatures (Hall and Patel, 1985). Yields are reported to range between 2500 kg ha^{-1} in Southern Africa to 4000 kg ha^{-1} at the International Institute for Tropical Agriculture (IITA) Ibadan, Nigeria (van Rij, 1997). In California, USA, under favourable conditions, yields ranging between 4000-7000 kg ha^{-1} have been reported (Sanden, 1993).

Major achievements in cowpea breeding in Africa include the development of productive early maturing cultivars, with a range of preferred seeds types and resistance to critical pests and diseases (Singh and Ntare, 1985). Such cultivars mature in 60- 70 days and can produce grain yields of 2000 kg ha^{-1} (Ehlers and Hall, 1997).

However, the adoption of these early cultivars has not been rapid; yields are still considerably low (300 kg ha^{-1}) (Ehlers and Hall, 1997). This can be attributed among other reasons to: (1) ineffective extension systems (2) lack of the high density sole-cropping and crop husbandry practices that are required for these modern cultivars to achieve high grain yield (Ehlers and Hall, 1997) (Farmers still prefer using locally adapted cultivars and low planting densities in traditional intercropping systems with cereal crops) (3) Resource poor farmers in the marginal areas of Africa attempt to grow crops under diverse environmental conditions, which are risk prone and are characterised by environmental stresses such as inadequate moisture availability and nutrient deficiencies and (4) The poor quality of seed used by farmers could also be a major limiting factor contributing to low yields.

It is likely that environmental stresses characteristic of the cowpea production areas during plant growth can interact with seed developmental processes and ultimately influence seed quality and yield.

1.2 Environmental effects on seed quality

Germination capacity and physiological vigour are the two most important indicators of seed quality, because they are intrinsic properties of the seed. Seed vigour gives an indication of the ability of the seed to emerge under a wide range of environmental conditions and is the most informative indicator of physiological seed quality. The maximum potential of this physiological quality is determined by the genetic constitution of the seed; and is fundamentally dependent on several endogenous and external factors which occur throughout seed development on the mother plant, at harvest time and during storage (Salisbury and Ross, 1991; Bewley and Black, 1994; Dornbos, 1995; Wulf, 1995; Hilhorst and Toorop, 1997; Tekrony, 2003; Powell *et al.*, 2005).

Studies on the effect of environmental conditions on seed development and quality are well documented (Delouche, 1980; Gutterman, 1992; Dornbos, 1995; Wulf, 1995). Temperature, photoperiod, water deficits and soil fertility are known for their effects on seed mass, number and quality (Gutterman, 1992; Bewley and Black, 1994; Dornbos, 1995; Wulf, 1995). Several workers cited in the literature have investigated the effect of nutrient deficiencies on seed quality. In pea (*Pisum sativum*), seeds that

were harvested from a boron deficient area produced abnormal seedlings when planted in sand (Legatt, as cited in Delouche, 1980). Misra *et al.*, (1991) reported that a foliar spray of boron at the reproductive stage of sorghum (*Sorghum bicolor*) varieties generally increased the phosphorous content and water soluble sugars of seed coatings. The effects of boron appear to be associated with an increase in seed yield and an improvement in the germination energy index (Misra *et al.*, 1991). Germination energy index could be used as an indicator of physiological vigour. Similarly, calcium is an important element because it is an essential component of cell wall synthesis and enhances cell wall integrity possibly as calcium pectate. Calcium deficiency caused a reduction in germination of carrot and pepper seed (Harrington, 1960). Osmopriming with $CaCl_2$ was reported to improve rice (*Oryza sativa* L) seed germination (Ruan *et al.*, 2002). Calcium osmopriming improved green bean seed germination and field establishment (Mazibuko and Modi, 2005). The effect of nitrogen fertilizer application on seed germination and vigour has also been investigated. In seeds of *Sisymbrium officinale*, the amount of nitrate in mature seeds was positively correlated with germination (Hilhorst, 1990). Rowarth *et al.*, (1999) observed that seed vigour improved with increased nitrogen application. Furthermore, increased N also resulted in to an increase in seed weight which had a direct effect on seed size. Larger seeds attain higher germination percentages and have an advantage with respect to time taken to emergence (Wulf, 1995). Rapid and uniform emergence is indicative of high seed vigour.

Temperature stress has also been reported to influence seed germination and vigour. High temperatures during seed development can cause early pod ripening and rapid seed maturation and result in small, poor quality seeds in common beans (*Phaseolus vulgaris*) (Siddique and Goodwin, 1980) and annual rye grass (*Lolium rigidum*) (Steadman *et al.*, 2004). In soybean (*Glycine max*), maximum daily phytotron temperatures of 33 and 38ºC during development reduced seed size, increased the number of shrivelled seeds and resulted in lower germination and vigour (Tekrony *et al.*, 2004). Furthermore, seed vigour evaluated after accelerated aging was significantly reduced when maturation occurred at 33ºC and was unacceptable at 38ºC (Tekrony *et al.*, 2004). On the other hand, in seeds of bean, wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), lower temperatures during and after seed filling significantly influenced the development of seed quality by extending the post

maturation phase, resulting in seeds with higher potential longevity (Ellis and Filho, 1992; Sanhewe and Ellis, 1996). Climatic changes may also alter seed composition. Thomas *et al.* (2003) observed that total non-structural carbohydrates (TNC) in soybean decreased as temperature increased and the proportion of soluble sugars to starch decreased , while the concentrations of N and P (crude proteins) increased with temperature to 40/30ºC, then decreased sharply.

Temperature and drought stress during seed filling were reported to have an influence on the germination and vigour of mature soybean seeds. Both types of stress decreased germination in the standard germination test as well as embryonic axis weight (Dornbos, 1995).

Responses to drought stress apparently are variable and may be indicative of different underlying physiological mechanisms. For example, sorghum seeds grown under mild drought stress displayed significantly much higher germination than those grown under normal conditions (Benech -Arnold *et al.*, 1991). Similarly, drought stress seemed to have improved seed quality in brassica (*Brassica campestris* [*rapa*] L.). Work done by Ellis *et al*. (2000) showed that in rapid cycling brassica, terminal drought resulted in enhanced water loss and low seed weight; however seed quality development was more rapid and there was a greater maximum seed quality with respect to potential longevity. Similarly, (Vieira *et al.*, 1992) reported that soybean seeds exposed to drought stress during seed development showed large reductions in yield and seed number; however drought stress did not have an effect on germination and vigour. The lack of an effect on germination and vigour may be attributed to adaptive responses to stress conditions. Plants are able to adapt to stress conditions and continue with their metabolic activities. In addition, plant responses to drought stress can be influenced by several factors including, the timing of the stress, duration, intensity and genotype. These factors can influence underlying physiological mechanisms and lead to variable physiological responses.

Interesting observations were made by Frascaroli *et al.* (2004) who reported that defoliation of the mother plants during kernel maturation had an improving effect on the cold tolerance of germinating maize (*Zea mays*) seeds. Results reported in this study indicated that reaction to defoliation could be used as a model for better

understanding of genetic control and physiological mechanisms that determine the basis of maize cold tolerance at germination. The variability in plant physiological responses to stress factors was clearly demonstrated by the work of Virgil (1989) who observed that defoliation during the stage of protein body formation of radicles of cotton seeds arrested vacuole filling and decreased the rate and percentage of the germination of the affected seeds.

Possibly, different physiological mechanisms can be induced by environmental stresses during development. These mechanisms may lead to variable physiological responses that may influence seed germination and vigour. A shortage of assimilates required to synthesize the storage compounds that subsequently drive the germination process may be related to loss of seed vigour (Dornbos and McDonald, 1986). Green *et al.* (1965) found that soybean plants from early planting dates which matured during hot weather produced seeds with low germination. These authors were able to show that high quality seeds could be produced from early or late planting dates if seed development and maturation did not occur under unfavourable weather conditions. Seed from later dates of planting which reached maturity after the hot dry conditions had ended were high in quality (Green *et al.*, 1965). The influence of pre-maturation events on seed viability and vigour was investigated by Siddique and Wright (2004). These authors reported that delayed sowing had relatively larger effect on germination and emergence of aged seeds of peas and flax (*Linum usitatissimum*) and noted that sowing date had relatively small effects on viability, but larger effects on seed vigour (Siddique and Wright, 2004). Differences in environmental conditions experienced during development and maturation, the length of seed filling period, concentrations of fat, soluble carbohydrates, proteins and starch were examined as potential causes of differences in seed vigour (Siddique and Wright, 2004). However, (Siddique and Wright, 2004) concluded that none of these variables could alone explain the observed variations in seed vigour with sowing date.

It is generally recognized that plant response mechanisms to environmental stress factors during development are diverse and can be complex; these can result in reduced seed number, size, germination and vigour. These mechanisms may be the basis of different developmental patterns resulting in variable physiological responses. Mechanisms that may limit the development of vigour are not clearly understood. It is

feasible that physiological mechanisms during seed development under different environmental conditions determine seed germination and vigour; and also limit maximum seed quality acquisition. In addition, it is likely that interactions between environmental conditions and seed developmental processes modify the ultra structure of the developing seed to ultimately impact on seed quality. Not many studies have identified the mechanisms involved during these interactions (Hilhorst and Toorop, 1997).

1.3 Physiology of seed development and stress tolerance

1.3.1 Plant hormones and the regulation of seed development

Besides the deposition of food reserves, seed development is characterized by the formation of a large number of important chemical compounds: growth regulators or hormones. Developing seeds are a rich source of cytokinins (CKs), auxins, gibberellins (GAs) and abscisic acid (ABA). Abscisic acid is the most widely studied seed phytohormes in relation to water stress and dessication tolerance (Walker-Simmons, 1987; Zeevart *et al.*, 1988; Wilen *et al.*, 1994; Vertucci and Farrant, 1995).

1.3.1.1 Abscisic acid and seed development

Abscisic acid is a universal stress hormone whose production is controlled or triggered by several mechanisms (Salisbury and Ross, 1991). It mediates the acclimation of plants to environmental stress (Salisbury and Ross, 1991) and interacts with other cellular metabolites and environmental factors in the regulation of stress responses (Salisbury and Ross, 1991; Wilen *et al.*, 1994). A major effect of water stress on seed quality is the reduction in seed number and seed size. During flowering under conditions of water stress, ABA may trigger responses that can reduce the number of primordia that are produced resulting in a reduction of the number of grains that can be formed (Bewley and Black, 1994). When water stress occurs during pod formation, ABA causes a decrease in the number of pods per plant, due to abortion and abscission when pods fail to expand, thus decreasing seed yield.

The primary function of ABA during seed development seems to be the inhibition of embryo growth, so that germination cannot commence. Precocious germination on the plant, termed vivipary, can occur in mutant embryos of Arabidopsis (*Arabidopsis thaliana*) (Koornneef *et al.*, 1989) and maize (*Zea mays*) (Neill *et al.*, 1986;

Robichaud and Sussex, 1986) that have reduced ability to synthesize ABA or have lower ABA sensitivity. A similar phenomenon to precocious germination, preharvest sprouting is prevalent in wheat cultivars that are deficient in Mo an element important for ABA synthesis (Modi and Cairns, 1994).

ABA increases during seed development in a wide range of species and reaches a maximum at approximately the same time as seed dry weight. At maturation there is a substantial decline in ABA as it is metabolized to phaseic and dihydrophaseic acids (Roberts and Hooley, 1988). Physiological and molecular studies have shown that the exogenous application of ABA is often accompanied by the expression of a number of genes and proteins in plant cells (Takahashi *et al.*, 2004; Chen *et al.*, 2006) and enhanced synthesis of dehydration-related proteins and other compatible solutes such as soluble sugars (Wang *et al.*, 2002). The observation that in Arabidopsis, the double mutant *aba, abi3* which is both ABA deficient and insensitive is not tolerant to desiccation, contrary to the single mutants, has led to the suggestion that the induction of desiccation tolerance is controlled by and is extremely sensitive to ABA (Hilhorst and Toorop, 1997). Exogenous application of ABA induces desiccation tolerance to developing embryos (Bartels *et al.*, 1988b; Anandarajah and Mackersie, 1990) and prolongs the desiccation tolerant phase in mature embryos (Blackman *et al.*, 1991). However, the mechanism by which ABA induces desiccation tolerance is not clear (Vertucci and Farrant, 1995).

1.3.2 Seed desiccation tolerance

During the final developmental stage, known as late embryogenesis, seed embryos mature and undergo desiccation (Bewley and Black, 1994). The desiccation phase marks the termination of development. The ability of cells to withstand stress imposed by an almost complete loss of cellular water during desiccation and to resume normal metabolic activities upon imbibition is described as desiccation tolerance (Kermode, 1995; Vertucci and Farrant, 1995; Hoekstra *et al.*, 2002). Embryos in desiccation tolerant (orthodox) seeds acquire tolerance to desiccation prior to the onset of desiccation (Sun and Leopold, 1993; Kermode, 1997) and lose it during germination (Berjak *et al.*, 1989; LePrince *et al.*, 1994), but the timing of dessication loss varies among species (Senaratna and Mackersie, 1983). Orthodox seeds are thought to have

developed strategies that enable them to survive the effects of water loss during desiccation. These mechanisms involve the protection of cellular structures against desiccation stress (Vertucci and Farrant, 1995; Kermode, 1997; Hoekstra *et al.*, 2001; Hoekstra *et al.*, 2002).

1.3.2.1 Metabolic stresses resulting from cellular dehydration during seed maturation

Desiccation tolerant seeds can withstand metabolic and biophysical stresses during maturation drying (Hilhorst and Toorop, 1997). As water is removed from the cells, the decrease in cellular volume causes crowding of cytoplasmic components; cell contents become gradually more viscous, increasing the chances of molecular interactions that can cause protein denaturation and membrane fusion (Hoekstra *et al.*, 2001). Changes in metabolic activities are thought to occur at specific moisture levels (Leopold and Vertucci, 1989). At moisture levels below 0.7 g g^{-1}(-1.5 MPa) tissues no longer grow or expand (Levitt, 1980); and changes occur in the patterns and synthesis of proteins and nucleic acids (Ingram and Bartels, 1996). The production of protectants such as straight chain polyols or cyclic polyols may also be induced (Bohnert *et al.*, 1995). Below 0.45 g g^{-1} (-3 MPa), protein synthesis ceases and repair processes become inoperative (Dell'Aquila, 1992). Whereas respiration can continue to moisture levels about 0.25 g g^{-1} (Farrant, 2000), it is estimated that mitochondrial respiration ceases at water contents lower than 0.25 g g^{-1} (Vertucci and Farrant, 1995). Below 0.25 g g^{-1}, catabolic processes can still proceed, producing high energy intermediates. Because of metabolic imbalances arising due to various reactions within metabolic pathways reacting differently to low moisture levels, the processing of these intermediates becomes impaired leading to the production of reactive oxygen species (ROS) including free radicals. Cells with high metabolic activity may die because of the increase in the quantity and activity of free radicals. These free radicals can also damage membrane structures (Wolff *et al.*, 1986).

Membrane structures are particularly susceptible to damage induced by water deficit stress (Crowe and Crowe, 1992; Oliver and Bewley, 1997; Crowe *et al.*, 1998). Production of ROS and free radicals increases during desiccation. Free radicals cause extensive peroxidation and de-esterification of membrane lipids, thus decreasing the fluidity of the membranes (Senaratna and Mackersie, 1983; Tetteroo *et al.*, 1996).

Peroxidation can lead to loss of cell membrane integrity and subsequent extensive leakage of cytoplasmic components upon imbibition.

Loss of membrane structure and function is also attributed to a process known as "demixing", a desiccation-induced compaction of molecules causing altered lipid phase transition (Crowe *et al.*, 1987; Wolfe, 1987; Bryant and Wolfe, 1989). Under hydrated conditions polar lipids spontaneously align to form micelles or bilayer membranes, depending on the polar head group of the lipid. Mobile acyl chains within the bilayers afford considerable fluidity to the structure and enable proteins and other constituents to be inserted. Upon drying, water molecules are removed from adjacent polar head groups resulting in close packing of acyl chains caused by increased Van der Waals interactions (Crowe *et al.*, 1990) and greater membrane rigidity. The nature of this phase transition is determined by the molecular geometry of the membranes and their polar lipid components, the presence of other membrane constituents (proteins and sterols), temperature and the extent of water stress (Bryant and Wolfe, 1989). Two types of phase transitions have been reported: Lamellar liquid crystalline phase to gel phase, which maintains the bilayer configuration and the lamellar liquid crystalline phase to the hexagonal phase in which a non-bilayer structure is formed. The lamellar liquid crystalline to gel phase is favoured by polar lipids with a rectangular-shaped head group such as phophatidylcholine (Quinn, 1985) and by saturated long chain fatty acids and saturated free fatty acids (Mckersie *et al.*, 1989). The gel phase is completely reversible upon rehydration. The formation of the hexagonal phases on the other hand has a deleterious effect, as demixing results in loss of some of the membrane constituents and causes a complete loss of compartmentation within the cell. These changes result in leaky membranes upon imbibition.

The products of lipid peroxidation are harmful to other macromolecules such as nucleic acids and proteins (Hilhorst and Toorop, 1997). Removal of water from cells alters the structure of these macromolecules as the hydrophilic and hydrophobic interactions that stabilise their conformation are weakened or lost (Crowe *et al.*, 1987). The mutagenic capacity of free radicals is due to the direct interaction of hydroxyl radicals with proteins (Wolff *et al.*, 1986) and DNA (Dizdaroglu, 1991).

Hydroxyl radicals damage DNA and proteins through modifications of bases/amino acid residues, DNA/protein cross-linking and fragmentation of DNA strands.

Membrane modifications and changes in the pattern of protein synthesis and/or expression can also occur as a result of drought stress (Bray, 1993; Bohnert *et al.*, 1995). It is possible that interaction between drought and desiccation stress can impair physiological processes during seed development and affect seed vigour.

1.3.3 Drought tolerance and desiccation stress

Drought tolerance can be considered as the tolerance to moderate dehydration, down to a moisture content below which there is no bulk cytoplasmic water present (~23 % water on a fresh weight basis, or ~0.3 g H_2O g dm^{-1} (Hoekstra *et al.*, 2001). Desiccation tolerance generally refers to the tolerance of further dehydration, when the hydration shell of molecules is gradually lost (Hoekstra *et al.*, 2001). Cellular processes that are affected by drought stress are those that are utilised by stress tolerant plants in adapting to stressful conditions and maintaining metabolism under those conditions. Under water stress, many cellular processes change. This may include changes in transcription, mRNA stability, protein turnover, membrane modifications, signalling pathways metabolite balance and osmolyte biosynthesis (Bray, 1993). Figure 1.3 illustrates the link between signalling pathways, regulatory processes, changes in metabolism and the effects this may have on cell membranes, osmotic potential and photosynthetic processes. These changes allow the plant to maintain metabolism and continue growth under water stress (Bohnert *et al.*, 1995).

Under drought conditions, membrane modifications may allow cell membranes to maintain optimum fluidity. However, under prolonged and severe drought, cell membranes may become less fluid to prevent the leakage of solutes and/or retain bulk water and enzyme activities. How this relates physiologically to the development of vigour during desiccation under drought stress is not clearly understood. Loss of membrane integrity is believed to be a fundamental cause of loss of vigour in mature orthodox seeds (Delouche, 1980).

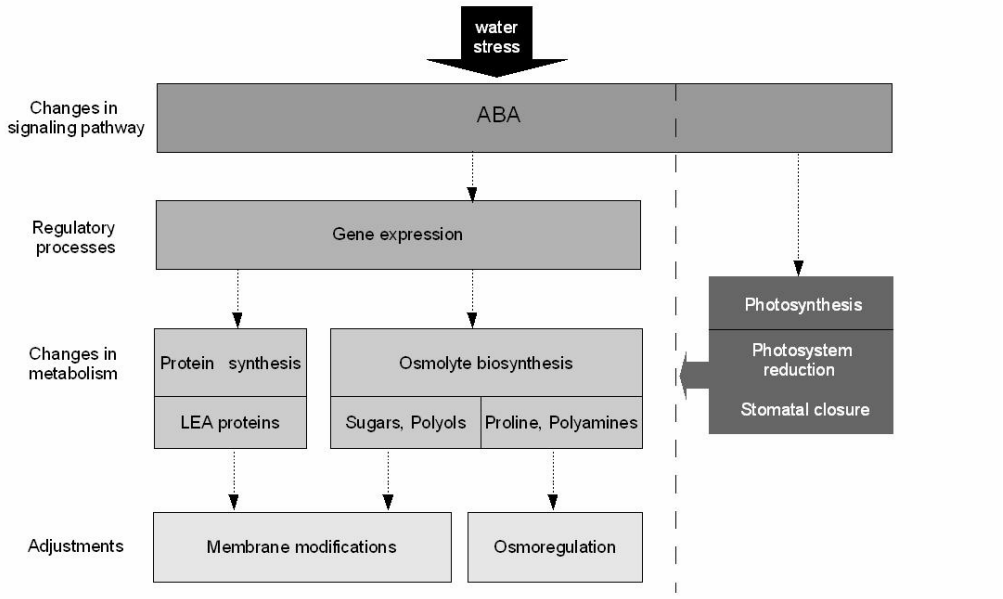

Figure 1-3 Linkages between changes in signalling pathway, regulatory processes, changes in metabolism showing adjustments at the cellular level that enable plant cells to cope with water stress.

1.3.4 Seed vigour in relation to seed desiccation

Embryos may acquire germinability soon after histodifferentiation; however germination may not be normal because the accumulation of food reserves is not yet completed (Hilhorst and Toorop, 1997). The ability to germinate may be considered as the first sign of seed quality development. Seed vigour improves with increase in dry matter accumulation (Miles, 1985); and progresses up to the point of maximum dry matter accumulation. This point coincides with the attainment of physiological maturity when seed quality is thought to be maximum (Harrington, 1972), although this does not appear to hold for all crops (Ellis and Filho, 1992; Sanhewe and Ellis, 1996). During programmed desiccation, while the seed is still on the mother plant, cells are filled with food reserves thus replacing intracellular water. If desiccation occurs during seed development and maturation under drought stress, different interacting physiological mechanisms may occur between cells exposed to water and desiccation stress and affect the development of seed vigour. Bulk water retention in response to drought stress during seed maturation may lead to increased active

metabolism. Cells may utilize some of these food reserves and cause an increase in compounds such as free radicals as has been mentioned earlier (LePrince *et al.*, 1994). This may cause changes and alterations in some of the accumulated storage compounds, for example, unsaturated lipids which are very susceptible. Damage can also occur to key enzymes; DNA (genome) and RNA. Drought and desiccation tolerance are correlated with the accumulation of substantial quantities of compounds such as sugars, compatible solutes and specific proteins such as the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) (Hoekstra *et al.*, 2001). These compounds are believed to play a role in the protection against desiccation damage (Dornbos and McDonald, 1986; Kuo *et al.*, 1988; Horbowicz and Obendorf, 1994); they are also implicated in cellular responses to drought stress as mentioned earlier. For many seeds, the timing of maximum dry matter accumulation and the acquisition of maximum desiccation tolerance of the embryonic axis occur within day's apart (Berry and Bewley, 1991; Demir and Ellis, 1992). Alterations in the quantities and composition of sugars and proteins may cause poor acquisition of desiccation tolerance and negatively affect seed vigour. The associations between desiccation tolerance, drought stress and seed vigour have been barely explored and our understanding of the relationships is scanty.

1.3.5 Protection mechanisms against desiccation in seeds

Several compounds are observed to accumulate in orthodox seeds when desiccation tolerance is acquired and degraded when the tolerance is lost. It is speculated that these compounds, which include soluble carbohydrates and proteins protect subcelluar surfaces and confer desiccation tolerance.

1.3.5.1 Soluble carbohydrates and seed response to desiccation

In orthodox seeds, sucrose, raffinose family of oligosaccharides (RFO) and monosaccharides contribute between 1-12% of the dry mass (Kuo *et al.*, 1988). Sucrose content ranges between 15-90% of the soluble carbohydrates and the monosaccharides are usually in trace amounts. RFOs are implicated in the protection of macromolecular structures during desiccation (Crowe *et al.*, 1987; Leopold *et al.*, 1994). The proposed mechanism of protection by these sugars is based on the hydroxyl groups forming hydrogen bonds with the phosphate group of the polar head group in phospholipids and with the carboxyl group of proteins (Crowe *et al.*, 1998),

thus preventing phase transitions of lipids and denaturation of proteins. This is the basis of the so called water replacement hypothesis (Vertucci and Farrant, 1995) whereby sugars replace water molecules during drying, thus maintaining the structural and functional integrity of membranes, proteins and other macromolecules. The second hypothesis proposes that these oligosaccharides may promote the formation of a vitreous (glassy) state that protects macromolecular structures during desiccation (Leopold *et al.,* 1994). Seed longevity has also been correlated with total RFO content and the ratio of RFO to sucrose (Horbowicz and Obendorf, 1994). On the contrary, different views have been proposed that RFO are not involved directly or are at least not the only factors necessary for desiccation tolerance and storability (Black *et al.*, 1999; Buitink *et al.*, 2000).

RFO biosynthesis proceeds by the introduction of galactosyl residues into oligosaccharides by a galactosyl transfer from UDP-D-galactose to myoinositol yielding galactinol and UDP (Peterbauer and Richter, 2001). The reaction is catalysed by the enzyme galactinol synthase. The formation of raffinose is catalysed by the enzyme raffinose synthase, which is specific to sucrose as a galactosyl acceptor (Lehle and Tanner, 1973). Raffinose is subsequently used for the synthesis of stachyose and verbascose by the enzymes raffinose synthase and verbascose synthase, respectively (Kandler and Hopf, 1982; Peterbauer *et al.,* 2001).

The concentration of initial substrates such as sucrose and myo-inositol and feed back loops can affect the final content of RFOs deposited in mature seeds (Peterbauer *et al.,* 2001). Sucrose, which acts as a galactosyl acceptor in RFO biosynthesis, is the major carbohydrate imported in to the developing seed. During photosynthesis, the first step in sucrose synthesis is the formation of sucrose phosphate from UDP-glucose and fructose-6- phosphate. The reaction is catalysed by sucrose phosphate synthase. This is followed by hydrolysis with a specific sucrose phosphatase (Matheson, 1984). Temperature and water stress during seed growth may alter metabolic pathways during photosynthesis that can result in alterations in the quantities and composition of the accumulated food reserves. Alterations in metabolic processes may lead to the synthesis of protective compounds such as polyols (Bohnert *et al.,* 1995). Lahuta *et al.,* (2000) reported that the composition of accumulated food reserves was altered by drought. Seeds produced under drought conditions had less

dry matter, contained less starch but more oligosaccharides, sucrose and reducing monosaccharides (Lahuta *et al.*, 2000). Polyols including straight chain metabolites such as mannitol and sorbitol (Bieleski, 1982) or cyclic polyols such as myoinositol (Loewus and Loewus, 1983) are known to accumulate in response to drought stress.

Myoinositol is formed after cyclisation glucose-6-phosphate catalysed by myoinositol-1-phosphatase. In addition to the earlier mentioned roles, myoinositol is also implicated in cell membrane biosynthesis and signal transduction mechanisms (Bohnert *et al.*, 1995). Significant changes observed in relative ratios of raffinose, stachyose and verbascose induced by water stress in field bean (*Vicia faba*) (Lahuta *et al.*, 2000) and cucumber (*Cucumis sativus*) (Widders and Kwantes, 1995) could probably be linked to the accumulation of myoinositol. In mature seeds of pea lines with different RFO composition, the synthesis of galactinol in the line with higher RFO content is probably supported by a higher myoinositol concentration during the initial phase of galactinol accumulation (Peterbauer and Richter, 2001). Similarly, levels of galactinol and raffinose were reported to be much lower in tubers of transgenic potato (*Solanum tuberosum*) with a reduced content of myoinositol (Keller *et al.*, 1998). No studies cited in the literature have examined the accumulation of myoinositol in developing seeds of cowpea produced under water tress in relation to RFO concentration.

There seems to be a lack of conclusive evidence in the literature on the role of RFOs in desiccation tolerance. Sinniah *et al.*, (1998) observed that in *Brassica spp.* seeds, there were no significant differences in the final concentration of RFOs in plants that were subjected to water stress and the controls. Similarly, wheat embryos that were induced to water stress tolerance by a 24-h water loss had no detectable raffinose; however, the carbohydrate was observed to accumulate at later times even in embryos in detached grains that had not become desiccation tolerant (Black *et al.*, 1999). These authors concluded that desiccation tolerance and the occurrence of raffinose in wheat embryos are not correlated. In cucumber, lupin *(Lupinus polyphyllus*) and soybean seeds, maturation temperature had little effect on the concentration of RFOs (Obendorf *et al.*, 1998). Although Lahuta *et al.*, (2000) relates changes in relative ratios of RFOs under conditions of water stress to a build up in desiccation tolerance in field beans. The study was not extended to examine how this would relate to seed

quality. The work of Sinniah *et al.,* (1998) and Black *et al.,* (1999) supported the argument that RFOs are not involved directly or are at least not the only factors necessary for desiccation. Based on a simple multiple regression model, Sinniah *et al.,* (1998) proposed that RFOs and proteins are equally likely to be required for seed quality development. However, the findings by Black *et al.,* (1999) in wheat challenges the assumption which is often made or implied that raffinose and other oligosaccharides play a role in the acquisition of desiccation tolerance. Sinniah *et al.,* (1998) concluded that differences in seed quality among different commercial seed lots are more likely to result from differences in heat stable protein accumulation than in sugars. This is because heat stable proteins tend to accumulate comparatively late during development; and the deleterious environmental effects such as high temperatures on seed quality tend not to be detected until comparatively late during seed development and maturation (Ellis *et al.,* 1993; Ellis and Hong, 1994). Proteins such as the late abundant embryogenesis (LEA) proteins are thought to participate in desiccation tolerance (Dure, 1993; Close, 1997; Kermode, 1997). These proteins are also implicated in plant responses to drought stress. Conceivably, they may also be implicated in interactions between seed developmental processes and environmental conditions during growth of the mother plant that may ultimately influence seed vigour.

1.3.5.2 The late embryogenesis abundant proteins

The late embryogenesis abundant (LEA) proteins were first discovered in the cotton plant (*Gossypium hirsutum*) (Galau *et al.,* 1986; Dure, 1993) and as the name suggests, are expressed at high levels during the later stages of seed development (Galau *et al.,* 1991; Blackman *et al.,* 1995; Oliver and Bewley, 1997). They constitute up to 4% of cellular protein (Roberts *et al.,* 1993); and are highly hydrophilic proteins with characteristic sequence motifs, which are largely unstructured in the hydrated state (Tunnacliffe and Wise, 2007). These proteins have been described as heat-stable proteins (Blackman *et al.,* 1991; Kermode, 1997), because they are thermo-stable and can withstand temperatures as high as 100^{o}C. They are rapidly degraded during germination as desiccation tolerance is lost (Galau *et al.,* 1991; Robertson and Chandler, 1992).

Based on amino acid/mRNA sequences and expression patterns, LEA proteins were initially classified into at least five groups (Cuming, 1999; Wise, 2003). However, using a newly developed bioinformatics tool known as peptide profile (POPP) analysis, these proteins are now classified in 3 groups only: Group 1 proteins are characterised by a hydrophilic 20 amino- acid motif (Cuming, 1999); Group 2 have at least two of three distinct sequence motifs named Y, S and K (Close, 1997); and Group 3 contain multiple copies of an 11-amino-acid motif (Cuming, 1999). Groups 4 and 5 have been eliminated when LEAs were classified using the POPP analysis and redistributed to groups 2 and 3 (Tunnacliffe and Wise, 2007).

Group I LEA has been predicted to have a water binding capacity (Litts *et al.,* 1991; Bray, 1993). The dehydrins, or group II LEAs, are characterised by a conserved 15 amino acid sequence (Bray, 1993; Close, 1997). This group is proposed to sequester ions during drying. They might also interact with exposed hydrophobic surfaces of partially denatured proteins to prevent protein-protein aggregation (Bray, 1993; Campbell and Close, 1997; Close, 1997). Group II LEA has received particular attention because they share extensive amino acid sequence with proteins synthesized in response to ABA and water stress (Bradford and Chandler, 1992; Finch-Savage and Blake, 1994; Farrant, 2000). Group III LEA proteins (D-7 family) and group V (D-29 family) are involved in sequestering excess ions (Dure, 1993). Group IV LEA (D-13) proteins were initially believed to stabilise membranes and macromolecules by forming a hydration shell around organelles (Bray, 1993).

The different groups have been proposed to contribute in various ways to protection from desiccation during embryo maturation. Most of the experimental evidence shows that LEA proteins that are over expressed in vegetative tissue can improve tolerance to various degrees of hyperosmotic stress (-1 to -6 MPa), induced by partial loss of water, salt or freezing (Imai *et al.,* 1996; Swire-Clark and Marcotte Jr, 1999). However, whether LEA proteins play a similar role in seeds as they do in drought tolerant systems is not clear (Boudet *et al.,* 2006). How this may relate to the development of seed vigour under conditions of drought is not well understood.

Dehydrins from maize embryos (Egerton-Warburton *et al.,* 1997) and wheat (Danyluk *et al.,* 1998) were found to be associated with cytoplasmic endomembrane(s) and (the)

plasma membrane(s), respectively, suggesting that they might be involved in the protection of these membranes against desiccation. Contrastingly, dehydrins have been detected in seeds that remain desiccation-sensitive at shedding (Kermode, 1997). Black *et al.,* (1999) observed that detachment of wheat grains induced the appearance of dehydrins at an earlier stage of development, even in embryos that had not been made desiccation- tolerant by incipient drying. These authors suggested that possibly there is a maternal suppression of dehydrins accumulation by embryos which is relieved by detachment from the mother plant (Black *et al.,* 1999) and concluded that dehydrins accumulation is not regulated by factors that specifically control the induction of desiccation tolerance. Similar conclusions were made by Boudet *et al.,* (2006) when investigating whether proteins in *Medicago truncatula* can be identified that are specifically linked to desiccation tolerance. These authors found several isoforms of dehydrins that were linked to drought-tolerance rather than desiccation-tolerance; and proposed that LEA proteins can be divided in to two groups. The first group containing LEAs that seem to be seed specific and are induced only in tissues that are desiccation tolerant whereas the second group contains proteins including dehydrins that are expressed in vegetative tissues (Boudet *et al.,* 2006).

The presence of dehydrins in recalcitrant seeds from temperate climates (Kermode, 1997), the absence of correlation between their amounts and seed longevity (Wechsberg *et al.,* 1994), together with the observation that dehydrins protect enzyme activities only at water potentials above -3 MPa (Reyes *et al.,* 2005) has led to the suggestion that dehydrins might play a protective role at high hydration levels (>0.8 g g^{-1}) (Boudet *et al.,* 2006). However, LEA proteins that may be specifically linked to desiccation tolerance might play a role at lower moisture levels corresponding to 0.3 g g^{-1} (Boudet *et al.,* 2006). At higher moisture levels, desiccation tolerant linked LEA proteins may act as compatible solutes that preferentially exclude reactive substances from the surface of macromolecules (Liu and Zheng, 2005; Reyes *et al.,* 2005). Similarly at water contents less than 0.3 g g^{-1} they might exert their protective effects by replacing water molecules by hydrogen bonding and /or forming a glass which stabilizes the system in the dry state (Hoekstra *et al.,* 2001).

Examination of proteins in *Medicago truncatula* revealed that several LEA proteins including Group 1 LEA proteins as seed specific (Boudet *et al.,* 2006). Group 1 LEA

proteins have been proposed to function *in planta* to protect against desiccation stress, by possibly functioning as water binding and /or replacement molecules (Gilles *et al.*, 2007). Gilles *et al.,* (2007) confirmed the existence of PII-like structure that could be significant with respect to the proposed role of Group I LEA proteins as water replacement molecules during the acquisition of desiccation tolerance in seed embryos. Analysis of Circular Dichroism (CD) spectra of a recombinant Group I LEA revealed the presence of an isodichroic point indicating a transition between two structural states (Soulages *et al.*, 2002): an extended helical or poly (Pro) II (PII) structure in the recombinant Group I LEA and little, ‹-helix. PII-like structures have been repeatedly implicated in protein-protein and protein-peptide interactions (Anderson and Gorski, 2005). The presence of this structural element in Group I LEA and the abundance of these proteins in seed embryos suggest that these proteins may be important in the amelioration of desiccation related stresses (Gilles *et al.*, 2007).

Despite the widespread phenomenon of desiccation tolerance in orthodox seeds and its importance in seed quality development, the mechanism(s) that lead to desiccation tolerance are poorly understood. In seeds, direct in *vivo* evidence for the role of LEA proteins in tolerance to complete water loss is not yet convincing (Boudet *et al.*, 2006; Gilles *et al.*, 2007).

1.4 Justification

Acquisition and loss of desiccation tolerance in seeds is a complex process influenced by many physiological processes during seed development and germination. These processes are influenced by environmental conditions during plant growth and/seed development (Figure1.4). Environmental conditions may modify the ultra structure of the seed during development and cause changes at the cellular level, which may ultimately influence seed performance. These interactions are not well understood.

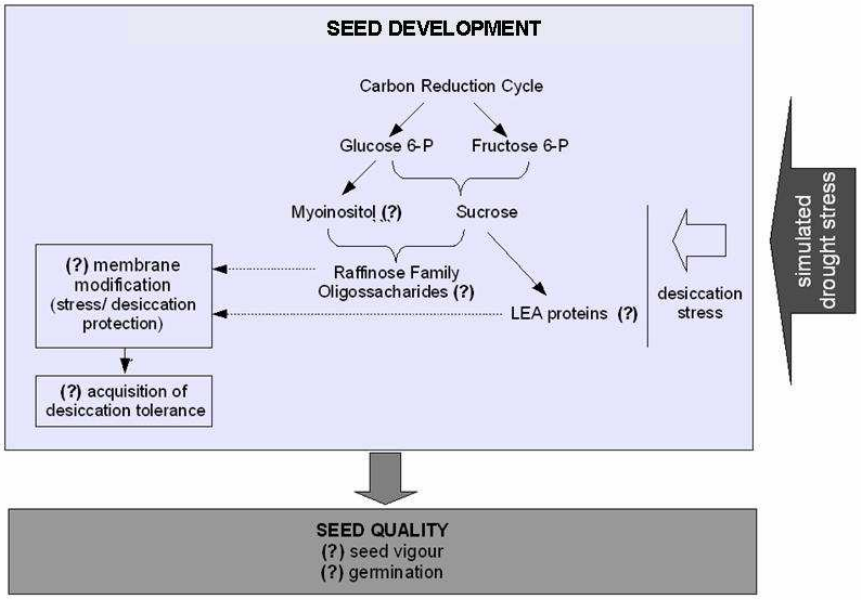

Figure 1-4 A hypothetical model summarizing the interaction between simulated drought and desiccation stress during seed development on the mother plant.

Cowpea is a drought-tolerant species; however, there are no studies that associate plant drought-tolerance with seed quality in response to water stress. It is possible that physiological mechanisms during seed development under different environmental conditions influence seed quality as determined by germination capacity and seed vigour. Environmental conditions may also limit maximum seed quality acquisition by influencing physiological processes during seed development and maturation. In addition, it is likely that interactions between environmental conditions and seed developmental processes modify the ultra structure of the developing seed to ultimately impact on seed quality. Not many studies have identified the mechanisms involved during these interactions.

Cowpea seeds exhibit a wide range of seed coat colours that may be significant in seed quality, with respect to germination capacity and longevity. Seed coat colour has been associated with seed propensity for leakage of substances during imbibition (Asiedu and Powell, 1998), but there have not been detailed studies on the influence of seed coat colour on seed performance. The relationship between seed coat colour and seed performance during germination needs further investigation.

Despite the widespread phenomenon of desiccation tolerance in orthodox seeds and its importance to seed quality development, the mechanism(s) that lead to desiccation tolerance are poorly understood. The raffinose family of oligosaccharides has been implicated in the acquisition of desiccation tolerance; however, there is no conclusive evidence in the literature on the role of RFOs in desiccation tolerance. Whereas RFOs have been shown to accumulate in seeds, no studies have shown their occurrence in cowpea. It is important to determine the relationship between desiccation tolerance, drought stress and seed vigour in cowpea, because the material (cowpea) represents an excellent experimental material for investigation into the nature of these associations.

Different groups of the late embryogenesis abundant proteins have been proposed to contribute in various ways to protection from desiccation during embryo maturation. These proteins are also degraded during germination, implying that they have a role in seed development and germination. However in seeds, direct in *vivo* evidence for the role of LEA proteins in tolerance to complete water loss is not yet convincing. The

expression of LEA proteins including dehydrins in vegetative tissue in response to drought and in temperate crop species has led to the speculation that there are LEA proteins that are specifically linked to desiccation stress rather than drought stress. Hence there is need to further investigate the proposition that different groups of LEA proteins might play protective roles at different hydration levels and that those that may be specifically linked to desiccation tolerance have a protective function at lower hydration levels (~ 0.3 g g^{-1}).

1.4.1 Aims and objectives

This study sought to understand the effect of production site and water stress on cowpea seed quality with respect to germination capacity and vigour. Patterns of RFOs accumulation and LEA proteins (dehydrins) accumulation and/or expression during seed development, maturation and germination were used to physiologically relate seed performance to water stress. The specific objectives of this study were:

1. To investigate changes in seed water content and acquisition of germination capacity during cowpea seed development at different production sites.
2. To determine the effect of simulated drought under controlled environment conditions on cowpea seed quality with respect to germination capacity and vigour.
3. To investigate whether there are differences in solute leakage in seeds of cowpea cultivars differing in seed coat colour and produced at two distinct production sites and whether these differences correlate with other aspects of seed performance during germination and influence the results of an accelerated aging test, which is traditionally used to discriminate between high and low vigour legume seeds.
4. To determine the patterns of RFO accumulation in relation to water stress and the development of seed quality in cowpea.
5. To investigate the effect of water stress and ABA on the accumulation of stress LEA proteins (dehydrins) in relation to seed quality development in cowpea.

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2 CHANGES IN SEED WATER CONTENT AND ACQUISITION OF GERMINATION CAPACITY DURING COWPEA SEED DEVELOPMENT AT DIFFERENT PRODUCTION SITES

2.1 Introduction

Studies into physiological mechanisms that regulate seed growth and development remain important, and several mechanisms have been proposed to explain the role of water status. Walbot (1978) suggested that embryo development may be regulated by changes in water potential. Adams and Rinne (1980) emphasized the role of desiccation as an important signal in switching development from maturation to germination. Galau *et al.,* (1991) and (Kermode, 1995) have reviewed how developmental gene expression programmes, abscisic acid (ABA) synthesis and perception and seed water status interact in determining the course of embryogenesis.

Seed water status can be evaluated using the total water potential (ψ_w) which is the sum of osmotic or solute potential (ψ_s) and pressure or turgor, potential(ψ_{wp}); however, seed water content is commonly used (Bradford, 1994). The general pattern in orthodox seeds is that seed fresh weight increases rapidly early in embryogenesis as cell division and expansion of the embryo occurs, resulting in a sharp increase in seed water content (g water^{-1} g seed) (Fraser *et al.*, 1982; Bewley and Black, 1994; Westgate, 1994). As dry weight subsequently accumulates, seed water content declines during seed filling (Bewley and Black, 1994). This developmental change in seed water content is due to the water volume being displaced from the expanded cells by the accumulation of storage materials (Bradford, 1994). Seed water content declines rapidly after the completion of maturation depending on species and weather conditions, until the seeds reach harvest maturity (HM) (Egli, 1998), to considerably low levels (< 0.1g water g seed) (Egli and Bruening, 2001). This progression of changes is a realistically good indicator of seed development, and water content is generally used as a measure of seed maturity (Harrington, 1972; Bewley and Black, 1994). In this study seed water content was used as a measure of seed development because it can give a practical indication of changes in dry matter accumulation during growth on the mother plant which may possibly be related to the acquisition of germination capacity and vigour under different environmental conditions.

In investigating the role of water in seed development and the effects of water stress thereon, Bradford (1994) emphasized the importance of recognizing that the effects of water deficits on the mother plant will be superimposed on the internal water relations within the developing seed. This emphasis is particularly significant when examining seed water relations during development under conditions of water stress in drought-tolerant crops such as cowpea. Water stress during seed growth and development on the mother plant in drought tolerant crops may elicit different responses depending on when the stress occurs, its duration and severity. In most crops generally, water stress occurring at or soon after pollination can induce embryo abortion and limit the total number of seeds produced (Bewley and Black, 1994). At later stages of development during dry matter accumulation, water stress may lead to reduced seed fill duration (SFD) (Egli, 2006). Quatter *et al*, (1987) and de Souza *et al.* (1997) reported that water stress reduced the SFD in maize (*Zea mays*) and soybean *(Glycine max)* respectively. The effect was probably due to the acceleration of leaf senescence, which may have interfered with assimilate supply. In a drought-tolerant crop such as cowpea, which exhibits traits such as delayed leaf senescence (DLS) (Ehlers and Hall, 1997), water stress on the mother plant during seed growth and development may probably not have a significant effect on SFD.

Seed growth is also characterized by the seed growth rate (SGR- the rate of dry matter accumulation during the linear phase of seed growth) (Egli, 2006). Variation in SGR can be genetic and is closely associated with seed size (Egli, 1981; Sung and Chen, 1990) in that large seeds usually grow rapidly and small seeds grow slowly. Seed growth rate is also influenced by environmental conditions during seed filling either through direct effects on the ability of the seed to accumulate dry matter or indirectly by affecting the supply of assimilates to the seed (Egli, 2006). Seed growth rate was shown to be relatively insensitive to direct effects of water stress on the seed with only minimal effects shown for soybean (Meckel *et al.*, 1984) maize (Westgate and Thompson Grant, 1989) and pea (*Pisum sativum*) (Ney *et al.*, 1994). However, severe water stress may reduce SGR as was shown for soybean (Meckel *et al.*, 1984) and maize (Quatter *et al.*, 1987). Reductions in SGR and SFD can have an effect on dry matter accumulation and eventually seed size, yield and quality. The ability of seeds to germinate is considered as the first sign of seed quality development; and seed

vigour has been shown to improve with increase in dry matter accumulation (Miles, 1985).

The relationship between plant drought tolerance, water stress and the pattern of seed quality development is not clearly understood. The production site can have an effect on the seed water content, which in turn may impact on assimilate production and affect dry matter accumulation by influencing both SGR and SFD. Information in the literature on how these changes may relate to the pattern of acquisition of germination and vigour under different production sites is scanty. In a study investigating the influence of water status on soluble carbohydrate accumulation, Modi *et al.,* (2002) demonstrated that soybean seed cotyledons and axes have similar patterns of water content and osmolality during seed development and germination, and that three major events of soluble carbohydrate occurrence are shared by soybean seed parts during development and germination. Soluble carbohydrates and specific proteins are believed to play a role in stabilizing the macromolecular organization of cell membranes during water loss when the seeds undergo maturation and become desiccation tolerant (Dornbos and McDonald, 1986; Kuo *et al.*, 1988; Horbowicz and Obendorf, 1994). Possibly, the changes in seed water content during development under different production sites may influence the synthesis of these compounds and affect the acquisition of desiccation tolerance. Changes in the ratios of soluble sugars (raffinose family of oligosaccharides) under conditions of water stress were related to a build up in desiccation tolerance in field beans (Lahuta *et al.*, 2000); however, it is not clear how this would relate to the acquisition of seed germination and vigour. Thus, it is surmised that the production site for cowpeas influences two basic determinants of seed quality development: seed water content and food reserve accumulation Further, it is postulated that seed water content and food reserve accumulation may be associated with desiccation tolerance during seed development and eventually mature seed germination capacity. Hence, the objective of this study was to compare cowpea seed development with respect to dry matter accumulation, seed vigour, germination and abnormal seedlings, at different stages characterized by seed water content at two production sites distinguished by dryland and irrigated conditions.

2.2 Materials and methods

2.2.1 Experimental site

The experiment was conducted at two sites in KwaZulu-Natal, South Africa, during the 2003/2004 growing season. The sites were at the University of KwaZulu-Natal (UKZN) Research Farm (Ukulinga) in Pietermaritzburg (29° 37' S 30° 16' E) and at Umbumbulu, (29^{o} 36'S 30^{o} 25'E) (Figure 2.1). The long-term mean annual and monthly rainfall and temperature for the experimental sites are presented in Table 2.1a and b. The experiment at Ukulinga was planted the on 27th February 2004 and harvested in July 2004. Planting at Umbumbulu was done on the 1st March 2004 and harvesting was done in June/July 2004.

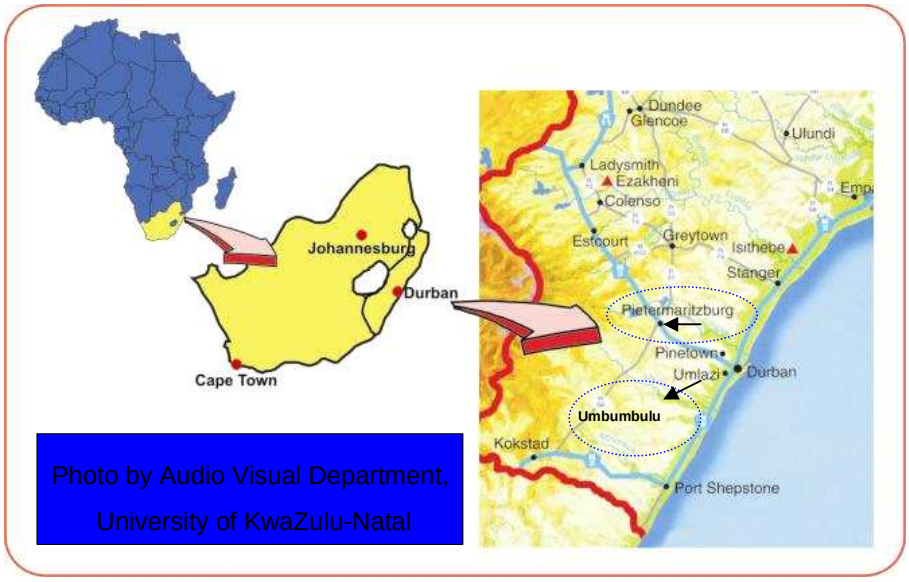

Figure 2-1 Field study areas, Pietermaritzburg and Umbumbulu, ~ 65 km apart.

Table 2-1a Long-term climatic data (rainfall and temperature) averages at Ukulinga from January to December (Camp, 1999)

	Annual	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Rainfall (mm)	738	116	98	92	48	27	10	10	30	51	67	90	99
Temperature (^{o}C)	18.1	21.9	21.9	21.1	18.7	16	13.4	13.4	15.2	17.1	18.3	19.5	21.2

Table 2-2b Long-term climatic data (rainfall and temperature) averages at Umbumbulu from January to December (Camp, 1999)

	Annual	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Rainfall (mm)	956	138	121	127	59	40	27	24	33	61	94	119	113
Temperature (^{o}C)	18.6	21.9	22.1	21.4	19.4	17.2	14.9	14.7	15.7	17.3	18.4	19.5	21.2

2.2.2 Plant materials, experimental design and crop establishment

Seeds of cowpea cultivar Brown Mix were purchased (Capstone Seeds, Howick, South Africa); cultivars Makonge and Nzovu were donated by the Environmental Action Team (EAT), Kitale, Kenya; cultivars M66 and K80 were purchased from the Basic Seed Unit of the Kenya Agricultural Research Institute (KARI), Machakos, Kenya; cultivar Kenkunde seeds were purchased from the Kenya Seed Company, Kitale, Kenya. Seeds were planted without conditioned storage or pretreatment within three to four weeks after they were received in the laboratory.

The two experiments were laid out in a randomized complete block design with three replications. Seeds were planted at a spacing of 75 cm between rows and 20 cm within rows (see field layout in Appendix 2.1). At Ukulinga, a compound fertilizer 2:3:2 (22) was applied at the rate of 600 kg ha^{-1}. At Umbumbulu (under dry land conditions) the experiment was fertilized according to the soil analysis (Appendix 2.2) using organic fertiliser (Neutrog®) (see Phiri and Modi, 2005). At Ukulinga site (Pietermaritzburg) the experiment was irrigated (~25 mm week^{-1}) until ~ 80 % crop senescence stages. At Umbumbulu the crop was grown under dryland conditions with no additional water application (Phiri and Modi, 2005). Weed control was done manually by hand-hoeing and no chemicals were used to control pests and diseases at both sites. Field emergence was determined two weeks after planting. Early pods from the bottom parts of the plants were tagged as they reached R3 stage of plant development (Figure 2.2), and harvested during six developmental stages (R4, R5, R6, R7, R8, R9) weekly until harvest maturity (R9). Seeds were used immediately (within 24h) after harvesting for germination tests (after room temperature storage) and those that were used for chemical analyses were freeze dried and stored at -70 ^{o}C

until they were needed for chemical analysis. At Ukulinga, seeds of different cultivars matured at different times; however they were all harvested at the same time. Environmental conditions during harvesting were wet. Harvested pods were kept in a dry well ventilated glasshouse and threshed. Seeds were removed and placed on benches in the glasshouse under dry conditions and allowed to dry to less than 12% moisture content. Mature dry seeds were then stored at 5^{o}C in the cold room until further analysis. At Umbumbulu environmental conditions during harvesting were dry and sunny. Dry pods were harvested separately according to the time taken to reach harvest maturity for each cultivar. Similarly, the pods were threshed and the seeds removed and allowed to dry to less than 12% moisture content in the glasshouse and then the mature dry seeds stored at 5^{o}C in the cold room until further analysis. Seed yield was determined at harvest maturity before storage.

Figure 2-2 Pods were tagged at R3 when they were ~ 25 mm long at first blossom (Fageria, 1992).

2.2.3 Determination of seed water content and dry matter accumulation

For each developmental stage, ten seeds, which had been dissected into separate seed tissues (embryo, cotyledons and seed coat), were weighed to four decimal places, frozen in liquid nitrogen and lyophilized for 72 h and then weighed again to determine the dry matter content. Seed water content (in g water^{-1} g seed) was calculated using the following formula:

$$Seed\ water\ content\,(g\ water^{-1}\ g\ seed) = \frac{Fresh\,weight - Dry\,weight}{Fresh\,weight}$$

2.2.4 Laboratory germination test and seed vigour

Seeds from each cultivar harvested from the replicates in the field experiments were bulked. Bulked samples were divided into five replicates of ten seeds and germinated using the paper towel method (ISTA, 1999). Seeds were arranged in a straight-line midway between the long edges of the paper towels. The paper towels were rolled up and placed in a polythene bag to reduce loss of moisture and then placed in plastic buckets. The buckets were placed in a germination cabinet set at a temperature of 30/20^{o}C with 8h light/16h dark conditions. Germination was assessed by counting seeds which had radicle protrusion, daily up to eight days. Normal, abnormal and dead seeds (ISTA, 1996) were assessed and recorded at the final count. Germination index (GI) was used to measure seed vigour. The test is based on the time taken to germinate and the total number of germinated seeds. GI was selected because the amount of seed material was limited and the test was able to provide an indication of seed vigour development over time. GI was determined according to Scott *et al.* (1983):

$$GI = \frac{\sum_{i=1}^{n} T_i N_i}{TN}$$

GI = germination index, T_i is the number of days after sowing starting with the final day, N_i is the total number of seeds germinated on day i, where i = day 1, 2, 3…. 8 and N is the total number of seeds that have germinated on the final count. The method calculates an index, which describes seed vigour on a scale of 0 to 1, with values close to 0 indicating low vigour and a value of 1 indicating maximum vigour.

2.2.5 Statistical analysis

Genstat® (8th edition, Rothamsted Experimental Station, UK) was used to perform analyses of variance. The data was subjected to analysis of variance after testing that they meet the assumptions underlying the use of this technique (Appendix 2.3). Germination percentage, germination index and field emergence (%) data were subjected to angular transformation prior to statistical analysis (Appendices 2.4a, b, c, d, f, g & j). Separate analyses were initially done for each site to compare variances (Appendix 2.4e) and subsequently the data was pooled for analysis. The differences between means were determined by LSD (least significant difference; P = 0.05) which was calculated as SED x t $_{(\text{error degrees of freedom, } 0.05)}$.

2.3 Results

The major difference between the two production sites used in this study was that one site (Ukulinga, Pietermaritzburg) was an irrigated site, representing resource-rich experimental station conditions, and the other site (Umbumbulu) was a dryland site, representing resources-poor small-scale farming conditions. The sites differed in environmental conditions. The climatic data were collected from both sites using basic equipment for rainfall and data loggers for temperature and humidity. The rainfall and temperature data for both sites during the growing season are shown in Figure 2.3. The total amount of rainfall throughout the growing season at Umbumbulu was 142.4 mm of which 88.4 mm constituting 62% of the total occurred in March 2004. At Ukulinga the total amount of rainfall during the growing season was 109 mm. However, the plants at Ukulinga were irrigated with ~25 mm water per week which adds up to a total of 500 mm for the whole growing period of 5 months. Thus the plants at Ukulinga received total moisture of ~ 609 mm of water during the growing season. The autumn and winter temperatures were not significantly different at the two sites, but Umbumbulu was slightly warmer than Ukulinga during the spring.

A separate analysis was initially done for each site and the F-statistic was used to test for the homogeneity of variances (Appendices 2.4e). The F-calculated was not greater than F-tabulated, at the 1% level of significance; therefore the Null Hypothesis was accepted and it was concluded that there is a common variance. Hence data from both sites were pooled for subsequent analysis. Statistical analysis showed that the data sets before and after angular transformation met the underlying assumptions for the analysis of variance (Appendix 2.3). However, the analysis of both germination percentage, germination index and field emergence data was performed on transformed data (Appendices 2.4a, b, c, d, f, h & j). The ANOVA for the separate analysis for both sites (Ukulinga and Umbumbulu) were consistent with those before transformation (Appendices 2.4a & b). Similarly, the ANOVA for the pooled data from both sites with respect to germination capacity, germination index and field emergence were consistent with those before transformation (2.4f, g. h. i &j). Therefore the original data was used in the presentation and discussion of the results.

Assessment of initial seed quality before planting (data not shown) showed that the seeds were of high quality. However, the production environment influenced the final emergence as evidenced by the highly significant differences ($P < 0.001$) between sites. The average number of plants that emerged under irrigated conditions was 89% at Ukulinga compared to 76% at Umbumbulu (Figure 2.4).

For all six cultivars, highly significant differences were observed between the stages of development ($P < 0.001$) and between production sites ($P < 0.001$) (Figure 2.5) with respect to seed water content. Seeds produced at Umbumbulu maintained consistently higher water content throughout development up to stage five (Figure 2.5). However, there were no significant differences in seed water content at harvest maturity (HM).

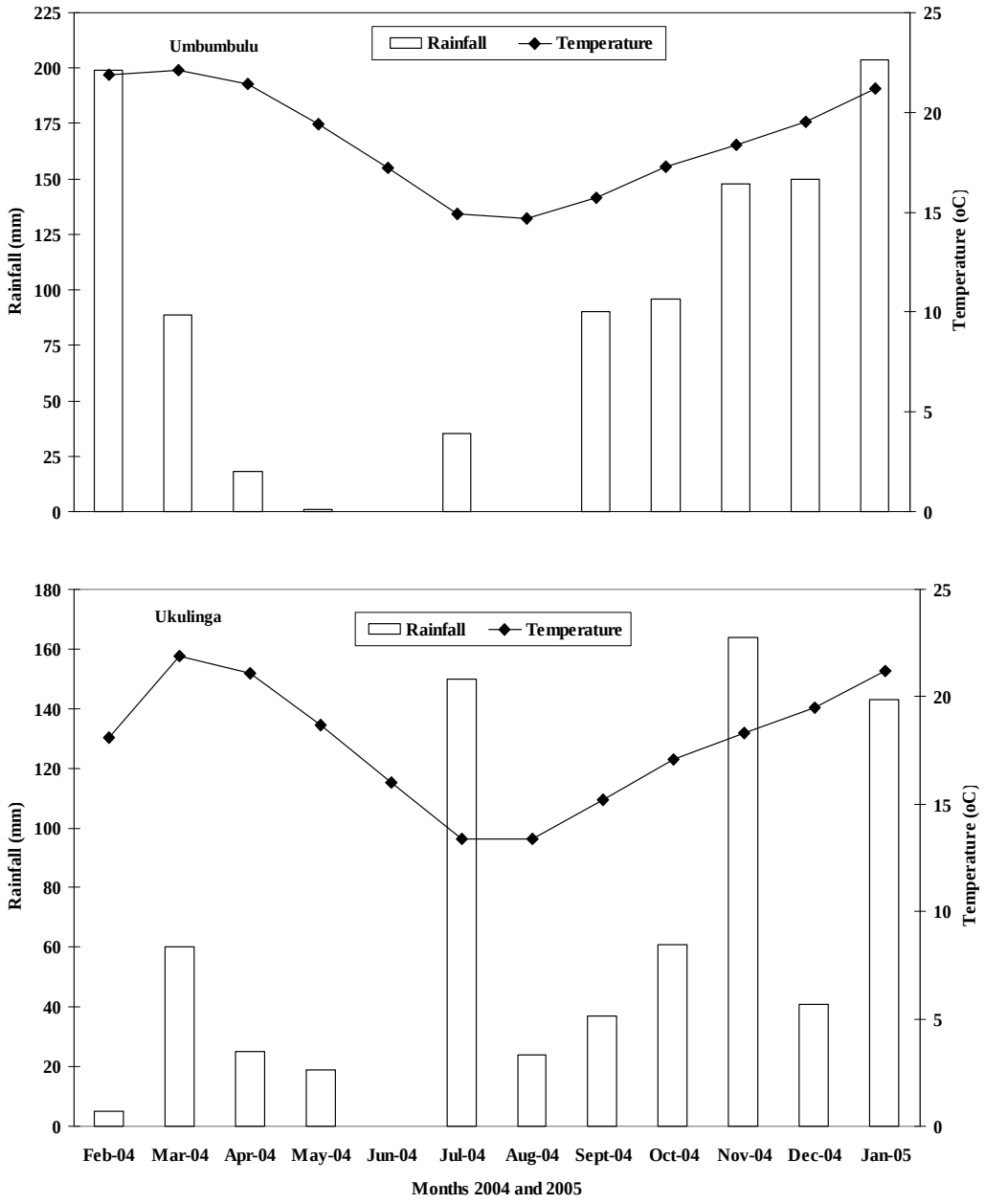

Figure 2-3 Temperature and rainfall data at Umbumbulu and Ukulinga experimental sites during the 2004 growing season.

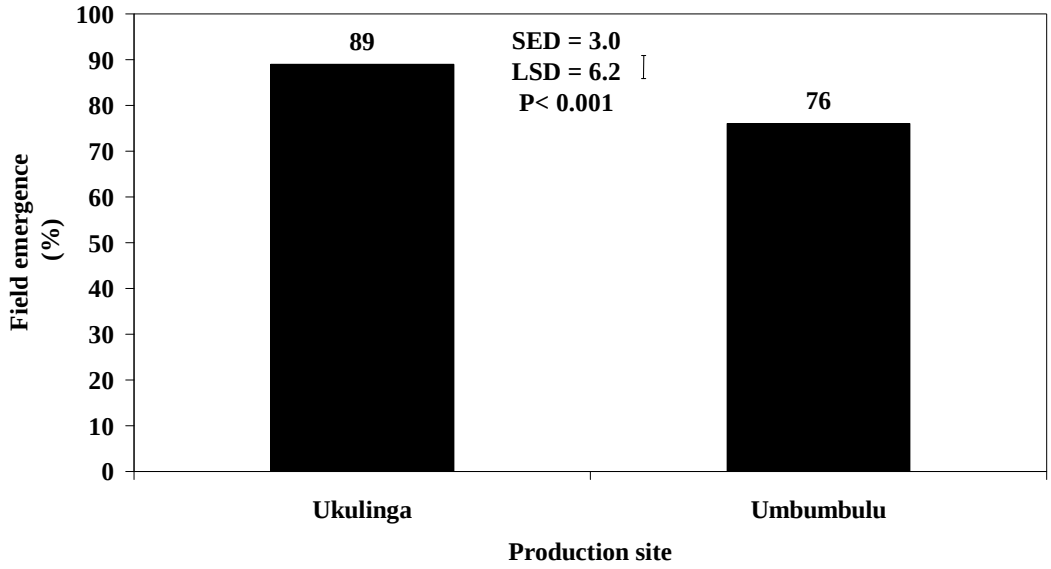

Figure 2-4 Average field emergence for six cowpea cultivars produced at two sites (Ukulinga and Umbumbulu).

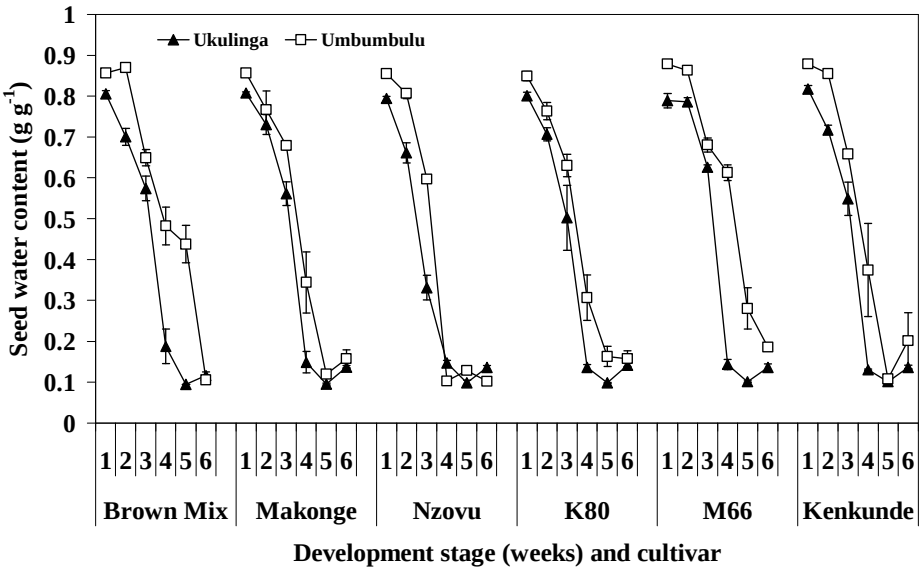

Figure 2-5 Seed water content during seed development and maturation for six cultivars of cowpea produced at two different sites (Ukulinga and Umbumbulu. Note: Development stage refers to weeks after R3 when pods were tagged for each cultivar.

Similarly, the interaction between seed development and the production site was highly significant ($P < 0.001$) (Figure 2.6 and 2.7). Developing seeds produced under

irrigation at Ukulinga showed an improvement in germination and vigour more rapidly than those produced at Umbumbulu (Figure 2.6). Germination capacity ranged from 45% to 88% for seeds produced at Ukulinga compared with 10% to 40% for seeds produced at Umbumbulu at development stage three. Germination capacity at Umbumbulu increased rapidly to 80% at the fourth stage of development and did not differ significantly at harvest maturity from that of seeds produced at Ukulinga. The delay in the development of vigour at Umbumbulu was highly significant ($P< 0.001$) during development stage three (Figure 2.7). However, despite this delay in physiological development, seeds of all cultivars produced at Umbumbulu showed a large improvement in vigour during development stage four and did not differ significantly from those produced at Ukulinga at development stage six (HM) (Figure 2.7).

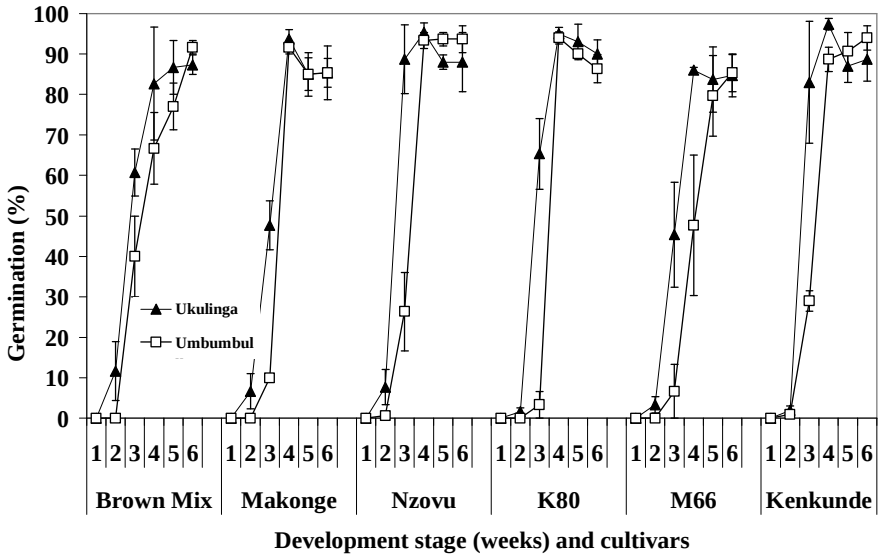

Figure 2-6 Germination capacity during seed development and maturation for six cultivars of cowpea produced at two different sites (Ukulinga and Umbumbulu). Note: Development stage refers to weeks after R3 when pods were tagged for each cultivar.

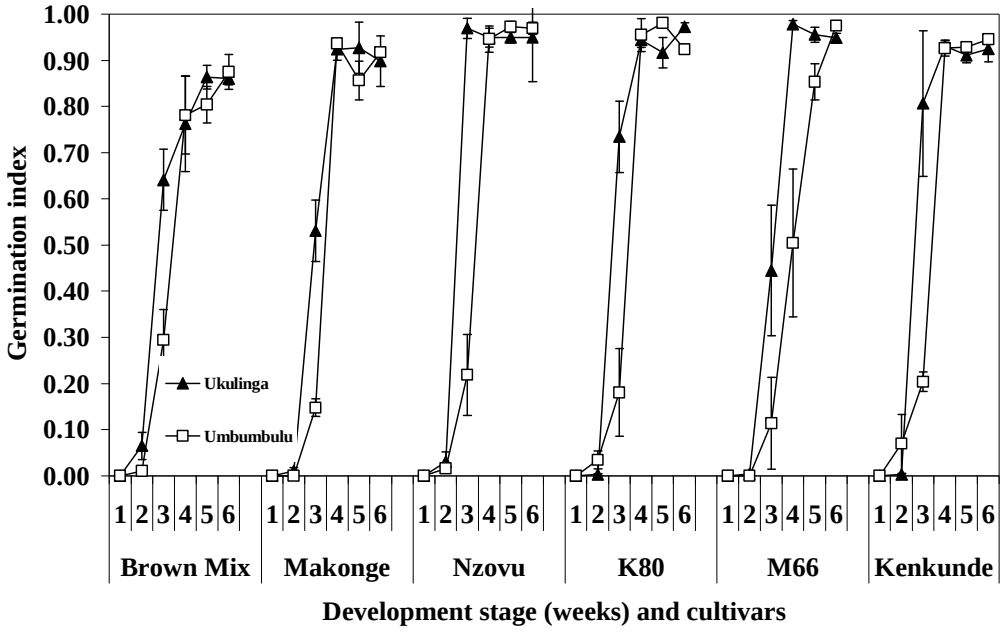

Figure 2-7 Germination index during seed development and maturation for six cultivars of cowpea produced at two different sites (Ukulinga and Umbumbulu). Note: Development stage refers to weeks after R3 when pods were tagged for each cultivar.

However, seed development was affected, as evidenced by the reduction in dry matter accumulation (Figure 2.8) and the large proportion of abnormal seedlings observed during development (stage three) of seeds produced at Umbumbulu compared with seeds produced at Ukulinga (Figure 2.10).Seed yield was also significantly affected (Figure 2.9) at Umbumbulu compared with those produced at Ukulinga. Highly significant differences were observed between the cultivars with respect to yield. Under well irrigated conditions at Ukulinga, the cultivars M66, K80 and Makonge showed the best performance than Kenkunde and Nzovu. Brown Mix performed the poorest under irrigated conditions. Dry land conditions significantly reduced the yields of all the cultivars. Although not significantly different from K80, Nzovu and Makonge, the cultivar M66 showed the best performance under dry land conditions.

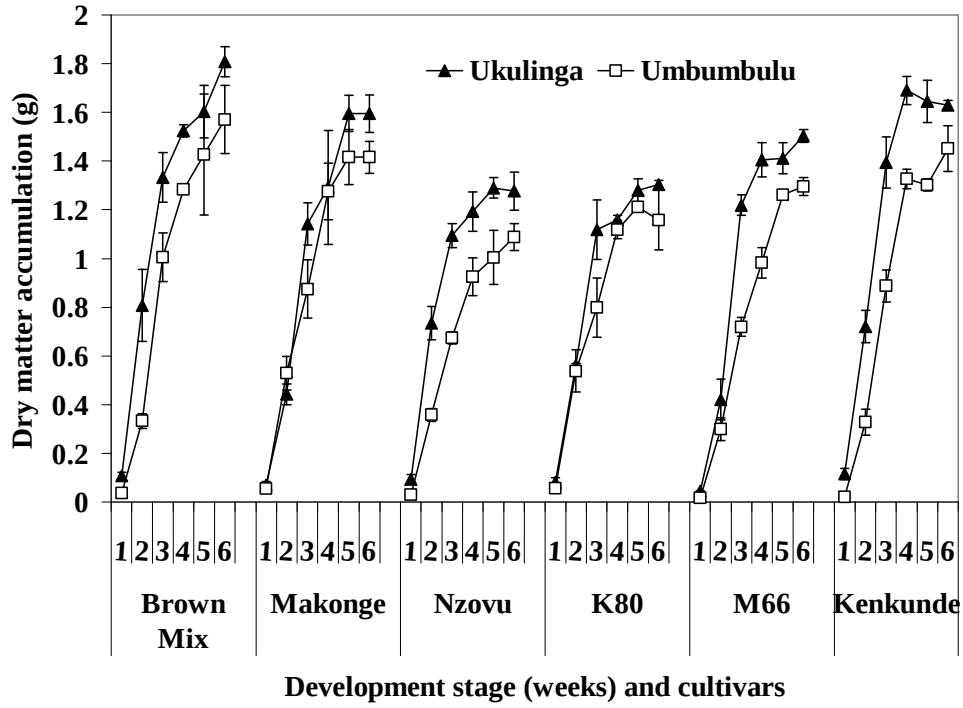

Figure 2-8 Dry matter accumulation during seed development and maturation for six cultivars of cowpea produced at two different sites (Ukulinga and Umbumbulu). Note: Development stage refers to weeks after R3 when pods were tagged for each cultivar.

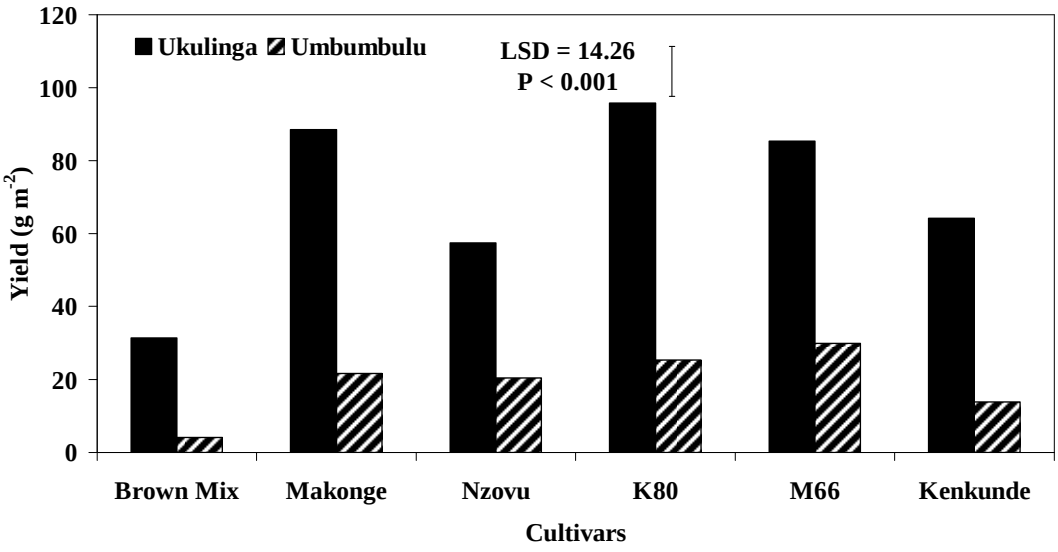

Figure 2-9 Seed yield of six cowpea cultivars produced at two sites (Ukulinga and Umbumbulu).

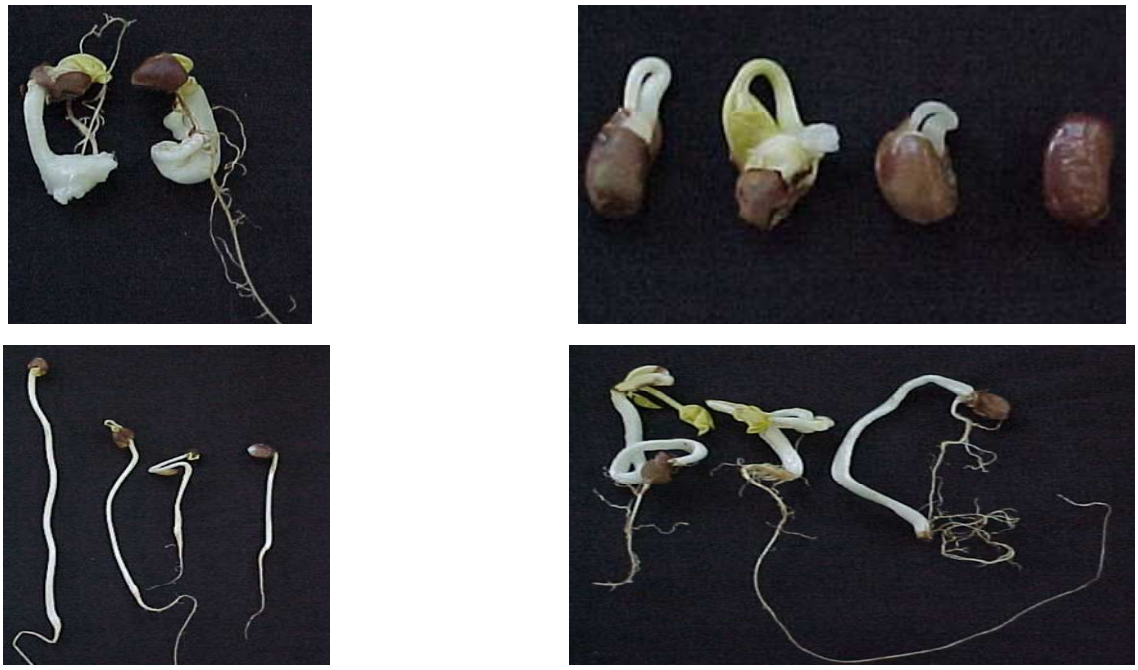

Figure 2-10 Typical seedling abnormalities observed at developmental stage three for seeds produced at Umbumbulu. Detailed descriptions of seedling abnormalities can be found in AOSA (1992).

There was a negative linear relationship between germination capacity and seed water , which was highly significant for both sites (r^2 = 0.9944, P < 0.001 and r^2 = 0.8956, P < 0.01 for Umbumbulu and Ukulinga, respectively). Moreover, the regression coefficients (131.26 for Umbumbulu) showed that the magnitude of change in seed water content was larger compared Ukulinga (124.14). These results indicated that the decline in seed water content at Umbumbulu was more rapid compared to Ukulinga (Figure 2.5). The accumulation of dry matter was also positively linearly correlated with germination capacity (P < 0.01, r^2 = 0.8529 and 0.9074 for Umbumbulu and Ukulinga, respectively). Similarly, there was a highly significant positive linear relationship between dry matter accumulation and the development of seed vigour (P < 0.01, r^2 = 0.8691 and 0.9098 for Umbumbulu and Ukulinga).

2.4 Discussion

The total rainfall in 2004 for Ukulinga (729mm) and Umbumbulu (1029.7mm) compared reasonably well with the long-term rainfall averages for Ukulinga (738mm)

and Umbumbulu (956mm) (Camp, 1999). However these figures are relatively much higher than the national average in South Africa, which is estimated to be 501mm (Bennie and Hansley, 2001). Planting at both sites was done much later during the rainy season in February and March 2004. The rainfall at Umbumbulu during this time (growing season) was relatively lower than the minimum rainfall requirements for cowpea production in Southern Africa which are estimated to be about 300 mm spread over the growing season (van Rij, 1997). The growing conditions at Umbumbulu were comparatively similar to cowpea production areas in the Sahel region, where yields of up to 1000 kg ha^{-1} have been recorded under conditions of limited moisture (181mm per year) and high temperatures (Hall and Patel, 1985). These conditions (at Umbumbulu), were generally hot and dry, and similar to dryland conditions characteristic of cowpea production areas in Africa. South Africa, with an approximate land area of 102.8 million ha and an average annual rainfall of 501 mm (Bennie and Hansley, 2001), can be considered as a dry region, which can be drought prone. Dryland crop production in South Africa covers an estimated 11 million ha and utilizes 12% of the potentially available rain water estimated to be 51.4 million m^3 (Bennie and Hansley, 2001).

The production environment influenced the final emergence as evidenced by the highly significant differences ($P < 0.001$) between sites. Seeds that were used for planting were assessed for germination capacity and vigour and were found to be of high quality (data not shown). The differences in emergence between sites may be partly attributed to inadequate moisture availability at Umbumbulu. Dry seeds at Umbumbulu may have been able to extract water from the soils with very little moisture and germinated due to the high seed matric potential. The germinated seeds may have been able to tolerate desiccation for a short period after seedling growth had begun (Finch-Savage, 1987). However, damage to the expanded tissues could have occurred if the supply of water did not increase for a long period. This may probably explain the poor seedling establishment and emergence at Umbumbulu. On the other hand, seeds at Ukulinga which had adequate soil moisture for the germination process and subsequent seedling emergence and establishment performed well.

The influence of the environment in determining seed quality development was evident by the close relationship between seed water content and quality. This

influence was more significant at Umbumbulu as seen from the regression coefficients which showed that the magnitude of change in seed water content was larger at Umbumbulu compared with Ukulinga. These results indicated that the decline in seed water content at Umbumbulu was more rapid compared with Ukulinga.

The higher seed water content observed in seeds produced at Umbumbulu compared with those produced at Ukulinga was probably related to differences in the stage of physiological development. Seed water status has been shown to be related to the physiological events associated with seed development (Perl, 1987; Modi *et al.*, 2002). Major events associated with development include the accumulation of dry matter and the acquisition of germination capacity and vigour.

The observed significant interaction ($P < 0.001$) between seed development and the production environment coupled with the delay in the acquisition of germination and vigour at Umbumbulu during stage three of development suggested that the production site influenced the physiological processes that regulate the development of germination capacity and seed vigour. It can be speculated that at Umbumbulu, two events occurred simultaneously; firstly the desiccation intolerant stage may have been prolonged as a result of the delayed physiological development. Walbot (1978) speculated that changes in water potential and membrane permeability will be ultimately shown to control embryo development. Perl (1987) proposed that sensitivity of seeds to drought could be linked to the cell membrane structure during its synthesis or to changes in activities at the genome level. Desiccation intolerance may also be accompanied by the process of DNA synthesis and cell division (Perl, 1987). Thus, the large proportion of abnormal seedlings observed during development stage three of seeds produced at Umbumbulu may be related to changes in regulatory processes triggered by the production environment.

Secondly, the dry conditions at Umbumbulu may have induced premature drying due to accelerated ontogeny (Bohnert *et al.*, 1995). This may have resulted in premature suppression of the developmental programme and a switch to germination related events (Kermode, 1995). Consequently, simultaneous developmental and germination-related synthesis may have occurred, which explains the large number of

abnormal seedlings and the slow improvement in germination and vigour (Figure 2.10). Water deficits can cause changes in ABA and GA sensitivity in seeds (Bewley and Black, 1994; Bohnert *et al.,* 1995). It has been hypothesized that sensitivity to growth hormones is due to the presence or absence of available specific receptor sites, which seem to be membrane located (Trewavas and Jones 1991). In their reviews Galau *et al.,* (1991) and (Kermode, 1995) discussed how developmental gene expression programmes, ABA synthesis and seed water status interact in determining the course of embryogenesis. Environmental conditions during this study may have influenced these physiological interactions, which may have affected seed water content and germination capacity.

Despite the delay in development at Umbumbulu, germination capacity increased rapidly at the fourth stage of development and did not differ significantly at HM from the seeds produced at Ukulinga. Differences in harvesting methods between the two sites, (at Ukulinga all the seeds were harvested on the same day and at Umbumbulu the cultivars were harvested individually as they attained maturity) did not result in to apparent differences in germination capacity between the two sites. Similarly, in a study on the comparative effects of date of sowing on the yield, viability and vigour of peas (*Pisum sativun*) and flax (*Linum usitatussimum*) Siddique and Wright (2004) observed that sowing dates (which influence the harvesting time) had relatively small effects on the germination of non-aged seeds, but much larger effects on the emergence of non-aged seeds and on the germination and emergence of aged seeds. In the present study differences in the time and method of harvesting between sites may have influenced other aspects of seed quality (vigour).

The findings in this study with respect to germination capacity are contrary to the report that temperature and drought stress during seed development decreased seed germination in soybean (Dornbos, 1995). It is important to note that, unlike the study by Dornbos (1995), the present study did not examine stress conditions under controlled environmental conditions. Plants are able to adapt to environmental stress (Salisbury and Ross, 1991). The adaptive mechanisms may account for the observation that while stress can reduce seed number and seed size, the seeds that are formed usually have high germination capacity. This observation is supported by the

reports that water stress did not result in reduced germination capacity in soybean (Dornbos *et al.*, 1989; Smiciklas *et al.*, 1989; Vieira *et al.*, 1992).

Seed development was, however, affected, as evidenced by the reduction in dry matter accumulation and seed yield at Umbumbulu compared with those produced at Ukulinga. The highly significant reduction in yield (Figure 2.9) clearly shows that although cowpeas are drought tolerant, the species is sensitive to drought stress. Cultivar differences were also evident in the tolerance to drought stress. The better performance of M66 under both irrigated conditions at Ukulinga and dry land conditions at Umbumbulu are consistent with the known characteristics of this cultivar which was bred for drought-tolerance in Kenya (Ehlers and Hall, 1997). Differences in drought-avoidance mechanisms were also evident between the cultivars. For example, M66 has the Delayed Leaf Senescence trait which is an adaptive trait for drought tolerance (Gwathmey *et al.*, 1992). M66 differed significantly from Nzovu with respect to yield under irrigated conditions at Ukulinga; however the yield difference between the two cultivars was not significantly different at Umbumbulu. It was observed that the cultivar Nzovu was the fastest growing. Probably the faster growth rate is a drought avoidance strategy that allows this cultivar to avoid drought stress by maximizing as much as possible on the little available moisture.

Seed development was also affected as has already been mentioned earlier; this was evident from the large proportion of abnormal seedlings observed during development of seeds produced at Umbumbulu compared with seeds produced at Ukulinga.

In conclusion, this study provided evidence that cowpea seed lots produced under different environmental, and possibly management conditions may not differ with respect to seed quality as determined by germination capacity. However, there were significant differences between sites with respect to seed maturation patterns determined by water content and dry matter accumulation. Water status influences the physiological events associated with seed development (Modi *et al.*, 2002). Therefore, it is likely that the differences in seed water content during development and between the developmental stage germination capacities observed in this study have significance in mature seed quality as determined by factors other than germination

capacity. The findings of this study merit further investigations into the physiological quality of cowpea seeds as influenced by production site. Future studies should examine seed vigour and storability. In addition different strategies that enable them to cope with drought stress should also be examined.

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3 EFFECT OF SIMULATED DROUGHT UNDER CONTROLLED ENVIRONMENT CONDITIONS ON COWPEA SEED QUALITY WITH RESPECT TO GERMINATION CAPACITY AND VIGOUR

3.1 Introduction

Vigour is a well-established characteristic of seeds and can be defined as the inherent ability of seeds within a seed lot to establish normal (or usable) seedlings under diverse growing environments (Hilhorst and Toorop, 1997). Poor seed vigour may be due to the inherent genetics of a cultivar, environmental conditions during seed development and post –maturation, handling during seed conditioning, seed storage conditions and a combination of these factors (Delouche, 1980). It is likely that drought stress on the mother plant may interact with seed developmental processes and influence vigour. Interaction between drought stress and seed quality in cowpea has not been satisfactorily explained.

In investigating whether there is a relationship between drought tolerance and the development of seed germination and vigour in cowpea, it is important to recognise that response mechanisms to drought stress at the whole plant level are effective in maintaining plant water status and avoiding cell desiccation (Tardieu, 1996). Cowpea plants are able to cope with drought stress by exhibiting an extreme dehydration avoidance strategy (Ludlow and Muchow, 1990; Petrie and Hall, 1992; Singh and Matsui, 2002). This allows the plant cells to avoid being stressed because plant functions are relatively unexposed to tissue dehydration (Tardieu, 1996). The avoidance strategy in cowpea may involve enhanced absorption of soil moisture , limited crop water loss and the retention of cellular hydration despite the reduction in plant water potential (Blum, 2005). This is achieved through several mechanisms, including stomatal closure, reduced leaf growth and leaf senescence, which lead to reduced water flux through the plant; or increased root growth which may lead to an increase in water uptake. The plant growth regulator abscisic acid (ABA) is also implicated in the complex responses to drought stress by crop plants. ABA accumulates in various plant organs in response to water stress (Salisbury and Ross, 1991) and plays a major role in stress avoidance through its effect on stomatal

closure, on reduction of leaf expansion and the promotion of root growth (Tardieu, 1996).

However, it is likely that a relationship between drought tolerance and the development of seed germination and vigour may exist at the cellular and molecular levels. Plant responses to drought at the cellular level can be divided into those that allow the plant cells to continue growth and development, those that allow plant survival and those that are a response to the lethal disruption of function (Mullet and Whitsitt, 1996). Probably the effect of drought tolerance on seed germination and vigour would occur as a result of changes at the cellular level, which can lead to a reduction of photosynthesis. An increase in ABA levels in response to drought may lead to stomatal closure, decrease in transpiration rates, carbon fixation and a decline in nitrate levels, thus resulting in less assimilate being produced and translocated to the developing seeds, which may eventually affect seed germination and vigour. An important cellular response to water deficits is the accumulation of osmotically active compounds that allow cells to re-establish turgor and extract additional water from the soil (Morgan *et al.*, 1991). Certain compounds such as the amino acid proline, polyols and various sugars and proteins may accumulate in cells subjected to drought stress (Bray, 1993; Bohnert *et al.*, 1995; Tardieu, 1996). Some of these compounds for example, sugars and proteins may also play a role in the stabilisation of the molecular organisation of cell membranes when seeds lose water during maturation (Bewley and Black, 1994). In orthodox seeds, water loss occurs in a programmed manner and serves as an indicator for the termination of development (Kermode, 1995). It is likely that an interaction may occur between drought tolerant mechanisms at the cellular level and seed developmental processes and influence germination and vigour.

In a study investigating cowpea response to different intensities of drought at different stages of growth Turk *et al.* (1980) reported that drought stress resulted in low pod density and small seeds, which considerably reduced cowpea seed yield. Interestingly, seeds produced under well watered and drought conditions were smaller than those produced when water supplies were intermediate; intermediate water supply produced the largest seeds (Turk *et al.*, 1980). Although these authors concluded that that “the ecological strategy of cowpea under stress in many circumstances involves an extreme reduction in pod number, which ensures that plump viable seeds are

produced", the study did not show whether germination and seed vigour tests were done to determine whether there were differences in quality between the heaviest and smaller seeds. It is possible that drought stress may have also caused alterations in the composition of the seed which may have influenced seed vigour.

There are numerous reports in the literature on the effect of drought stress on seed germination and vigour. Temperature and drought stress during seed filling decreased germination in the standard germination test as well as embryonic axis weight in mature soybean *(Glycine max)* seeds (Dornbos, 1995). In contrast, sorghum (*Sorghum bicolor*) seeds grown under mild drought stress displayed significantly much higher germination than those grown under normal conditions (Benech -Arnold *et al.*, 1991). Similarly, drought stress seemed to have improved seed quality in brassica (*Brassica campestris* [*rapa*] L.). Work done by Ellis *et al*. (2000) showed that in rapid cycling brassica, terminal drought resulted in enhanced water loss and low seed weight; however seed quality development was more rapid and there was a greater maximum seed quality with respect to potential longevity. Viera *et al.* (1992) reported that soybean seeds exposed to drought stress during seed development showed large reductions in yield and seed number; however drought stress did not have an effect on germination and vigour. Clearly, responses to drought stress are variable and range from negative effects to positive effects and no effects on seed germination and vigour (Chapter 1). These variable responses may be indicative of different underlying physiological mechanisms or complex interactions between seed developmental processes, plant genotypes and environmental factors.

It has been suggested that the effect of the maternal environment can be carried over and influence the performance of subsequent generations. Wulf (1995) postulated that "the success of an individual plant in a population may depend not only on the characteristics of that particular individual but also on the previous generation, particularly on the conditions experienced by the mother plant during seed development and maturation". In a study investigating the residual effect of temperature on the growth of peas Highkin and Lang (1966) observed that when peas were grown for several generations under adverse temperature conditions , each generation (up to about the fifth) grew more poorly than the previous one. It has been suggested that the developing embryo or accumulated food reserves can be modified

by interaction between the environment and the mother plant so that effects carry over through a number of succeeding generations (Salisbury and Ross, 1991). These modifications may arise through cytoplasmic factors in the transmission of organelles, by endospermic effects and by phenotypic effects which include the influence of both the maternal genotype and the maternal environment (Roach and Wulf, 1987). The mechanisms by which these effects are induced, maintained and transmitted, sometimes along several generations, are not well understood.

There is, to a large extent, information about the mechanisms of drought adaptation of cowpea at the whole plant and cellular level, and their influence on seed yield (Turner, 1979; Turk *et al.*, 1980; Ludlow and Muchow, 1990; Petrie and Hall, 1992; Petrie *et al.*, 1992; Hall *et al.*, 2003; Hall, 2004), but less knowledge and understanding on how these mechanisms influence seed germination and vigour. Information in the literature on the effect of environmental stress factors on seed germination and vigour seems to contain contradictory conclusions (Vieira *et al.*, 1992; Bewley and Black, 1994; Dornbos, 1995; Tekrony *et al.*, 2004). Differences between plant species in response to drought stress are considerable and well established and may probably account for some of these contradictory conclusions. In addition, cultivar differences may also exist. The magnitude and consequences of cultivar differences with respect to germination and vigour in drought tolerant crops have not always been assessed. Variations in the duration, intensity and timing of the stress and experimental techniques in the imposition and determination of water stress may also influence results and conclusions.

Cowpea seeds were initially produced at two distinct production sites, Umbumbulu and Ukulinga, and used in this study to investigate the effect of the maternal environment and simulated drought under controlled environmental conditions on seed germination capacity, vigour and yield. The use of a soil water sensor to monitor changes in soil matric potential and water content in a pot experiment was compared with normal gravimetric methods.

3.2 Materials and methods

3.2.1 Soil characteristics

The following soil characteristics were determined from soil samples, which were taken from Ukulinga (see chapter 2, section 2.2). Prior to the analysis the soil samples were air-dried, ground and passed through a 2 mm sieve. Samples were analysed to determine particle size, particle density, % total porosity and bulk density.

3.2.1.1 Particle size analysis

Particle size distribution of the soil samples was performed using the pipette method (Johnson, 2000). A 20 g soil sample was dispersed chemically by the calgon solution (sodium hexametaphosphate and sodium carbonate dissolved in de-ionized water) and mechanically by ultrasound at a maximum output for three minutes. The suspension was then passed through a 0.053 mm sieve to a 1 L measuring cylinder and filled with distilled water. When the temperature of the suspension had reached room temperature, the suspension was agitated properly and a 20 ml sample was taken immediately by pipette to determine the amount of coarse silt. The second sample was taken from 100 mm depth at a pre-calculated time based on Stoke's law to determine silt and clay. To represent the clay content a 20 ml sample was taken at 75 mm below the surface based on the same principle. Each sample was then oven dried for 24 h at 105 ^{o}C to determine the mass of the soil particles. Finally, the sand fraction was determined from the soil sample that was left over after sieving by 0.053 mm sieve. After oven drying the soil was sieved through a nest of sieves consisting of 0.500 mm for coarse, 0.250 mm for medium and 0.0106 mm for fine and very fine sand, using a sieve shaker for five minutes.

3.2.1.2 Soil particle density

Soil particle density determination was done according to the method of (Blake and Hartge, 1986b). A 50 g soil sample was added to a pre-weighed 100 ml volumetric flask (M_W) and weighed again with the soil sample *(*M_S). The water content of a duplicate sample was determined by oven drying at 105 ^{o}C. To remove the entrapped air the flask was boiled for several minutes on a hotplate with frequent but gentle agitation of the contents to prevent soil loss by foaming. The flask and its contents was allowed to cool to room temperature and then boiled, cooled and distilled water

was added to fill it up and the weight taken *(M_{SW})*. Finally, the soil was removed and the flask was weighed by filling with boiled cooled and distilled water *(M_W)*. The particle density was then calculated using the following formula (Johnson, 2000).

$$\rho_p = \frac{\rho_W (M_S - M_A)}{[(M_W - M_A) - (M_{SW} - M_S)]} \quad (3.1)$$

where ρ_w is the density of water in kg m^{-3} at temperature observed, M_S is mass of flask plus soil sample corrected to oven dry water content, M_A is mass of flask, M_{SW} mass of flask filled with soil and water, and M_W is mass of flask filled with water at the temperature observed.

3.2.1.3 Bulk density

The bulk density of the soil was determined using the core method. Undisturbed soil cores sampled were taken from the two sites at Ukulinga (Site 1- the trial site and Site 2- near the Agricultural Engineering Department). The mass of the dry soil was determined after oven drying at 105 ^{o}C for 24h. The volume of the soil was assumed to be equal to the volume of the core sampler, since the soil was trimmed at the edge of the core at both ends. The internal diameter (*D*) and height *(H)* of the core were measured using a vernier calliper to calculate the volume of the core, $V = \pi(D/2)^2 H$ and the bulk density of the soil was calculated using the following equation:

$$\rho_b = M_{ds} / V \quad (3.2)$$

where ρ_b is the bulk density of the soil (kg m^{-3}), M_{ds} mass of dry soil (kg) and *V* the volume of the core sampler (m^3).

3.2.1.4 Total porosity

The total porosity was calculated using Eq.3.3 which considered only the soil bulk density (ρ_b) and the particle density (ρ_s) of the soil. The value can be used as an estimate of the soil water content at saturation.

$$P = 1 - \rho_b / \rho_s \quad (3.3)$$

where *P* is the total porosity of the soil (%), ρ_b is soil bulk density (kg m^{-3}), and ρ_s is the soil particle density (kg m^{-3}) (Danielson and Sutherland, 1986).

3.2.1.5 Mass water content

This method involved the measurement of soil water content by weighing the wet sample, oven drying at 105 ^{o}C for 24 h, and reweighing the sample to determine the amount of water removed. Soil water content can be expressed either as a ratio of the mass of water to mass of dry soil (mass water content) or by multiplying the ratio with bulk density. The result may be expressed on volumetric water content (Gardner, 1986):

$$\theta = \frac{W_w Y_d}{W_d Y_w} \qquad (3.4)$$

where θ is volumetric water content (m^3 m^{-3}), W_w weight of water (kg), W_d dry weight of soil (kg), Y_d oven-dry bulk density (kg m^{-3}) and Y_w density of water (kg m^{-}).

3.2.1.6 Soil water retention characteristics

The soil water retentivity characteristics represent the relationship between soil water content (θ_m or θ_v) and the matric potential (ψ_m). Water retention over a range of matric potentials was measured on undisturbed soil cores sampled. Cores were saturated by standing them in a trough, with gradual addition of water (over about one hour) until the water level was about 3 mm from the top of the core; these were soaked overnight until they became saturated when the upper soil surface glistened.

Water retention in the 0-1 m (0-10 kPa) tension range: The core and lid were removed from the water and "while dripping wet", weighed on a top pan balance. This provided a measure of the saturated water content. The core was then transferred to the tension table, with a tension set at 0.10 m. The top of the tension table was covered with a Perspex sheet to minimize evaporation. The core was weighed after 48 h and replaced on the tension table and a slight pressure applied to achieve a good soil bed contact. The tension was increased to 0.20 and the exercise repeated and reweighing done after 48h. This procedure was repeated at further tensions of 0.30, 0.40, 0.50, 0.75 and 1.00 m.

Water retention in the 0-1m (0-10 kPa) tension range: The cores were transferred to a pressure pot and each core placed on a saturated disc of filter paper on a 100 kPa ceramic plate. After closing the lid of the chamber, a pressure of 50 kPa was applied

by adjusting the pressure regulator. The outlet from the ceramic plate was connected to an external burette to allow for the monitoring of water outflow from the soil cores in response to the applied pressure. When no change in the water level in the burette could be detected over an 8h period, the negative water pressure in the soil (i.e. matric potential) was assumed to match the positive air pressure indicating that equilibrium has been reached. (The level of water in the burette must be similar to the elevation of the ceramic plate). The air pressure was then released and the lid removed and the core samples weighed. The core samples were returned to the chamber and the process repeated at a pressure of 100 kPa.

Water retention in the -1500 kPa: Retaining rings (10 mm high x 50 mm diameter) on discs of filter paper were placed on a ceramic plate set up in a high-pressure chamber. The rings were filled with air-dried < 2 mm soil, and compacted slightly with a rubber bung. Water was applied to the ceramic plate to allow the soil to wet up slowly by capillary movement. This was allowed to soak for 30 min. and then the chamber was closed and a pressure of 1500 kPa was applied. The water level in a burette connected to the outlet from the ceramic plate was observed to determine when the samples equilibrated with the pressure. When equilibrium had been reached, the pressure was released and the lid removed from the pressure chamber. The soil was transferred from each sample to a 50 ml beaker and water content measured by oven drying.

Developing the soil water retention curve: Volumetric water content at the various matric potentials were calculated for each core and a retentivity curve plotted with volumetric water content on the y-axis (log scale) and matric potential on the x-axis (log scale). The formula for calculating matric potential and water content was based on the retentivity function developed by (Gardner *et al.*, 1970):

$$\theta_v = \left(\psi_m / a \right)^{1/b} \qquad (3.5)$$

where θ_v = volumetric water content ($m^3\ m^{-3}$), ψ_m = matric potential (-kPa), *a* and *b* are empirical constants, which can be developed from the regression line of $\ln\theta$ vs. $\ln\psi$:

$$\ln \theta_v = \left(\frac{-1}{b}\right) \ln \psi_m + \left(\frac{1}{b}\right) \ln a \quad (3.6)$$

from the trend equation (Figure 1)

$$y = -0.0888x + 3.6198 \quad (3.7)$$

$$b = {}^{1}\!\!\diagup\!\!{}_{slope} \quad (3.8)$$

$$Intercept = \left(\frac{1}{b}\right) \ln a \quad (3.9)$$

Or

a = exp (b*intercept) (3.10)

b = 11.2612613

a = 5.0509E+17

Estimation of volumetric water content θ_v (% m^3 m^{-3}) was derived from equation 3.5 as follows:

$$\theta_v = \left({}^{\psi_m}\!\!\diagup\!\!{}_{5.0509 * 10^{17}} \right)^{\left({}^{-1}\!\!\diagup\!\!{}_{11.261262}\right)} \quad (3.11)$$

Or

$$\theta_v = \left(-0.19798 * 10^{17} \psi_m\right)^{-0.088799} \quad (3.12)$$

Or

$$\theta_v = 37.32865\left(-\psi_m\right)^{-0.088799} \quad (3.13)$$

Soil matric potential ψ_m (-kPa) was determined as follows:

$$\psi_m = \exp(-11.261388 \ln\left({}^{\theta_v}\!\!\diagup\!\!{}_{37.331} \right) \quad (3.14)$$

Estimated values of θ_v using Eq.3.5 were compared with measured θ_v values to determine the mean square error (% m^3 m^{-3}).

3.2.1.7 The ThetaProbe soil moisture sensor: theoretical considerations

The relationship between volumetric water content and soil matric potential was determined using a soil moisture sensor. The ThetaProbe developed by Macaulay Land Use Research Institute, Aberdeen, Scotland and Delta-T Devices, Cambridge, England is essentially a frequency domain reflectometry sensor (FDR) that is used to determine volumetric water content by generating an electromagnetic field to the soil.

The probe generates a 100 MHz sinusoidal signal which is applied to a specially designed internal transmission line that extends into the soil by means of the array of four rods. The impedance of this array varies with the impedance of the soil, which has two components - the apparent dielectric constant (ε) and the ionic conductivity. The 100 MHz signal frequency has been chosen to minimise the effect of ionic conductivity, so that changes in the transmission line impedance are dependent almost solely on the soil's apparent dielectric constant.

The dielectric constant is a complex quantity and a measure of polarity that determines the velocity of an electromagnetic wave or pulse through the soil (Topp and Davis, 1985). The working principle of dielectric methods is on the basis that soils are composite materials made up of different components like minerals, water and air. Because the dielectric constant of liquid water (81) is much larger than that of the other soil constituents (2 to 5 for soil minerals and 1 for air), the dielectric constant for soil is primarily dependant on soil water content.

A third order polynomial of the FDR sensor analog output *V* (in volts) can be used to estimate the square root of the dielectric constant $\sqrt{\varepsilon}$ as (Delta-T Devices, 1999):

$$\sqrt{\varepsilon} = 4.70V^3 - 6.40V^2 + 6.40V + 1.07 \qquad (3.15)$$

Work published by Whalley (1993), and Topp *et al.*, (1980) shows almost linear correlation between the square root of the dielectric constant, $\sqrt{\varepsilon}$, and volumetric moisture content, θ_v ($m^3\ m^{-3}$) for many soil types.

$$\theta_v = \left(\sqrt{\varepsilon} - a_0\right) / a_1 \qquad (3.16)$$

where $a_0 = \sqrt{\varepsilon_0}$ is the square root of the apparent dielectric constant (Eq.3.15) obtained using the ThetaProbe voltage measured in an air-dry soil. The term $a_1 = \sqrt{\varepsilon_w}$ is the difference between the square root of the dielectric constant of saturated soil (Eq.3.15 for the corresponding voltage) and dry soil divided by the soil water content at saturation.

$$a_1 = \left(\sqrt{\varepsilon_w} - \sqrt{\varepsilon_0}\right) / \theta_{vs} \qquad (3.17)$$

Factory values for a_0 and a_1 of 8.4 and 1.6 for mineral and 7.8 and 1.3 for organic soils are used, respectively. The output signal is between 0 and 1V DC for a range of soil dielectric constant, ε, between 1 and 32, corresponding to approximately 0.5 m^3 m^{-3} volumetric soil moisture content for mineral soils. The calibration curve or equations are usually developed for general soils and to make the calibration site specific, the ThetaProbe sensors were calibrated for the specific soil type that was used in the pot experiment.

3.2.2 Plant materials

Seeds of cowpea cultivar Nzovu were initially donated by Environmental Action Team (EAT), Kitale, Kenya. Cultivar M66 and Kenkunde were also originally purchased from the Basic Seed Unit of the Kenya Agricultural Research Institute (KARI), Machakos, Kenya and from the Kenya Seed Company, Kitale, Kenya, respectively. Seeds were planted at two different sites in Ukulinga and Umbumbulu under different production conditions to produce own fresh seed with known quality characteristics (Chapter 2). Mature seeds harvested from these two sites were used in this experiment.

3.2.3 Controlled environment experimental conditions

A pot experiment was conducted under simulated drought conditions in a tunnel at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The tunnel was enclosed in clear polythene sheeting. Temperature, solar radiation (PAR) and relative humidity (RH) were monitored electronically using HOBO 2K Loggers (Onset Computer Corporation, Bourne, USA).

3.2.4 Experimental design, potting procedure, water stress treatments

The experiment was laid out using a factorial arrangement in a randomized complete block design with the following three factors: production site (two levels- seeds produced at Ukulinga and Umbumbulu sites), cultivar (three levels- cultivars Nzovu, Kenkunde and M66) and water stress [three levels- No stress, Intermittent stress at 30 % field capacity (FC) and Terminal stress at 30% FC) (Explanation of water stress treatments is given in the paragraph following this one.) The total number of treatment combinations was eighteen. These were replicated five times giving a total

of 90 experimental units (undrained polyethylene pots each with a 20 litre capacity, an internal diameter of 340mm and a holding capacity of 20 kg of soil).

Pots were placed individually on a weighing balance and a precise quantity of water was added until the required mass equivalent to 17.47% gravimetric water content at FC corresponding to a matric potential of -33 kPa was attained. After the water had seeped into the soil until none was left on the surface, the weight of the pot and the wet soil were recorded and written on the pot. Several pots were randomly chosen and then emptied on to a polythene sheet spread on a dry surface to confirm that there was no excess water which had settled at the bottom.

Drought conditions were simulated based on two contrasting moisture environments typical of semi-arid tropics where cowpea is grown and were defined as follows: Intermittent stress, characteristic of the wet season in the monsoonal semiarid tropics , when stress can occur at any time and with varying intensities between emergence and maturity; and Terminal stress where crops are grown relying only on stored moisture and where crops grow and mature progressively on depleted soil moisture profile (Ludlow and Muchow, 1990). Intermittent stress was imposed during the vegetative stage of growth only whereas terminal stress was imposed throughout development from emergence to maturity.

For the treatments at 30% FC (Intermittent and Terminal stress), individual pots were placed on a weighing balance and a precise quantity of water was added until the required mass equivalent to 12.26 % gravimetric water content at permanent wilting point (PWP) corresponding to a matric potential of -1500kPa was attained. Plant available water capacity (PAWC) from the -33 and -1500 kPa water contents was determined gravimetrically (mass) by calculating the difference between the % gravimetric water content at -33 matric potential and -1500 kPa. An additional quantity equivalent to 30% PAWC was calculated and added to each pot to attain the required soil mass at 30% FC. The pots were individually emptied in to a concrete mixer and mixed thoroughly so as to achieve a uniform distribution of soil and water mixture inside the pot and the weights recorded and written on the pots.

Treatments were assigned randomly to pots within each block. Five seeds were planted in each pot by placing four equidistantly and one at the centre. The seeds were planted at a depth of 10 mm. A compound fertilizer 2:3:2 (22) was applied during planting at the rate of 600 kg $ha^{-1}$and soil water content in the pots monitored as described below. The experiment was sown on 29th March 2005 and harvested in August 2005.

3.2.5 Monitoring of soil water content

Individual pots were placed on a weighing balance and weighed, and then water added until the required moisture content at FC and 30% FC was attained. In order to estimate the mass of the growing plants and to make allowance for increased mass when watering; a few extra pots with plants separate from the experiment were used to verify calculations and estimates.

Volumetric water content was also monitored using ThetaProbe soil water sensors. For each of the three water stress treatments, soil water content was measured every 15s and averaged for each 24 h interval at a depth of 60 mm using two ML2 and one ML1 ThetaProbe sensors. In addition, soil water content was measured in all pots at weekly intervals and averaged for each water stress treatment using one ML2 ThetaProbe. Each sensor has a 125- mm long and 40-mm wide housing electronics, and four 60-mm long sensing rods with three distributed uniformly around the 26.5-mm circumference and one at the centre. The sensors were inserted vertically into the soil in the pots. Sensors were connected to a CR7X datalogger (Campbell Scientific, Logan, Utah) and sensed using a differential voltage instruction assigned for higher resolution management. Measured voltages were transformed to the square root of the apparent dielectric constant using the data logger third order polynomial instruction.

3.2.6 Data collection

Pods were tagged at R4 stage of plant development, and harvested during three developmental stages fortnightly until harvest maturity (Fageria, 1992).

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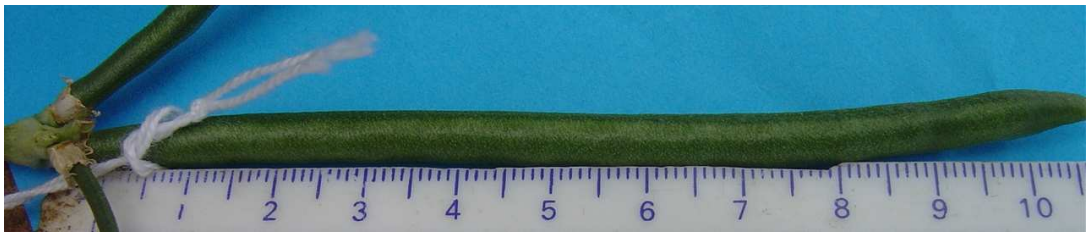

Figure 3-1 Pod tagged at R4 stage (~11cm long; seeds were not discernible).

3.2.7 Determination of early seed vigour, seed number and mass

Early seed vigour was assessed by determining mean emergence time (MET), final emergence (FE) seedling length and seedling dry weight. The mean time to emergence was calculated using the method of (Bewley and Black, 1994):

$$MET = \frac{\sum(fx)}{\sum f} \qquad (3.18)$$

where MET is mean time to emergence, f the number of newly germinating seeds at a given time (each day or hour) and x the number of days or hours counted from the day of sowing. MET measures the speed of emergence and faster emergence is a sign of high vigour. Seedling dry weight is a seedling growth and evaluation test and higher seedling weight is an indication high vigour; hence faster growth and a rapid and uniform emergence which can lead to vigorous and healthy seedlings and good seedling establishment. Final emergence was done by counting the emerged seedlings after 10d. Seedling growth rate was assessed by determining the seedling dry weight. At the end of the emergence period, normal seedlings were separated from the seed or cotyledons and placed in a drying oven at 80 ^{o}C for 24h. The dried seedlings were weighed and their weight divided by the number of normal seedlings per given test. Seed number and yield were determined at harvest maturity. Dry pods were harvested separately according to the time taken to reach harvest maturity for each cultivar. The pods were threshed and the seeds removed and allowed to dry ~ 12% moisture content in the tunnel before the determination of seed yield .Mature dry seeds were stored at 5^{o}C in the cold room until further analysis.

3.2.8 *Determination of seed water content (fresh mass basis) and dry matter accumulation*

For each developmental stage, seeds were weighed to 4 decimal places, frozen in liquid nitrogen and lyophilized for 72 h and then weighed again to determine the dry matter content. The lyophilized seed samples were stored at -20^{o}C in a deep freezer until further chemical analysis. Seed water content (g water g seed^{-1}) was calculated using the following formula,

$$Seed\ water\ content = \frac{Fresh\,weight - Dry\,weight}{Fresh\ weight} \qquad (3.19)$$

3.2.9 *Laboratory germination test and seed vigour*

Mature seeds were germinated using the paper towel method according to ISTA (1999). (For details germination testing see Chapter 2). Germination was assessed by counting seeds which had produced 2mm of radicle daily up to 8 days. Normal, abnormal and dead seeds (ISTA, 1996) were assessed and recorded at the final count at 8d. Seedling dry weight was determined as described above at the end of the germination test period. Germination Index (GI) was determined by the following formula (Scott *et al.*, 1983),

$$GI = \frac{\sum_{i=1}^{n} T_i N_i}{TN} \qquad (3.20)$$

Where GI = germination index,

T_i is the number of days after sowing starting with the final day, N_i is the total number of seeds germinated on day i, where i = day 1, 2, 3…. 8 and N is the total number of seeds that have germinated on the final count. The method calculates an index, which describes seed vigour on a scale of 0 to 1, with values close to 0 indicating low vigour and a value of 1 indicating maximum vigour.

3.2.10 *Morphological characteristics*

The initiation and development of successive nodes, plant height and the number of leaves was assessed weekly until flowering. Plant height was taken as the distance from the base of the stem to the apical bud. For the destructive measurement of leaf

area (cm^2), leaves were measured using a portable leaf area meter, LI-COR, LI-3000 manufactured by LI-COR-Inc, Lincoln, Nebraska.

3.2.11 Leaf water potential

Leaf water potential was determined using the Scholander pressure chamber (Scholander *et al.*, 1965). The first pair of trifoliate leaves was used for leaf water potential determination. Measurements were taken randomly from plants in each pot. A fresh leaf was removed from the plant using a sharp razor blade. The leaf was wrapped in cling film and quickly sealed in the chamber. Pressure was increased slowly at approximately the rate of 100 kPa s^{-1} using nitrogen gas from a cylinder until sap droplets appeared at the position of the of the xylem vessels at the cut surface. These droplets were examined using a hand lens. The endpoint was checked by decreasing the pressure and then slightly increasing it again. The leaf water potential is the negative of the applied pressure. Mid-day leaf water potential was determined between 1200-1300h Greenwich Meridian Time (GMT) on a hot day. Predawn leaf water potential were determined daily for 12 weeks from planting just before between 0400-0500h GMT.

3.2.12 Chlorophyll fluorescence

Chlorophyll fluorescence was determined using the Plant Efficiency Analyser (PEA), manufactured by Hansatech Instruments Ltd, Norfork, England. The operating principle of the PEA is based on the Kautsky effect (Kautsky and Hirsch, 1931). If a leaf is kept in darkness for several minutes and then is brightly illuminated, fluorescence rapidly rises from a low level (F_0) via an intermediate level (I) to a peak level (P) and then gradually decays through several intermediate maxima to a level close to the original F_0 level (T). If the light is of saturating intensity, the peak value P will be the maximum achievable fluorescence level at a given light intensity known as F_m. The difference between the maximal fluorescence signal F_m and the low level signal F_0 is said to be the variable component of fluorescence (F_v). The ratio F_v/F_m can then be calculated from the values obtained. A decline in F_v/F_m in dark adapted plants is a good indicator of photoinhibitory damage to plants subjected to stress.

Similarly, the first pair of trifoliate leaves was used for chlorophyll fluorescence determination. Light weight leaf clips were placed on the measuring sites chosen randomly on the leaves. For each water stress treatment, five different pots were sampled. The leaf clip shutter was then closed to allow for dark adaptation for 30 minutes. During measurement the PEA sensor was held in place over the clip. The shutter blade of the clip was then slid back to expose the dark adapted leaf to the sensor unit. Measurements were recorded according to user programmed settings of light level and record length selected on the sensor unit.

3.2.13 Description of statistical analysis

Genstat® 9 edition was used to perform analyses of variance and the differences between means were determined by LSD (least significant difference) (P = 0.05). Germination percentage and germination index data were subjected to angular transformation prior to statistical analysis (Appendices 3.1a & b). The differences between means were determined by LSD (least significant difference; P = 0.05) which was calculated as SED x t (error degrees of freedom, 0.05).

3.3 Results

Selected physical characteristics of the soil used in this study are shown in Table 3.1. A soil water content of 17.47% (gravimetric) at a soil water potential of -33 kPa was taken as FC as is recommended for finer textured soils. A soil water content of 12.26% (gravimetric) at a soil matric potential of -1500 kPa was taken as PWP. The PAWC for the soil was calculated to be 114 mm m^{-1}.

The relationship between volumetric water content and matric potential (log scale) is shown in Figure 3.1. The R^2 of 0.9909 clearly shows the degree of accuracy of estimation of matric potential using the regression equation. The trend line equation was used to derive the two empirical constants *a* (5.0509E+17) and *b* (11.2612613) which were used to estimate θ_v (Eq.3.5) (Gardner *et al.*, 1970). The calculated values of θ_v using Eq.3.5 were a good estimate of the measured values as evident from the error mean square of 0.354 % m^3 m^{-3}. The measured θ_v plotted on the y-scale against the matric potential (x-scale) and the formula for deriving both θ_v and ψ_m is also shown in Figure 3.1.

Table 3-1 Physical characteristics of soil used for the water stress experiment

Bulk density ($kg\ m^{-3}$)	% Gravimetric water content at selected matric potentials		
	Near Saturation	Field Capacity	Permanent Wilting Point
	-1 kPa	-33kPa	-1500kPa
1562	23.73	17.47	12.26

Particle size distribution					
Clay (%)	Silt (%)	Sand (%)			
		Coarse	Medium	Fine	Very Fine
31.86	48.94	10.35	2.20	2.50	4.15

% Total Porosity	Plant Available Water ($mm\ m^{-1}$)
41.06	114

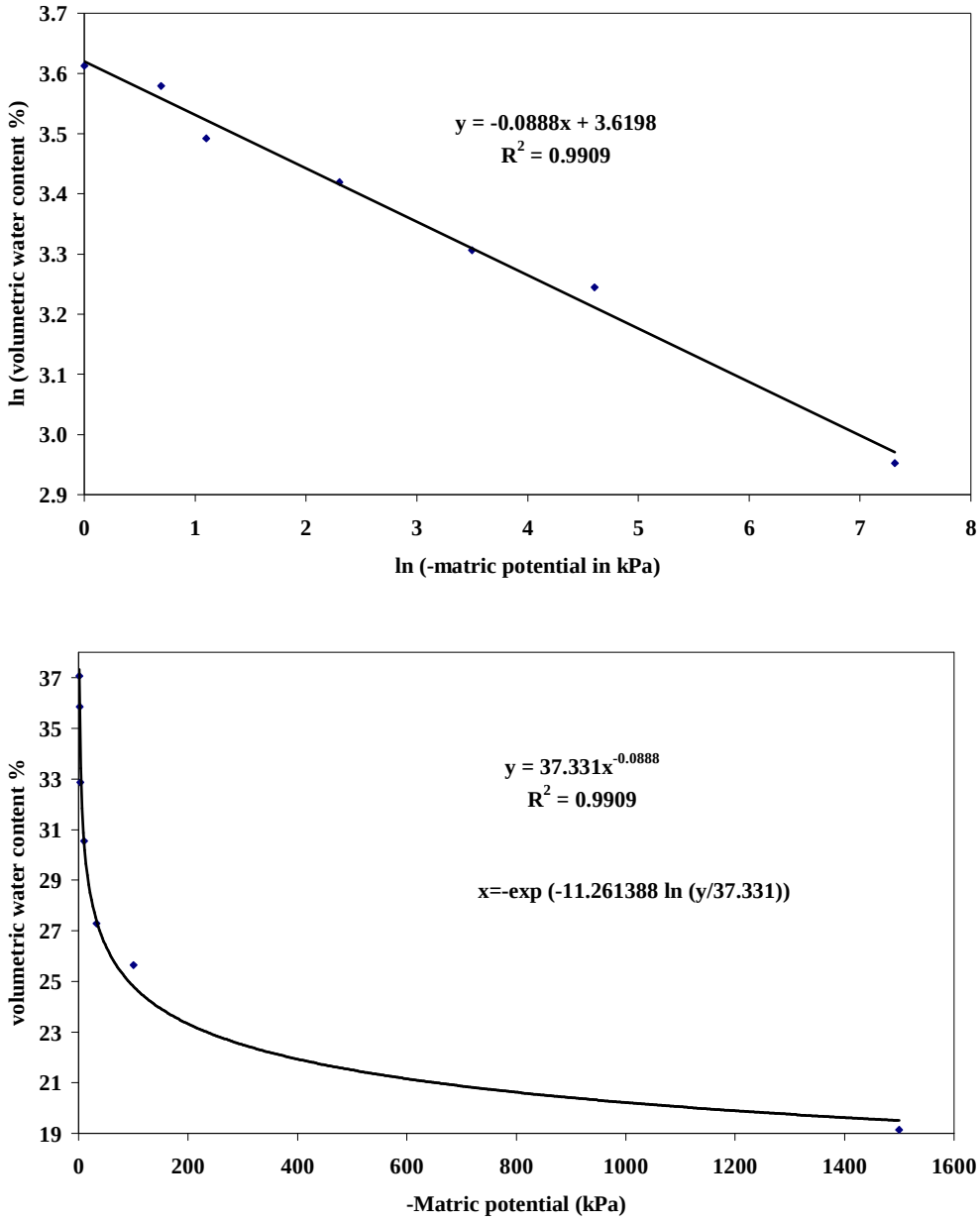

Figure 3-2 Relationship between volumetric water content and matric potential of the studied soil expressed logarithmically (top) and directly (bottom).

From the results obtained (Table 3.1,Figure 3.2), the retentivity curve showed high water retention and a gradual decrease in water content with increasing matric potential. The soil had a mean water content of 37 % at near saturation (Figure 3.1) which gradually decreased by approximately 10% at a matric potential of -33 kPa (FC). A matric potential lower than - 400 kPa corresponding to a water content of ~22

% for this particular soil profile is generally considered not stressful. Thus stressed plants were subjected to matric potentials larger than - 400 kPa (Figure 3.2).

The matric potential and corresponding water contents are shown in Figure 3.3 A, B and C. Changes in matric potential were monitored gravimetrically by weighing the pots weekly. Under terminal stress matric potential and soil water content decreased gradually from week 1 up to week 5 to almost -1500kPa (Figure 3.3A). The application of water during week 6 resulted into a rapid increase in both soil water content and matric potential. Water was withheld for the treatments under terminal stress for another five weeks after which the plants were watered again during week 12 and 13. The period between week 5 and week 12 was characterized by considerable stress which was reflected in the plant responses with respect to growth and development.

Under intermittent stress, plants were subjected to water stress during the vegetative growth phase only. This is clearly reflected in the changes in matric potential and water content shown in Figure 3.3B. A similar pattern to that observed under terminal stress occurred where matric potential and water content decreased during the first five weeks when water was withheld. Similarly matric potential was lowered and water content increased after plants were watered during week 6. Frequent watering was resumed for the intermittent stress treatment and this led to the matric potential and water content being similar to the control treatment (No stress) (Figure 3.3C). For the control treatment (No stress) matric potential was generally close to FC since the pots were watered frequently.

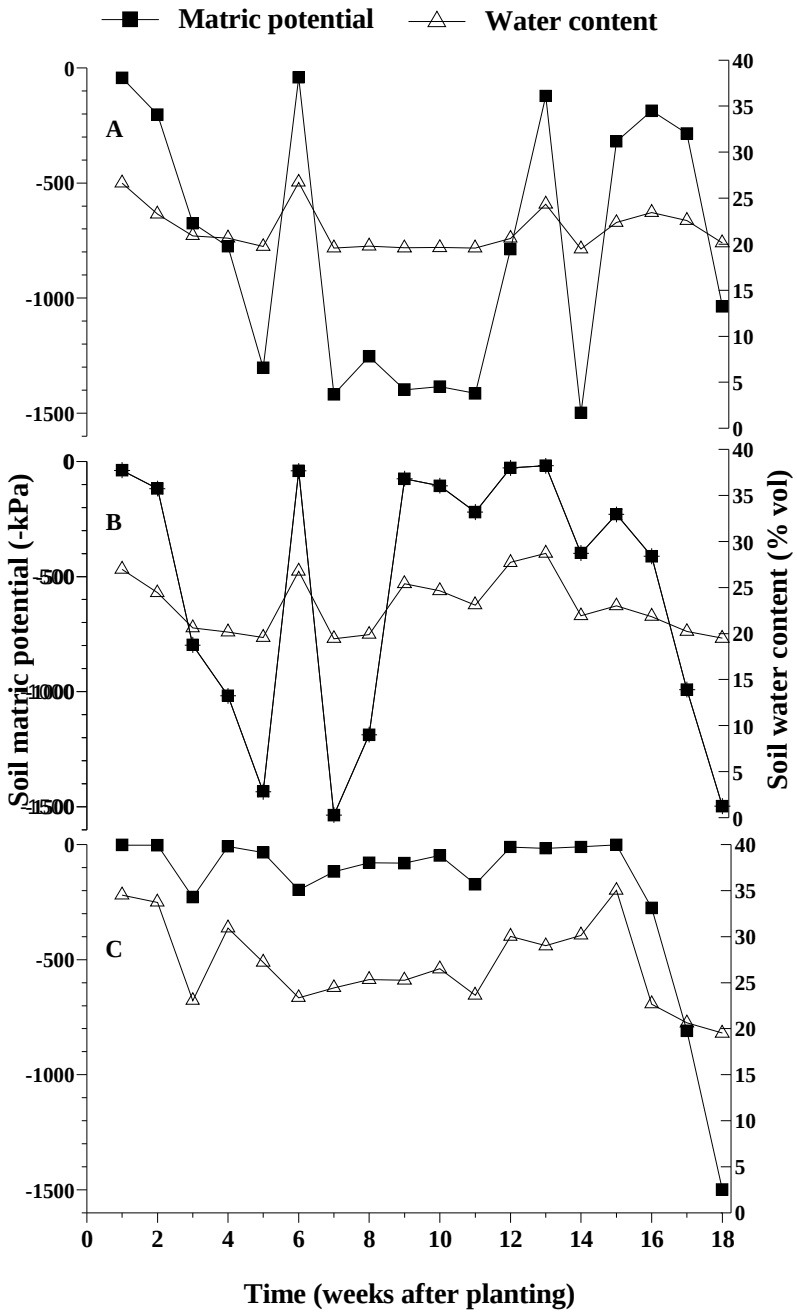

Figure 3-3 Changes in soil water content (%) and soil matric potential monitored gravimetrically during cowpea growth and development under simulated drought conditions: A (Terminal stress), B (Intermittent stress) and C (No stress).

Comparable results were observed when matric potential and water content were monitored using ThetaProbes (Figure 3.4 A, B and C). Similarly, under conditions of terminal stress matric potential decreased gradually from about -33 kPa in week 1 to

close to -1500kPa week in week 5 (Figure 3.4A). Matric potential rose to about -33 kPa when plants were watered in week 6 and was maintained to levels below -1000 kPa from week 7 to week 9 when plants were watered again in week 10. The pattern of changes in matric potential clearly reflected soil water content during growth and development and showed that plants under terminal stress were subjected to stressful conditions for a long a period.

Similar trends were observed for the intermittent stress treatment (Figure 3.4 B). Matric potential and water content both decreased during the first 4 weeks when watering was withheld and increased after watering in week 5. The resumption in frequent watering after the end of the vegetative growth phase resulted in to lower matric potential and higher water content from week 7. Similarly the ThetaProbe were effective in monitoring matric potential and water content which were consistently maintained at levels corresponding close to FC for the control (No stress) treatments (Figure 3.4C).

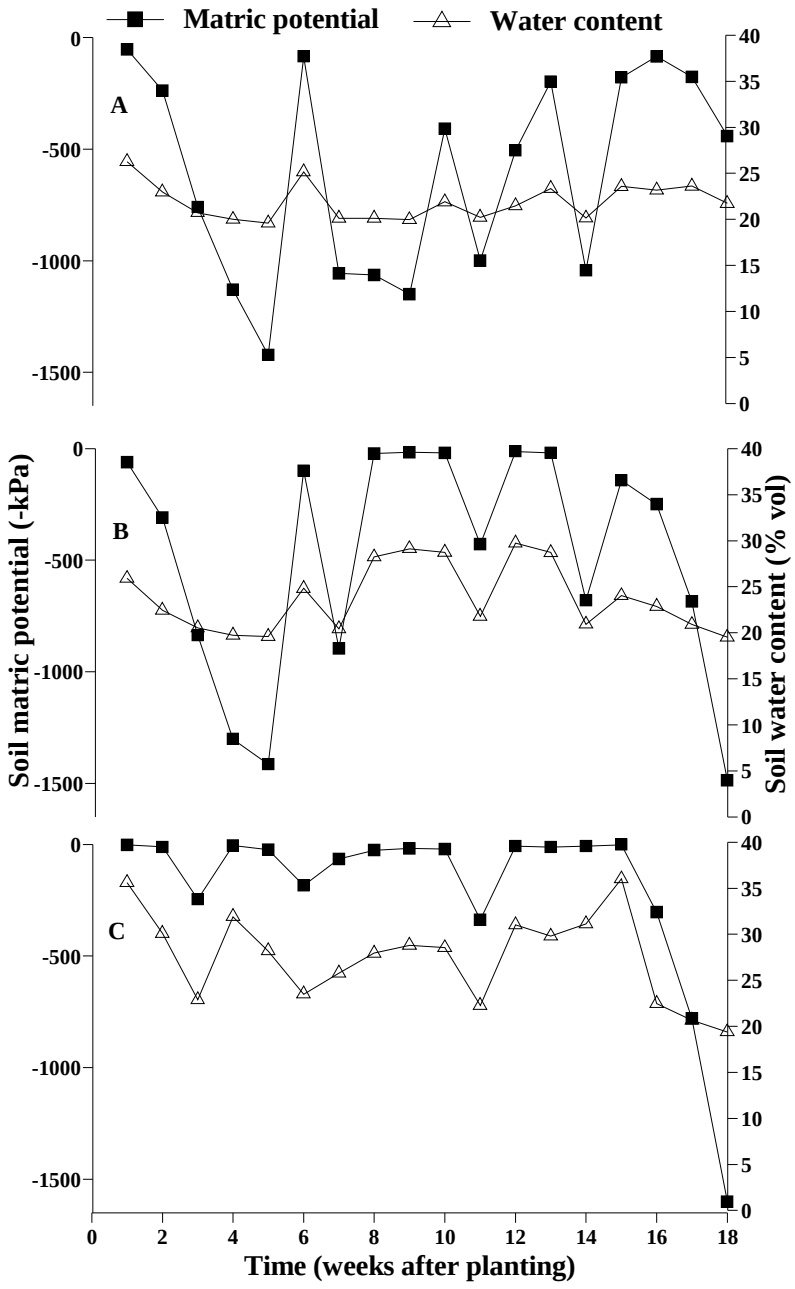

Figure 3-4 Changes in soil water content (%) and soil matric potential using ThetaProbes during cowpea growth and development under simulated drought conditions: A (Terminal stress), B (Intermittent stress) and C (No stress).

The results obtained from measurements taken on predawn leaf water potential over a period of 12 weeks during growth and development was more or less consistent with data recorded for soil matric potential and water content (Figure 3.5). Clear

differences were observed in predawn leaf water potential between stressed and non-stressed plants for all three cultivars.

Predawn leaf water potential for the non-stressed plants was generally lower than -0.3 MPa during growth for all cultivars. The cultivars M66 and Kenkunde showed the most consistent pattern of predawn leaf water potential for non-stressed plants which generally ranged between -0.3 and -0.5 MPa. However Nzovu, which initially recorded similar predawn leaf water potential as M66 and Kenkunde for non-stressed plants showed a high degree of fluctuation in predawn leaf water potential, ranging from -1.1 MPa to -0.66 MPa at the later stages of development.

Predawn leaf water potential for stressed plants in all the cultivars became more negative gradually from week 1 to week 4. Similarly, cultivar differences were discernible in predawn leaf water potential. Stressed plants of Kenkunde showed the most negative values for predawn leaf water potential (-1.1 MPa) during the first four weeks of development. Predawn leaf water potential for stressed plants of Nzovu also showed similar sharp fluctuation at later stages of development. M66 showed the least fluctuations in predawn leaf water potential

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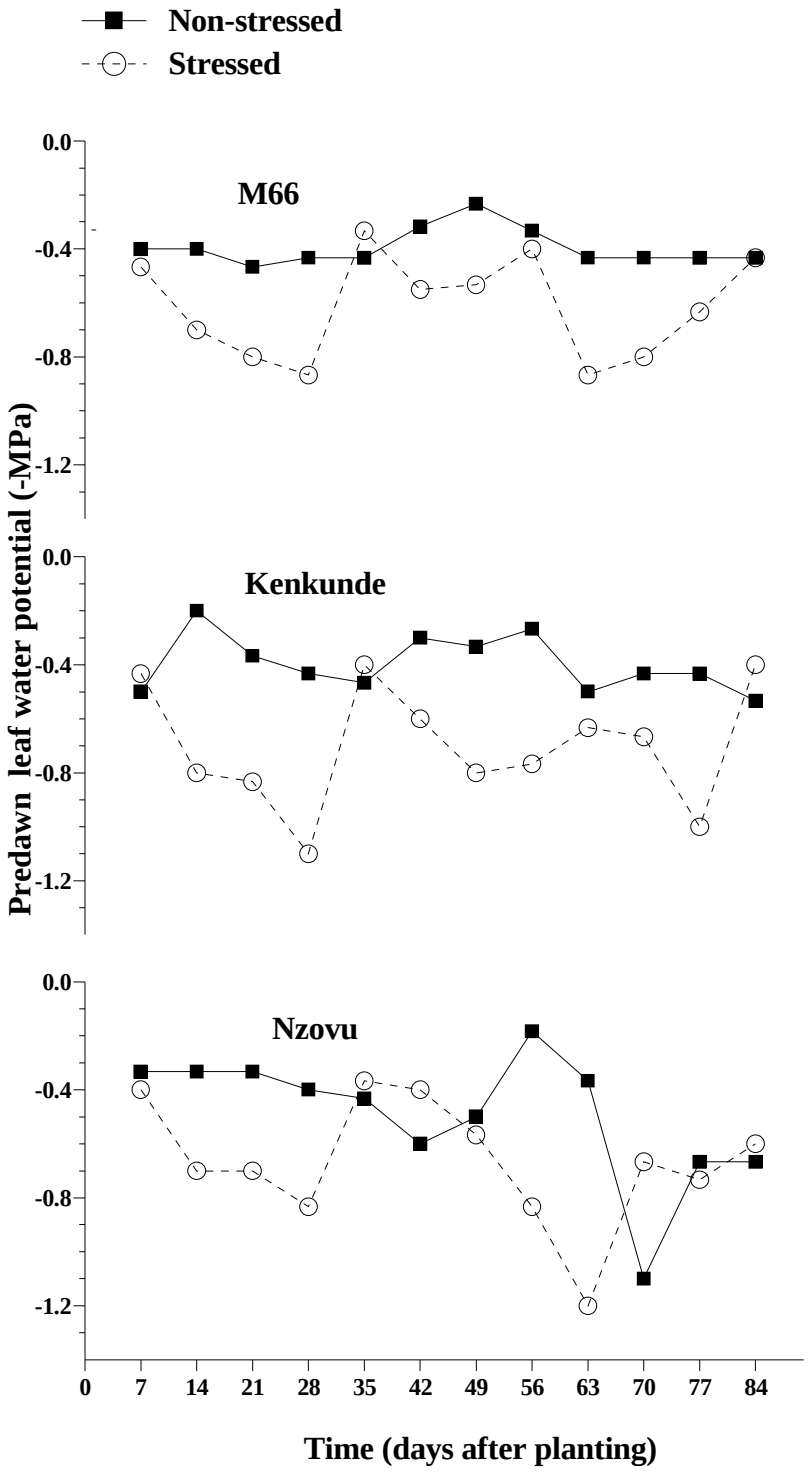

Figure 3-5 The general pattern of predawn leaf water potential (MPa) taken on three cowpea cultivars at weekly intervals during growth and development until harvest maturity. Clear differences between the non-stressed and stressed plants were observed over period of 84 days.

The effect of stress on mid-day water potential was also investigated. Under well watered conditions, mid-day leaf water potential ranged between -0.5 and -0.6 MPa and did not differ significantly between the cultivars. For the stressed plants, the mid-day leaf water potential ranged between -1.5, -1.2 and -1.3 MPa for M66, Kenkunde and Nzovu respectively (Fig 3.6).

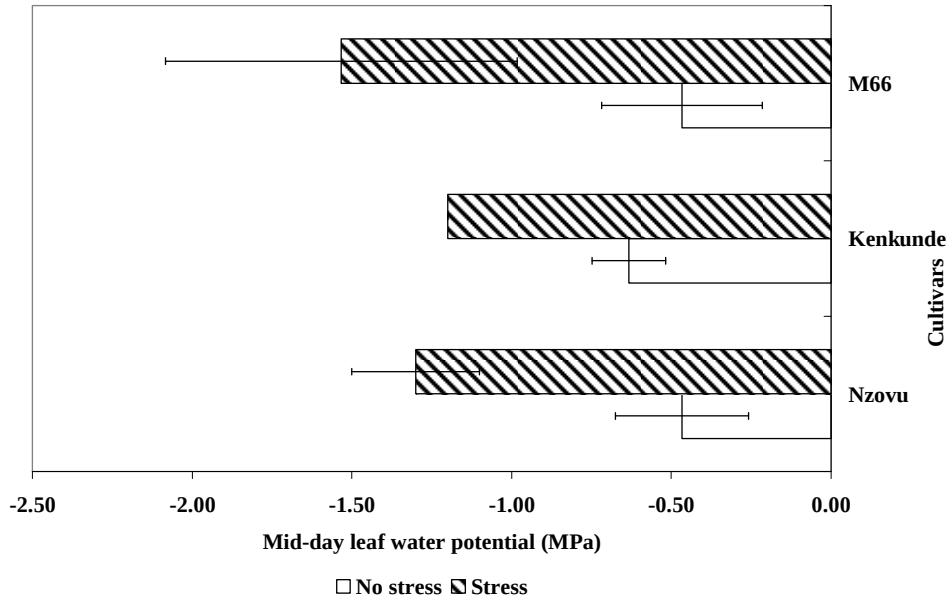

Figure 3-6 Mid-day leaf water potential for cowpea cultivars subjected to simulated drought conditions.

Withholding water for periods of 5 weeks in the terminal stress and intermittent stress treatments resulted in considerable retardation of growth (Figure 3.7). Plants that were subjected to terminal stress had fewer leaves with reduced leaf area than those subjected to no stress (Table 3.2). Similarly terminal and intermittent stress resulted in fewer flower buds formed by the cultivars Nzovu and Kenkunde; however, both terminal and intermittent stress severely inhibited bud development in cultivar M66, which produced very few flowers and consequently fewer seeds. It is important to note that M66 matured slowly compared with Nzovu and Kenkunde and the cultivar (M66) had not produced any buds during intermittent and terminal stress at the time of scoring for bud formation. Water stress also had an effect on the time taken to emergence. Seeds that were planted under no stress conditions emerged faster than

those that were subjected to stress. The cultivar Nzovu was the fastest to emerge under both stress and no stress conditions (Table 3.2).

Table 3-2 Effect of water stress on vegetative growth and mean emergence time (MET)

	Cultivar	Water stress treatment #		
		No stress	Intermittent stress	Terminal stress
Leaf area (cm^{-2})	Nzovu	255	76	83
	Kenkunde	237	87	77
	M66	221	66	72
No of buds	Nzovu	9	4	3
	Kenkunde	8	3	4
	M66	1	0	0
MET (days)	Nzovu	3.6	4.4	4.2
	Kenkunde	4.7	5.1	5.1
	M66	4	4.7	4.4

No stress = pots maintained at near FC
Intermittent stress = pots watered up to 30% FC during vegetative growth
Terminal stress = pots maintained at 30% FC from emergence to maturity

The effect of water stress on plant growth and development was clearly evident in all cultivars (Figure 3.7). The unstressed plants grew vigorously and had a lot of foliage. The stressed plants started showing signs of early senescence evident in the yellowing of leaves. Cultivar differences were also observed in response to stress. The cultivar Kenkunde appeared to be highly sensitive to water stress as evident from the wilting leaves (Figure 3.7).

Figure 3-7 Effect of terminal stress on the growth and development of three cowpea cultivars, Kenkunde (Top), Nzovu (Middle), and M66 (Bottom). Leaves of Kenkunde are showing signs of wilting.

The effect of water stress on photosynthetic processes was assessed using chlorophyll fluorescence (Figure 3.8). Highly significant differences ($P < 0.001$) were observed between treatments with respect to chlorophyll fluorescence (Figure 3.8). The Fv/Fm ratio for non-stressed plants (0.827) and intermittently stressed plants (0.820) were not significantly different. However both non-stressed plants and intermittently stressed treatments differed significantly from the terminal stress treatment (0.807) (Figure 3.8).

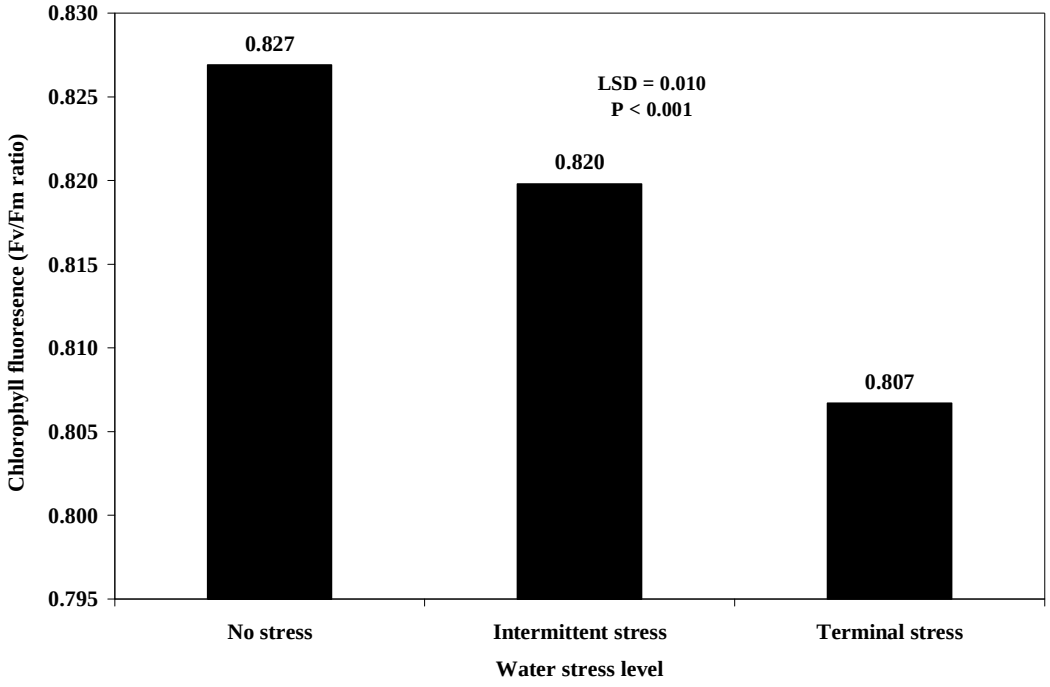

Figure 3-8 Chlorophyll fluorescence for all three cultivars subjected to simulated drought conditions.

The effect of production site (whence planted seed was harvested) on seedling vigour with respect to seedling dry weight, seedling length, root length, emergence time and final emergence was investigated (Table 3.3). Seeds which had been produced from Ukulinga had more dry weight, larger seedlings with deeper roots and emerged faster (although not significantly) than those which had been produced from Umbumbulu. However, there were no significant differences in the final emergence between seeds produced at both sites.

Table 3-3 Assessment of early seedling vigour with respect to seedling dry weight, seedling length, root length, emergence time and final emergence 10 d after planting across all cultivars.

Production site	Early seedling vigour				
	Dry weight (mg)	Seedling length (mm)	Root length (mm)	Mean Emergence Time (days)	Final emergence (%)
Ukulinga	122.3	139	39.9	4.4	97
Umbumbulu	97.7	119	36.4	4.6	98
P	<0.01	<0.01	<0.05	>0.05	>0.05
LSD	15.1	9	5.6	ns	ns

Both intermittent and terminal water stress had a considerable effect on seed development in the cultivars, M66 and Kenkunde; hence a comparison of changes in seed water content could only be done using the cultivar Nzovu. Nzovu was able to produce seeds during all the three stages of development and also under all stress conditions. Although the decline in seed water content was more rapid under terminal stress than intermittent and no stress; there were no significant differences in seed water content during the developmental stages (Figure 3.9).Seed water content was generally above 75 % for all the treatments at development stage 2 and decreased to approximately 60 % at stage 4. The decrease in seed water content from development stage 4 to 6 was more rapid. At maturity seed water content for all the treatments was approximately 9 %.

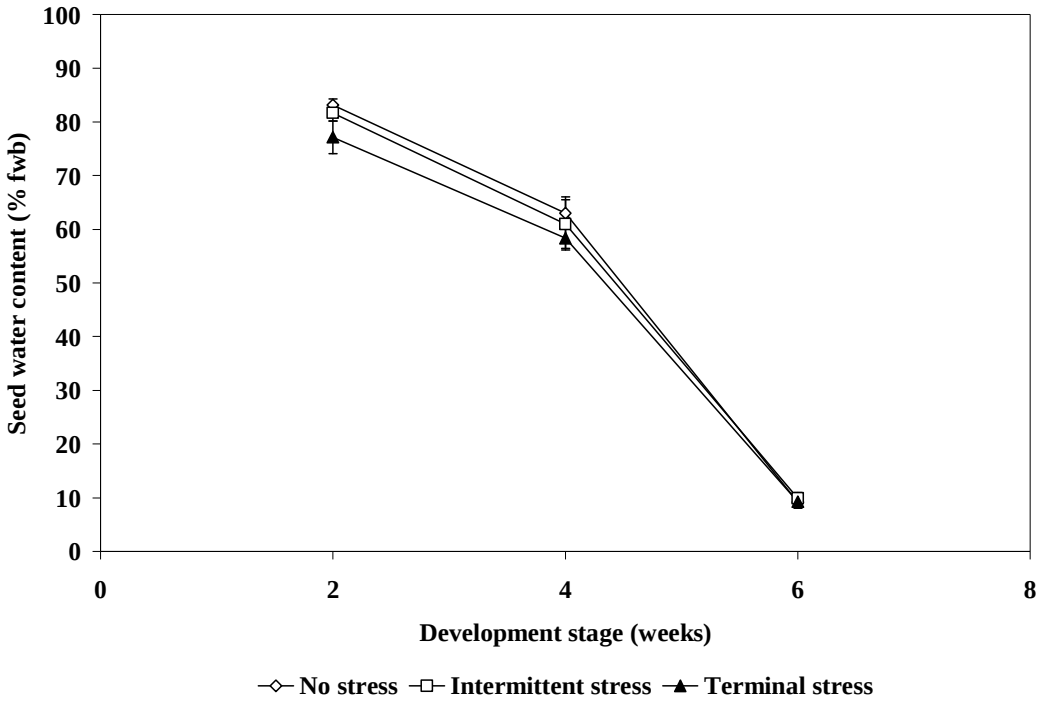

Figure 3-9 Changes in seed water status during development for the cowpea cultivar Nzovu grown under simulated drought conditions.

Highly significant differences ($P < 0.001$) were observed between the well-watered (non-stressed) treatments and both intermittent and terminal stress with respect to the effect of simulated drought conditions on the rate of dry matter accumulation (Figure 3.10). Developing seeds produced under well-watered conditions showed the highest rate of and final dry matter accumulation (based on pooled means for all the cultivars). Both intermittent stress and terminal stress significantly reduced the rate and final dry matter accumulation when means of all cultivars were pooled for each developmental stage.

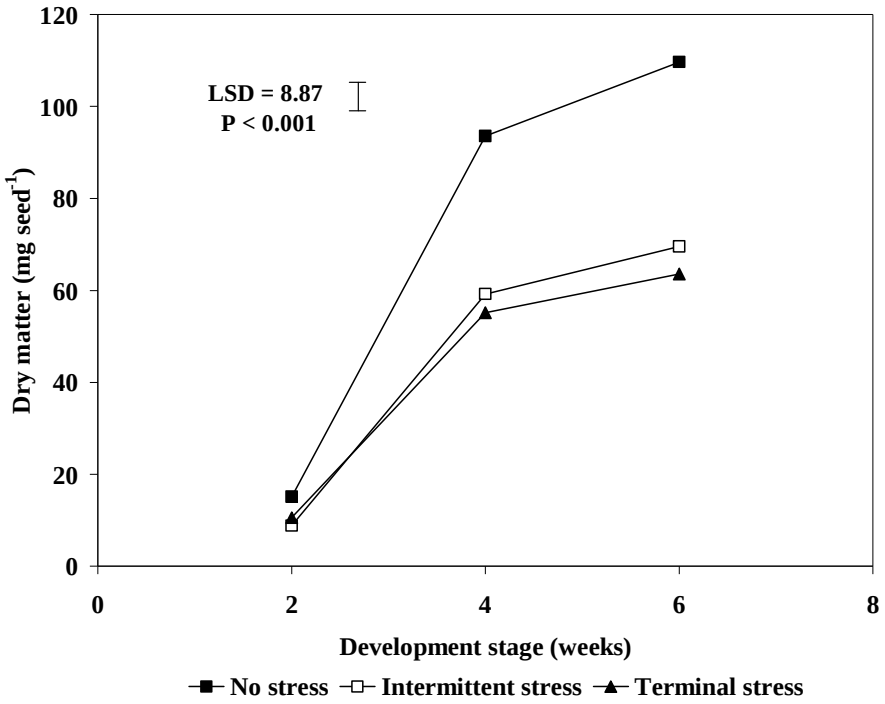

Figure 3-10 Effect of simulated drought conditions on the rate of dry matter (based on pooled means of all three cultivars for each developmental stage)

However, when the cultivars were compared separately with respect to mean dry matter accumulation (mg seed^{-1}), all three cultivars showed distinctly different responses to simulated drought conditions (Figure 3.11). Interaction between cultivar, stress and developmental stage with respect to mean dry matter accumulation (mg seed^{-1}) was highly significant ($P < 0.001$) (Figure 3.11). Although seeds of Nzovu subjected to terminal stress showed a barely noticeable higher mean rate of accumulation than for non-stressed seeds; both intermittent and terminal stress did not have a significant effect on the rate and final mean dry matter accumulation (mg seed^{-1}). However, highly significant differences ($P < 0.001$) in mean dry matter accumulation (mg seed^{-1}) were observed between Kenkunde seeds subjected to terminal stress and those grown under intermittent and no stress. For the cultivar M66, intermittent and terminal stress had severely inhibited bud formation, and consequent flowering. Hence very few seeds were produced. These were not adequate for sampling at various developmental stages and were left to be harvested at maturity and analysed for seed quality. However, non-stressed seeds of M66 showed the highest mean dry matter (mg seed^{-1}) at maturity.

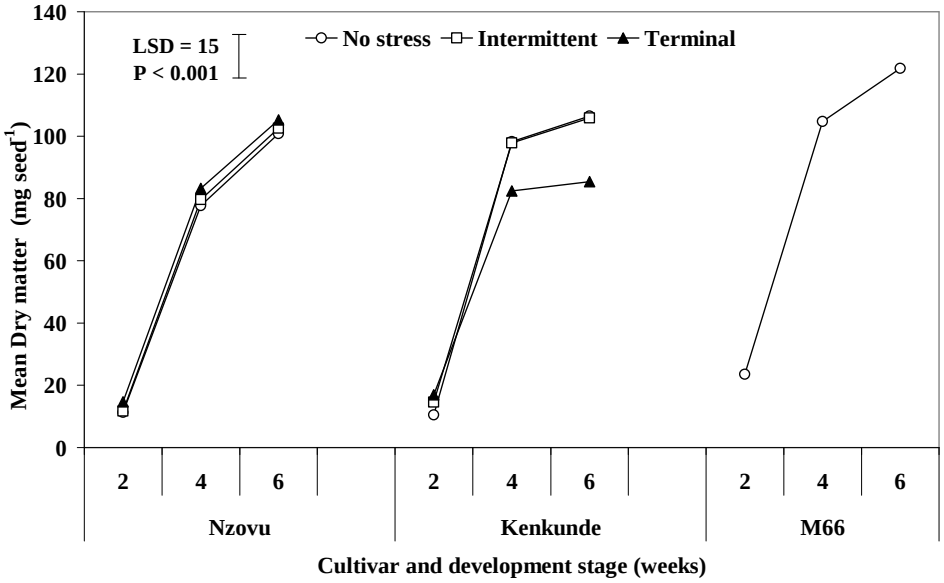

Figure 3-11 Mean dry matter (mg seed^{-1}) accumulation for cowpea cultivars (Nzovu, Kenkunde and M66) grown under simulated drought conditions.

Overall, dry matter accumulation at maturity was compared between sites, cultivars, stress treatments and control. A significant difference ($P < 0.05$) with respect to the accumulation of dry matter was observed between production sites. Seeds that had been produced at Ukulinga had more dry matter (56.5mg seed^{-1}) than those produced at Umbumbulu (51.3mg seed^{-1}) (Table 3.4). Cultivar differences were also highly significant ($P < .001$) with respect to dry matter accumulation (Table 3.4). Seeds of Kenkunde showed the highest dry matter (68.7mg seed^{-1}) for all treatments. This was followed by Nzovu (65.2mg seed^{-1}) and M66 (27.8mg seed^{-1}) which was the lowest. Water stress had a highly significant effect on dry matter accumulation ($P < 0.001$). Water stress reduced seed dry matter content by 39% for all cultivars. Dry matter for non-stressed seeds averaged 72.8 mg seed^{-1} compared with intermittent stress (45.8mg seed^{-1}) and terminal stress (43.1mg seed^{-1}) (Table 3.4).

Table 3-4 Effect of site, cultivar differences and simulated drought conditions on overall dry matter production (mg seed^{-1}).

Site	Ukulinga	Umbumbulu		Significance
	56.5	51.7		P< 0.05
Cultivar	Nzovu	Kenkunde	M66	
	65.2	68.7	27.8	P< 0.001
Stress	No stress	Intermittent stress	Terminal stress	
	72.8	45.8	43.1	P < 0.001

Mature seeds were harvested from stressed and non-stressed plants grown in the tunnel and assessed for germination capacity, germination index, and mean germination time, seedling dry weight, number of seeds and yield (Table 3.5). These plants had been grown from seeds that had been initially planted at two different production sites at Ukulinga Research farm and Umbumbulu in a previous experiment. Therefore "unstressed, intermittently stressed and terminally stressed" refers to harvested seeds from the tunnel experiment; production site (Umbumbulu and Ukulinga) refers to where the seeds planted in the present experiment were produced.

For the cultivar M66, as has been mentioned earlier, intermittent and terminal stress had severely inhibited bud formation, and consequent flowering. Hence very few seeds were produced. These were not adequate for sampling at various developmental stages and were left to be harvested at maturity and analysed for seed quality (Table 3.5). Germination data was analysed based on 5 replicates of 10 seeds. However, for M66, some treatments had very few replicates (in some cases only one replicate). Hence germination tests were based on a minimum of a single replicate (10 seeds); however, the statistical functions in the analysis of variance were used to estimate missing values (Appendices 3.1a & b). This provided reasonable and reliable estimates for the germination percentage and germination index.

A significant interaction between stress level, production site and cultivar ($P < 0.05$) was observed with respect to germination capacity. Germination capacity for all the water stress treatments, between and within production sites and cultivars ranged between 88- 100%. Seeds harvested from unstressed plants of Nzovu from Umbumbulu had significantly higher germination capacity (96%) than those from Ukulinga (88%). Contrastingly, seeds from unstressed plants of Kenkunde and M66 did not show significant differences with respect to germination capacity between the two sites. Seeds from intermittently stressed plants of Kenkunde and M66 from Umbumbulu performed significantly better than those from Ukulinga. Similarly, although not significantly different, seeds harvested from intermittently stressed plants of Nzovu from Umbumbulu which had germination capacity of 96% performed better than those from Ukulinga (90 %). Germination capacity of seeds for all the cultivars under terminal stress was very high (> 95 %) and did not differ significantly between the two sites.

Similarly a significant interaction between stress level, production site and cultivar ($P < 0.001$) was observed with respect to germination index (Table 3.5). Germination index for all the water stress treatments, between and within sites and cultivars ranged between 0.85-0.98. Seeds from unstressed plants of Nzovu and M66 from both production sites did not differ significantly with respect to germination index. However, seeds from unstressed plants of Kenkunde from Umbumbulu had significantly lower germination index than those from Ukulinga. Seeds from intermittently stressed plants of M66 from Umbumbulu performed significantly better than those from Ukulinga; however, seeds of Nzovu and Kenkunde produced from both sites were not significantly different with respect to germination index. Seeds from Nzovu and M66 plants from Umbumbulu subjected to terminal stress showed a germination index of 0.98 which was significantly higher than those from Ukulinga (0.93 and 0.85 for Nzovu and M66 respectively). Germination index for seeds of Kenkunde produced from the two sites was similar (0.90).

The interaction between stress level, production site and cultivar was similarly highly significant ($P < 0.001$) with respect to mean germination time (Table 3.5). The cultivar Nzovu generally emerged fastest for all stress treatments and between and within production sites. No significant differences with respect to mean germination

time were observed in seeds from unstressed plants of the three cultivars from both production sites. Seeds from intermittently stressed plants of M66 from Umbumbulu showed a more rapid germination rate than those from Ukulinga. However, seeds from intermittently stressed plants of Nzovu and Kenkunde from the two sites did not differ significantly with respect to germination index. Under terminal stress, seeds from Nzovu and Kenkunde plants which had originated from Umbumbulu germinated more rapidly than those from Ukulinga. However, seeds from Kenkunde plants subjected to terminal stress and originating from both sites did not differ significantly with respect to mean germination time.

Interactions between stress levels, production site and cultivars were also significant with respect to seedling dry weight (Table 3.5). Seeds harvested from unstressed plants of Nzovu and Kenkunde from both production sites did not differ significantly with respect to seedling dry weight. However, seedling dry weight of seeds from unstressed M66 plants from Umbumbulu was significantly higher than from those from Ukulinga. Seeds from intermittently stressed plants of Nzovu from both sites did not differ significantly with respect to seedling dry weight. However, under intermittent stress, seeds harvested from Kenkunde and originating from Ukulinga had significantly higher seedling dry weight, than those from Umbumbulu. On the contrary, seeds from intermittently stressed M66 plants from Umbumbulu showed significantly higher seedling dry weight than those from Ukulinga. Seedling dry weight of seeds from plants of all three cultivars which originated from Ukulinga and that were subjected to terminal stress was significantly reduced compared to those originating from Umbumbulu. The cultivar M66 produced the heaviest seedlings when subjected to terminal stress which was significantly higher than seedling dry weight under both intermittent and no stress.

Similar trends were observed with respect to the number of seeds. The interaction between stress level, production site and cultivar was significant ($P < 0.05$). Seeds from unstressed plants of Nzovu from Umbumbulu produced the highest number of seeds (133) compared to those from Ukulinga (112). Seed number harvested from unstressed plants of Kenkunde from Ukulinga (59) and Umbumbulu (54) was not significantly different. However, the number of seeds harvested from unstressed plants of M66 from Ukulinga was significantly lower than those from Umbumbulu.

Intermittent stress significantly reduced seed number for all cultivars. Seed number per plant from intermittently stressed plants of Nzovu from Ukulinga was reduced to 37 which did not differ significantly from Umbumbulu (44). Similarly, only 17 and 11 seeds were produced per plant from intermittently stressed plants of Kenkunde from Ukulinga and Umbumbulu respectively. Intermittent stress had a considerable effect on the seed number of the cultivar M66 from both sites. Only one seed was counted on average per plant. Again the effect of terminal stress on the seed number for plants from all the three cultivars was substantial. Terminal stress reduced the seed number in plants of Nzovu to 14 and 8 from seeds originating from Ukulinga and Umbumbulu respectively. The effect of terminal stress on seed number in plants of Kenkunde and M66 originating from both sites was considerably reduced and ranged between 2-4 seeds. As has been indicated earlier, the effect of terminal stress and intermittent stress on mean seed number (based on an average of 5 replications) with respect to the cultivar M66 was considerable (Table 3.5).). Few or no seeds were harvested from some replicates. In order to address the statistical implications (risk of Type I Error) of the large differences with respect to seed number between the treatments, the analysis of variance was carried out on all three cultivars (Appendix 3.1e). Data for M66 was deleted and re-analysed with respect to seed number and yield (Appendix 3.1g & h). Both analysis showed that cultivar x stress interactions were highly significant ($P < 0.001$).Similarly the CV (%) for all treatments with all three cultivars was reasonably low (10.2%) and increased slightly with the exclusion of M66 (11.8%) (Appendix 3.1e & g).

The determination of seed mass revealed clear differences between stress levels, production site and cultivars. The mass of seeds from unstressed plants of Nzovu was highest (>12,000 mg plant^{-1}) in the two production sites and were not significantly different. This was followed by Kenkunde where the mass of seeds from unstressed plants of Kenkunde from Ukulinga (8124 mg plant^{-1}) and Umbumbulu (6624 mg plant^{-1}) was also not significantly different. Seeds from unstressed plants of M66 had the lowest seed mass which differed significantly for the two sites (6978 and 3316 mg plant^{-1} Ukulinga and Umbumbulu respectively). The effect of intermittent stress on seed mass was highly significant ($P < 0.001$). Intermittent stress reduced seed mass in Nzovu by 62 and 59% for seeds from Ukulinga and Umbumbulu respectively. The reduction was much more significant in seeds of intermittently stressed plants of

Kenkunde (72 and 81 % for Ukulinga and Umbumbulu). Seed mass was reduced by a margin of 99 % for seeds from intermittently stressed plants of M66 from both sites. The effect of terminal stress on seed mass was similarly more pronounced. Under terminal stress seed mass was reduced further by 87 % and 92 % for seeds of Nzovu plants from Ukulinga and Umbumbulu respectively. A similar observation was made for seeds of Kenkunde plants subjected to terminal stress. A reduction in seed mass of 95 and 89 % for seeds of Kenkunde originating from Ukulinga and Umbumbulu and subjected to terminal stress was observed. Although mass for seeds from plants of M66 from both sites and subjected to terminal stress was higher than for intermittent stress, the differences were not significant. The percentage reduction in the mass of seeds from M66 plants subjected to terminal stress was similarly considerably high (95 and 89 % for seeds originating from Ukulinga and Umbumbulu respectively).

Table 3-5 Effect of water stress on seed performance of seeds of three cowpea cultivars produced from two sites (Ukulinga and Umbumbulu).

Stress level	Site	Cultivar	Seed Performance					
			Germination capacity (%)	Germination Index	Mean Germination Time (days)	Seedling dry weight (mg seedling^{-1})	Number of seeds (plant^{-1})	Seed mass (mg plant^{-1})
No Stress	Ukulinga	Nzovu	88	0.94	1.44	55	112	12288
		Kenkunde	90	0.89	1.86	79	59	8124
		M66	96	0.90	1.78	65	50	6978
	Umbumbulu	Nzovu	96	0.93	1.38	56	133	12592
		Kenkunde	92	0.86	1.92	73	54	6624
		M66	98	0.90	1.80	75	25	3316
Intermittent Stress	Ukulinga	Nzovu	90	0.93	1.56	65	37	4622
		Kenkunde	90	0.89	1.86	70	17	2294
		M66	91	0.87	2.06	65	1	30
	Umbumbulu	Nzovu	96	0.92	1.60	67	44	5162
		Kenkunde	100	0.90	1.83	60	11	1240
		M66	100	0.92	1.64	77	1	6
Terminal stress	Ukulinga	Nzovu	96	0.93	1.50	48	14	1514
		Kenkunde	100	0.90	1.82	52	3	342
		M66	95	0.85	2.22	47	2	270
	Umbumbulu	Nzovu	98	0.98	1.19	65	8	960
		Kenkunde	95	0.90	1.82	79	4	716
		M66	100	0.98	1.22	87	2	244
P			<0.05	<0.001	<0.001	<0.05	<0.05	<0.10
LSD			8	0.02	0.15	8	16	1764

3.4 Discussion

The results obtained in the present study are consistent with information in the literature that cowpea plants are able to withstand long periods of water deficits and continue with their normal metabolic activities because of drought avoidance mechanisms (Ludlow and Muchow, 1990; Blum, 1996; Hall, 2004; Blum, 2005). Nicole Vartanian (1996) described drought avoidance as a strategy whereby the "plant raises barriers between the external and internal environments in order to avoid dehydration and maintain a high water potential in plant tissues". This strategy was evident in the considerable reduction in leaf area and number observed in all the three cultivars and leaf movements observed in Kenkunde and Nzovu. Reduction in leaf area may limit crop water loss by minimizing transpiration losses (Blum, 2005). Leaf movements can increase the avoidance to dehydration by shedding radiation incident on leaves, reducing leaf temperatures and water loss (O'Toole *et al.*, 1979). The cultivar M66 maintained a stay-green leaf colour throughout development under both intermittent and terminal drought. M66 which was developed by the Kenya National Breeding Programme was bred for drought resistance (Ehlers and Hall, 1997) and may be used as a model species to study plant drought tolerance in relation to seed germination and vigour. The stay-green leaf colour observed in M66 could be probably attributed to delayed leaf senescence (DLS). It has been suggested that DLS may contribute to drought tolerance in cowpea during the reproductive-stages (Gwathmey *et al.*, 1992). The DLS trait, which appears to be controlled by one gene, enables cowpea to recover after drought and produce a second flush of pods that compensate for the low yield by the first flush of pods (Gwathmey and Hall, 1992).

Both intermittent and terminal stress did not influence photosynthesis, as was evident from the chlorophyll fluorescence results (Figure 3.8). Although F_v/F_m values were significantly reduced by terminal stress; the values were still above 0.807 on a maximum scale of 1.000, thus indicating that the photosynthetic capacity of the leaves was still intact (Figure 3.8). The concept of photosystem-II or the "photosynthetic capacity of leaves" has been reported to be relatively tolerant to desiccation and that damage is caused at very low leaf relative water content of 40% or less (Blum, 1996). Relative water content or water potential at which leaves die is an expression of the extent plants are able to withstand desiccation without dying (Ludlow and Muchow,

1990). The criterion for deciding to measure critical values may be based on when 50 % of the leaves of the plant are dead, or when 50 % of the surface area of a leaf is dead, or when there is only one leaf one a plant subjected to slow drying cycle (Ludlow and Muchow, 1990). In the present study, it was very interesting to note that terminal stress reduced the number of leaves to less than 40 % as seen from the result obtained; however, there was probably no impairment of photosystem II.

Further evidence of mechanisms of drought avoidance in cowpea was observed in predawn and midday leaf water potential. The consistency between predawn leaf water potential and measurements of soil water content and matric potential agreed with the suggestion by Turk and Hall (1980) that cowpeas avoid drought by mechanisms which result in only slow extraction of soil water as the soil begins to dry out. In the present study, the observed values were above -1.2 MPa and were similar to those observed by Petrie *et al.* (1992), who reported that cowpea plants subjected to severe stress conditions maintained predawn leaf water potential values above -1.8MPa, which were much lower than those of other species like pearl millet, sorghum and peanut which can develop water potentials as low and negative as - 4 to -9 under similar conditions. The midday leaf water potential, which was generally above -1.5 MPa did not vary much from the predawn leaf water potential and agree with the observations made by Turk and Hall (1980) and (Westgate and Thompson Grant, 1989) who reported similar values in mid-day leaf water potential for cowpea and soybean respectively. This was thought to be indicative of the ability of cowpea to avoid both atmospheric and soil drought (Turk and Hall, 1980).

The considerable cultivar differences observed during flower bud formation, flowering and seed development under simulated drought conditions are indicative of the complex and diverse nature of plant responses to drought stress that may exist even within a species. These differences may be due to a combination of several factors which may include phenology, the role of ABA and interaction between physiological processes and drought stress. Crop phenology has a dominant effect on plant growth, response to and productivity under drought stress (Blum, 1996) and may account for the further delay in bud formation that was observed in the cultivar M66 in response to intermittent and terminal stress. The delay in bud formation by the cultivar M66 in response to drought stress could also be partly attributed to ABA

accumulation (Blum, 1996). ABA may delay or advance flowering time depending on the species and the time when ABA is in effect (Trewavas and Jones 1991) and possibly the rate of stress (Blum, 1996). Contrastingly, the cultivar Nzovu, a local landrace, showed a different response to simulated drought conditions. Although drought stress delayed bud formation in the cultivar Nzovu, the cultivar was still able to produce seeds under both intermittent and terminal stress conditions. Similarly genetic differences may also account for the inability of the cultivar Kenkunde (developed by the Kenya Seed Company for high yield) to withstand terminal stress after 4 weeks and the failure to produce enough seeds for sampling after 4 and 6 weeks of development. Seed development under both intermittent and terminal stress may have been limited by embryo abortion which often occurs when water is limiting at or soon after pollination (Westgate and Peterson, 1993). In the present study it was observed that the cultivar Nzovu was prolific in the production of flowers even under conditions of simulated drought than the other two cultivars. This may also explain why Nzovu was still able to produce a reasonable amount of seed at all developmental stages even if embryo abortion occurred soon after pollination.

Water status of the developing seeds may have not been altered by simulated drought conditions acting on the mother plant as was evident from the lack of significant differences in seed water content between the well-watered plants and the intermittent and terminal stress treatments. However, it is important to recognize that seed water content is a poor indicator of the physiological status of water in the developing seeds, since it is changing continuously due to dry matter accumulation (Bradford, 1994). The total water potential which is the sum of osmotic or solute potential and the pressure or turgor potential may provide a better indication of the physiological status of water in the developing seed (Bradford, 1994). However, the observation that seed water content was not altered by simulated drought conditions is consisted with the report by Westgate and Thompson Grant (1989) who showed that water potential, osmotic potential and turgor of developing soybean seeds is largely unaffected by severe short-term water deficits that develop in the maternal plant during linear seed growth. The relative independence of the seed water potential from the mother plant has been interpreted as evidence for a high resistance to water movement between maternal and embryonic tissues (Bradford, 1994) and has led to the concept that “the maintenance of a favourable seed water status within the seed may be an important

prerequisite for conserving seed growth rate under adverse environmental conditions" (Westgate and Thompson Grant, 1989). Thus under terminal stress, long-term water deficits may have not affected seed water status because the seeds did not differ with respect to water content from those under well watered conditions. This may have allowed the seeds to continue with normal metabolic functions which determine seed growth rate (SGR).

SGR which is defined as the rate of dry matter accumulation during the linear phase of seed growth (Egli, 2006), was most likely not influenced in the cultivar Nzovu during development under both intermittent and terminal stress. This may probably explain why seeds subjected to these conditions (intermittent and terminal drought) did not differ from those produced under well-watered conditions with respect to mean dry matter accumulation. Variation in SGR can be genetic and is closely associated with seed size (Egli, 1981; Sung and Chen, 1990). It is possible that the relative insensitivity of the cultivar Nzovu to the direct effects of water stress may be related, firstly, to genetic factors. Nzovu was always the first to emerge and the earliest cultivar to reach maturity. The ability to emerge quickly and grow and mature faster may have limited its exposure to simulated drought conditions when compared with Kenkunde and M66. In addition, SGR is also influenced by environmental conditions during seed filling either through direct effects on the ability of the seed to accumulate dry matter or indirectly by affecting the supply of assimilates to the seed (Egli, 2006). This could be related to the observation that developing seeds are much stronger sinks than vegetative tissue during seed filling (Bradford, 1994); and drought stress may have further improved the sink capacity thus ensuring that seeds subjected to stress were able to accumulate sufficient dry matter. Another possibility that may explain the lack of significant differences in mean dry matter accumulation is that the production and translocation of assimilates to the developing seeds was not affected as evident from the results of chlorophyll fluorescence discussed above which showed that photosystem II was intact. Genetic factors related to SGR may also account for the differences in mean dry matter accumulation observed in Kenkunde and M66. The reduction in mean dry matter in Kenkunde seeds subjected to terminal stress could be probably due to the cultivar being relatively sensitive to drought stress and also the longer time it took to mature, thus exposing it for a longer period to terminal stress than Nzovu. Cultivar differences may also account for the higher degree of sensitivity

to drought stress and long maturity period observed in M66. The sensitivity to drought stress and the long maturity period may also explain why seeds of M66 which is "supposedly bred for drought tolerance" took the longest period to mature and did not produce sufficient seed under both intermittent and terminal drought. Reports can be found in the literature on the effects of water stress on SGR in several crops. For example, SGR has been shown to be relatively insensitive to direct effects of water stress on the seed with only minimal effects shown for soybean (Meckel *et al.*, 1984) maize (Westgate and Thompson Grant, 1989) and pea (*Pisum sativum*) (Ney *et al.*, 1994).

The observation that both intermittent and terminal stress considerably reduced seed number and yield but did not have an effect on the development of cowpea seed germination and vigour is consisted with a number of reports cited in the literature. Water stress has been shown to reduce seed number and size in soybean; however, the seeds that are usually formed have high germination quality (Dornbos *et al.*, 1989; Smiciklas *et al.*, 1989; Vieira *et al.*, 1992). Benech-Arnold *et al.*, (1991) reported that mild drought stress improved germination in sorghum and Ellis *et al.*, (2000) showed that drought stress can improve potential longevity in rapid cycling Brassica. Similarly, as has been noted earlier, the ability of developing seeds to act as stronger sinks than vegetative tissue during seed filling period may probably account for the observation that despite the reduction in seed size and number due to drought stress, the seeds that are formed are usually of high germination quality (Bradford, 1994). The development of high seed quality has also been associated with the accumulation of certain sugars and proteins. For example, in the experiment by Ellis *et al.*, (2000) on the effect of drought stress on seed quality development in Brassica, the authors suggest that the accumulation of certain oligosaccharides and certain heat stable proteins are "equally likely (or unlikely) to be required for the development of high quality seed". In the present study it may be possible that the high cowpea seed quality with respect to germination capacity and vigour that was observed may be associated with plant drought tolerance mechanisms. Plant drought tolerance mechanisms that operate at the whole-plant level have been shown in this study to be efficient in enabling cowpea plants to avoid drought stress; however, those that occur at the cellular level may influence seed germination and vigour. Cellular responses such as osmotic adjustment that allow plant cells to continue normal metabolic

functions that determine growth and development may not influence germination and vigour. However, those that allows plant survival such as reduction in photosynthesis and transpiration rates may affect the production and translocation of assimilates to plant cells and consequently seed number and size. Responses to lethal disruption of function at the cellular level may ultimately influence germination and vigour. Protection against lethal damage in seeds as a result of drought is correlated with the accumulation of sugars and proteins (dehydrin) (Blackman *et al.*, 1991; Close *et al.*, 1993). The presence of disaccharides such as sucrose and oligosaccharides are believed to play a role in stabilizing cell membranes during drying (Crowe and Crowe, 1992). It is thought that these compounds can interact with polar groups on cell membranes and effectively replace hydrogen bonding that is normally contributed by water (Kermode, 1995; Vertucci and Farrant, 1995; Kermode, 1997). During seed development, orthodox seeds also undergo a programmed water loss, acquire desiccation tolerance and also accumulate sugars and proteins which serve to protect the molecular organisation of cell membranes as the seeds lose water (Bewley and Black, 1994; Vertucci and Farrant, 1995). It is probable that an interaction may have occurred between seed developmental processes and drought stress implicating these sugars and influenced seed germination and vigour. The findings of this study merit further investigations into the molecular and biochemical aspects of the quality of cowpea seeds as influenced simulated drought conditions.

The highly significant interactions observed between the production site, cultivar and stress levels with respect to germination capacity, germination index, mean germination time, seedling dry weight, and seed number and mass suggests that the initial production site may have had an effect on the subsequent performance of the seeds produced under simulated drought conditions; however, these effects were influenced by cultivar differences and the levels of stress. Unlike in the study by Highkin and Lang (1966), who reported that adverse temperature conditions caused pea seeds to grow poorly, in the present study seeds that had been initially produced at Umbumbulu under very minimal rainfall and subsequently subjected to simulated drought stress did not differ with respect to germination and vigour from those initially produced at Ukulinga under optimal conditions. The underlying assumptions in the suggestions made by Salisbury and Ross (1991) and Wulf (1995) on the effect of the maternal environment which can be carried over and influence the performance

of subsequent generations is probably based on the belief that the maternal environment is often characterised by sub-optimal conditions and that the effects will lead to poor growth or quality. These assumptions may also inform the widely accepted belief among seed scientists that environmental conditions during seed development can not only influence the seeds viability but also the physiological vigour potential (Tekrony, 2003). However, the nature of such effects on the viability and vigour of the seed may be variable. Plant response mechanisms to stress factors are known to be complex may be the cause of different developmental patterns resulting in variable physiological responses. It may be possible that interaction between the production site and seed developmental processes may have modified the developing embryo or the accumulated food reserves and resulted in high seed quality. On the other hand no such modifications may have occurred and cowpea being a drought tolerant crop, mechanisms at the whole plant level may have been effective in allowing the species to adapt to adverse conditions at Umbumbulu and under simulated drought stress to continue with its normal plant functions at the cellular level. Hence the high quality of seeds observed from both production sites and in response to simulated drought conditions. There is clearly a need to investigate whether the simulated drought stress caused alterations in the accumulated food reserves relevant to seed performance (e.g. soluble carbohydrates) under simulated drought stress and whether such changes are related to germination and vigour.

Contrasting results have also been reported by other workers, on the effect of environmental stress factors such as drought on seed germination and vigour. For example, Dornbos (1995) reported that drought and temperature stress decreased germination in the standard germination test as well as embryonic axis weight in mature soybean seeds. In addition, maximum daily phytotron temperatures of 33 and 38^{o}C during development reduced seed size increased the number of shrivelled seeds and resulted in lower germination and vigour in soybean (Tekrony *et al.*, 2004).

These contradictory conclusions found in the literature on the effect of environmental stress factors on seed germination and vigour could be due to several factors which may include the methods used in imposing and quantifying the intensity and duration of stress. In the present study attempts were made to simulate drought conditions to be as close as possible to drought stress under field conditions by firstly using large 20kg

pots and secondly mixing the soil and water from individual pots thoroughly using a concrete mixture. A major criticism of pot experiments probably contributing to the contradictory conclusions found in the literature on the effect of water deficits on seed yield and quality has been the problem of watering pots periodically to moisture contents below field capacity (Anonymous, 1978). When water is added to a dry well drained soil, a downward movement takes place behind a wetting front (Zur *et al.*, 1994). If the amount of water being added is below field capacity, a situation may arise whereby there are two clear sections of wet and dry soil. This may probably account for the observation that in many occasions plant responses to rapid depletion of water in small pots may be considerably different from plant responses to drought in many field environments (Hall *et al.*, 1979).

Soil water content and matric potential in the pot were also monitored using a ThetaProbe soil water sensor and compared with the results obtained from the use of gravimetric methods. Gravimetric methods were found to be accurate and clearly reflected differences between the three water stress treatments. Munoz-Carpena (2004) noted that gravimetric method are inexpensive and accurate in determining soil water content, however, they are destructive, slow time-consuming and do not allow for making repetitions in the same location. In the present study gravimetric methods proved to be very laborious and tedious. The results obtained from the ThetaProbe were similarly accurate and more sensitive in determining soil water content and matric potential in the pots. Soil water content sensors such as the ThetaProbe are widely used to schedule irrigation (Campbell and Campbell, 1982) and to save water and nutrients from deep percolation, and to optimize crop production due to timely application and the efficient use of water, including rainfall (Lukangu *et al.*, 1999).

In conclusion, this study has demonstrated that adverse maternal environmental effects on the subsequent performance of seeds in a drought tolerant crop may not necessarily lead to poor performance and must be separated from inherent genotype related factors which may confound these effects. Cultivar differences in response to simulated drought conditions at the whole plant and tissue level can be considerable and highly variable; however, these differences may not have adverse effects the germination and vigour of the seeds. Drought avoidance mechanisms at the whole plant level in cowpea are quite efficient in allowing the species to adapt to simulated

drought conditions. These mechanisms may allow the cowpea cultivars to maintain metabolism and restore conditions for their continued growth under water stress; and produce few seeds but of high germination capacity and vigour. It is possible that mechanisms at the cellular level may modify the embryo and/or accumulated food reserves to influence the development of seed germination and vigour under water stress. There is, therefore, a need to investigate whether changes occurred in the composition of sugars and proteins at the cellular level which may be associated with seed germination and vigour.

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4 CONDUCTIVITY TEST OF COWPEA (*VIGNA UNGUICULATA* (L.) WALP) SEED IN RELATION WITH SEED COAT COLOUR

4.1 Introduction

Cowpea is an important food grain legume, generally grown worldwide in tropical and sub-tropical regions. The crop exhibits a wide range of seed coat colours. Seed coat colour has not been widely investigated as a factor influencing seed performance. The few available studies on this aspect do not provide consistent evidence about the association between seed coat colour and seed performance during germination (Wien and Kueneman, 1980; Mugnisjah *et al.*, 1987; Asiedu and Powell, 1998; Pimpini *et al.*, 2002). Solute leakage, seed germination and vigour have been associated with seed coat colour. In cowpea (Asiedu and Powell, 1998), long bean (*Vigna sesquipedalis*) (Abdullah *et al.*, 1993), soybean (*Glycine max*) (Mugnisjah *et al.*, 1987), and radicchio (*Cichorium intybus*) (Pimpini *et al.*, 2002), seeds referred to as pigmented, coloured or dark showed better performance than unpigmented, light or cream/beige seeds. Pigmented seeds showed lower solute leakage, slower decline in seed vigour and good storage potential, whereas unpigmented seeds showed higher solute leakage, rapid decline in vigour and poor storage potential. However, contrasting observations were made by Aiazzi *et al.*, (2006) who reported that in *Atriplex cordobensis*, light brown seeds from medium dispersion units and reddish seeds from medium and large dispersion units showed the highest quality with respect to germination capacity and seedling dry weight, and produced more vigorous seedlings than dark brown seeds. Similarly, brown coloured seeds of berseem clover (*Trifolium alexandrium*) were reported to be significantly inferior to yellow seeds with respect to germination capacity , emergence percentage rate , seedling elongation and seedling length (Dalianis, 1980). Furthermore, Dalianis (1980) concluded that high percentages of dark brown seeds in seed lots of berseem and Persian clover (*Trifolium resupinatum*), and of yellow seeds in Persian clover is an easy visual indication of inferior seed quality. In the literature, reports are also found which suggest that there is no association between seed coat colour and seed quality. For example, in an experiment conducted to evaluate the combined effects of plant colour and seed colour in sorghum (*Sorghum bicolor* (L.) Moench) on germination, emergence and agronomic performance, Pederson and Toy (2001) reported that no differences in standard warm germination or seed vigour test results could be

attributed to seed coat colour. Similarly, soybean cultivars could not be discriminated for storage potential on the basis of seed coat colour (Wien and Kueneman, 1980).

A seed vigour test may reflect underlying changes at the physiological and biochemical level, which may involve food reserve utilization, enzyme activity, metabolic changes in energy or storage compounds and membrane integrity (Tekrony, 2003). Membrane integrity is the basis of the electrical conductivity test for seed vigour, and it measures solute leakage (Mathews and Bradnock, 1967; ISTA, 1995). The electrical conductivity test has been used as an estimate of vigour in a number of crop species, including soybean (ISTA, 1995), with significant correlations reported between bulk conductivity and field emergence (Oliveria *et al.*, 1984; Loeffler *et al.*, 1988). Recent work by Midrad *et al.,* (2006) examining the potential of the bulk conductivity test to assess germination in cauliflower and cabbage showed a clear correlation between measurements of leachate conductivity and germination in both species. These authors suggested that there is potential for the rapid assessment of germination from conductivity readings. The conductivity test has also been extended to measure solute leakage at the individual seed level (Steere *et al.,* 1981) and consequently, the quality of individual seeds (McDonald and Wilson, 1979; Siddique and Goodwin, 1985). Assessment of the quality of whole seed lots using single seed conductivity was based on critical levels of electrical conductivity determined from correlations between data on percentage germination and individual electrical conductivity values (Siddique and Goodwin, 1985). Problems with the use of critical levels of individual electrical conductivity values to differentiate germinable and non-germinable seeds have been however identified in soybean, peas (Hepburn *et al.*, 1984) and Brassica (Thornton *et al.*, 1990). Electrical conductivity was not found to be closely associated with individual seed germination in pea (*Pisum sativum*) (Hepburn *et al.,* 1984) and seedling growth and emergence in soybean (Egli *et al.*, 1990). Hamman *et al.,* (2001) also failed to establish a relationship between conductivity of individual soybean seeds and their emergence performance; and observed that seeds with low conductivity performed poorly while those with high values performed well. These observations were attributed to other seed characteristics that might influence solute leakage and seed vigour (Hamman *et al.*, 2001), which may include variation in the physical, genetic and physiological composition of the seeds. Additional factors that may contribute to variation in

individual solute leakage may include environmental conditions during maturation and storage, seed moisture content, test conditions including soak temperature and the precise timing of the recording (Tekrony, 2003).

Variation in individual electrical conductivity can cause a lack of uniformity in test results and lead to contradictory conclusions. It is therefore, important that the variability in individual electrical conductivity is quantified and explained. Statistical parameters have been used to quantify variation in seed lots. The coefficient of variation was used by Illipronti Jr. *et al.,* (2000) to quantify uniformity in seedling performance in different soybean seed lots. Hacisalihoglu *et al.,* (1999) used standard deviation to quantify germination uniformity in lettuce. Other statistical parameters that have been used to quantify variation include the range which was used to measure the time for 10-90 % radicle emergence in lettuce (Tomas *et al.,* 1992) and 25-75 % range to measure uniformity of germination in cabbage (Jalink *et al.,* 1998). Recently, Muasya *et al.,* (2006) used the mean-median, range, skewness, kurtosis and box-whisker plots in addition to other statistical parameters to evaluate the variation found in individual seed electrical conductivity in common bean, and to explore whether cultivar or production environment affected the variation. These authors observed that individual electrical conductivity was highly variable over seed lots; and no single parameter related to all aspects of variation could be used as the sole parameter for quantifying variation in individual seed electrical conductivity. However, the use of box-whisker plots in exploring variation in individual electrical conductivity between production sites, aged and unaged seeds of cowpea cultivars differing in seed coat pigmentation has not been investigated. Information in the literature on associations between seed coat colour and variation in individual electrical conductivity values, and how the variation may influence the results of the single seed electrical conductivity test to discriminate between high and low vigour seeds in cowpea has not been clearly explained. There are also contradictory reports on associations between seed coat colour and seed performance. Furthermore, in many studies, seed coat colour is usually determined by visual inspection, which is subjective and may also have an effect on results.

The objectives of this study were (i) to evaluate the use of image analysis as a method that can be used to objectively determine seed coat colour variation in cowpea (ii) to

explore the statistical variation in individual seed's solute leakage for cowpea cultivars differing in seed coat colour and produced under different environmental conditions (iii) to correlate seed conductivity test with other aspects of seed performance during germination (iv) to compare conductivity test with accelerated aging test, with respect to seed performance.

4.2 Materials and methods

Seeds of cowpea cultivars Brown Mix Makonge, Nzovu, M66, K80 and Kenkunde were produced as explained in chapter 2 (section 2.2.2). Seeds of cowpea lines Black, Brown and White were donated by Professor Rob Melis of the African Crop Improvement Centre, University of KwaZulu-Natal. Cultivars were ranked visually, on the basis of seed coat colour, from the lightest (1) to the darkest (9). Image analysis was performed to determine seed coat colour variation among cultivars. Image analysis was carried out using Soft Imaging System, analySIS® PRO. Version 3.2 manufactured by Soft Imaging System, GmbH, Germany. Images were captured using a JVC KY30 3CCD video camera attached to a Kaiser RB 5000 natural lighting copy stand. Images were captured sequentially against a blue background with no alteration of the lighting on the copy stand and camera. Measurements of pixel values were recorded by randomly clicking at five different points on five individual seeds and hue, saturation and intensity were determined.

Seeds were harvested at harvest maturity (HM) and assessed for seed performance (electrical conductivity, germination capacity and germination index) before and after the accelerated aging (AA) test (AOSA, 1983). The accelerated aging test is a stress test which was developed to determine the storage potential of seeds, but it is also used now to predict the emergence potential for a wide range of larger seeded species. Seeds were aged using the AA method by maintaining samples at 41^{o}C and 100% relative humidity for 72h. Forty millilitres of water was added to plastic germination boxes measuring (11.0 x 11.0 x 3.5 cm). Inside the plastic box, a 10.0 x 10.0 x 3cm wire mesh screen (mesh 14 x 18) was placed. Seed samples (sample size was based on the number of seeds required for further tests) were weighed up to two decimal places then placed on top of the wire mesh tray and spread (approximately one layer deep). The lids were then secured on each box and the inner chamber (containing box, water, wire mesh tray and seed) was placed in a germination cabinet set at a temperature of 41^{o}C. Seeds were removed after 72h and

subjected to electrical conductivity and germination tests. Electrical conductivity (μS cm^{-1} g^{-1}) was determined according to ISTA (1995) for ten individual seeds (replicates) using a CM100 automatic seed analyser manufactured by Reid and Associates, Durban. Unaged and aged seed samples were divided into five replicates of twenty seeds and germinated using the paper towel method according to ISTA according to ISTA (1999) (For details on germination test see Chapter 2). Normal, abnormal and dead seeds (ISTA, 1999) were assessed and recorded at the final count on the eighth day. Germination Index (GI) was determined according to (Scott *et al.*, 1983):

$$GI = \frac{\sum_{i=1}^{n} T_i N_i}{TN}$$

where GI = germination index, T_i is the number of days after sowing starting with the final day, N_i is the total number of seeds germinated on day i, where i = day 1, 2, 3…. 8 and N is the total number of seeds that have germinated on the final count. Sensitivity to stress was determined by using the Aging Stress Differential Index (ASDI), which was determined by the difference between electrical conductivity, germination capacity and germination index values before and after AA.

Genstat® (9^{th} edition) was used to perform analyses of variance. Differences between means were determined by Least Significant Difference (LSD; P = 0.05)). Microsoft Excel was used to calculate ASDI. Histograms and box -whisker plots were used to determine statistical variation in individual electrical conductivity values. The box plot indicates the variability between the 25^{th} and the 75^{th} percentile which contains the middle 50 % of the data (Tukey, 1977). The range between the 25-75 % is known as the inter-quartile range. The line in the box plot indicates the median value of the data. If the median within the box plot is not equidistant from the upper hinge (75%) and the lower hinge (25%), then the data is skewed. The ends of the vertical lines or "whiskers" indicate the most extreme values within a reasonable distance from the end of the box. Box plots display variable locations and spread at a glance and provide some indication of the data symmetry and skewness. By using box plots for each variable side by side, it was possible to explore the variability in individual electrical conductivity values between production sites, unaged and aged seeds, and between cultivars at different sites.

4.3 Results

Highly significant differences ($P < 0.001$) between cultivars for hue and intensity were observed (Figure 4.1 A and B). Hue decreased progressively from the darkest (black seeded) to the lightest (white seeded) cultivar whereas the reverse was observed for intensity. Visual inspection showed that the brown seeded cultivar was darker than Kenkunde and Makonge. However, image analysis indicated that the brown seeded cultivar should be classified after Kenkunde and Makonge with respect to hue. Image analysis can be used to categorize the seeds in this study into three distinct groups based on hue and intensity. Group one consists of seeds with high pixel values for hue and low for intensity; these include Nzovu and the black seeded cultivar and can be defined as highly pigmented or dark. Group two includes Kenkunde, Makonge and Brown with lower values for hue and moderate intensity and can be defined as moderately pigmented or moderately dark. Brown Mix, K80 and M66 which had the lowest pixel values for hue and highest intensity can be defined as unpigmented or light coloured and form group three. In this study, seeds of Black, Brown and White cultivars were inadequate for planting. Therefore, further tests were done on the remaining six cultivars. However, the results of the accelerated aging test were based on five cultivars, because the cultivar Brown Mix did not perform well under field conditions and had poor yields. Thus, dark- coloured seeds refer to Nzovu, Kenkunde, and Makonge and light-coloured seeds refer to Brown Mix, K80 and M66.

Solute leakage was measured over a 24 h period and clear differences in solute leakage were observed at the start (1 h) between seeds produced at Ukulinga (Figure 4.2). Makonge, which has dark-coloured seeds, showed the highest solute leakage whereas Brown Mix (light-coloured) had the lowest. As expected, solute leakage increased for all cultivars with increase in time; however, K80 showed the fastest solute leakage increase with the highest value at 24 h. The general pattern of solute leakage was K80 > Nzovu > Makonge > Kenkunde > M66 > Brown Mix. For the seeds produced at Umbumbulu, there were no cultivar differences 1 h after conductivity test (Figure 4.2). The trend of cultivar leakage showed that Nzovu > M66 > Makonge > K80 ≅ Kenkunde > Brown Mix.

Individual seed electrical conductivity for different cowpea cultivars was generally not normally distributed (Figure 4.3). The patterns generally showed a positive skew tending towards high values and could not be associated with seed coat pigmentation

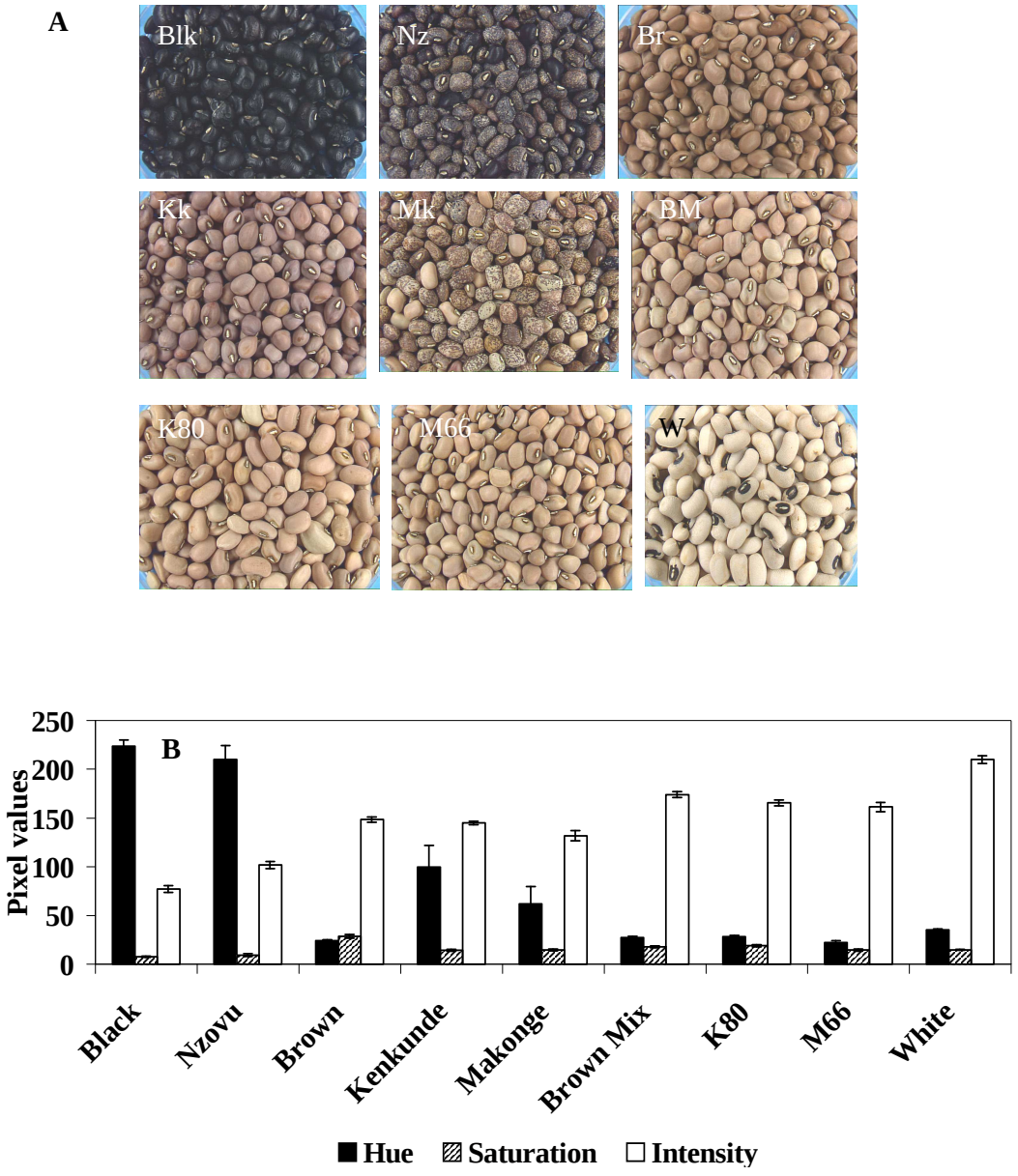

Figure 4-1 (A) Seeds of nine cultivars of cowpea ranked visually (from the darkest Blk- Black-9, Nz-Nzovu-8, Br- Brown- 7, Kk- Kenkunde- 6, Mk- Makonge-5, BM-Brown Mix-4, K80-3, and M66-2 to the lightest W-White-1); (B) Seed coat colour determination (hue, saturation and intensity) was done using image analysis.

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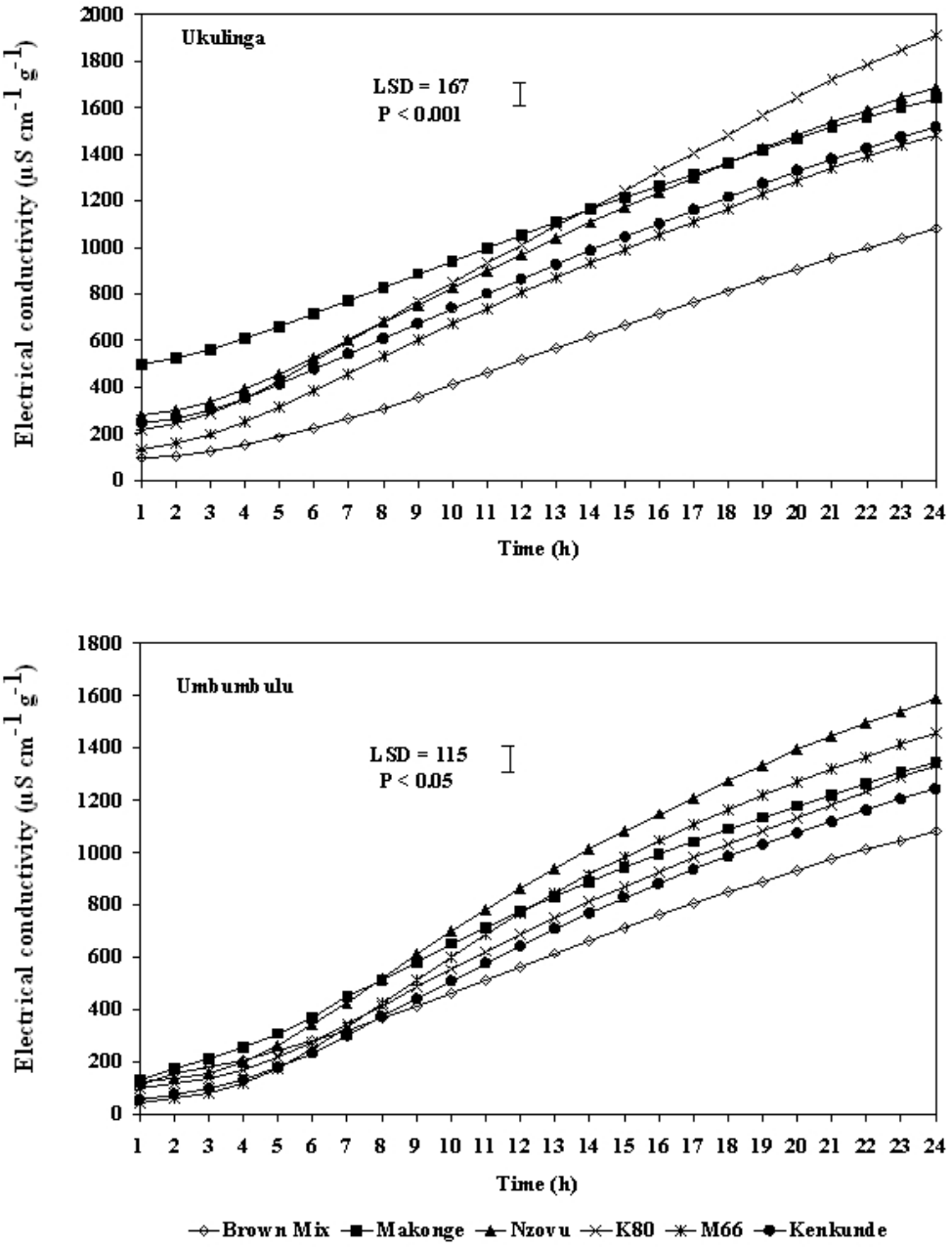

Figure 4-2 Differences in the rate of solute leakage for harvested seeds of cowpea cultivars differing in coat colour and produced at two sites (Ukulinga and Umbumbulu). Solute leakage was determined hourly up to 24h.

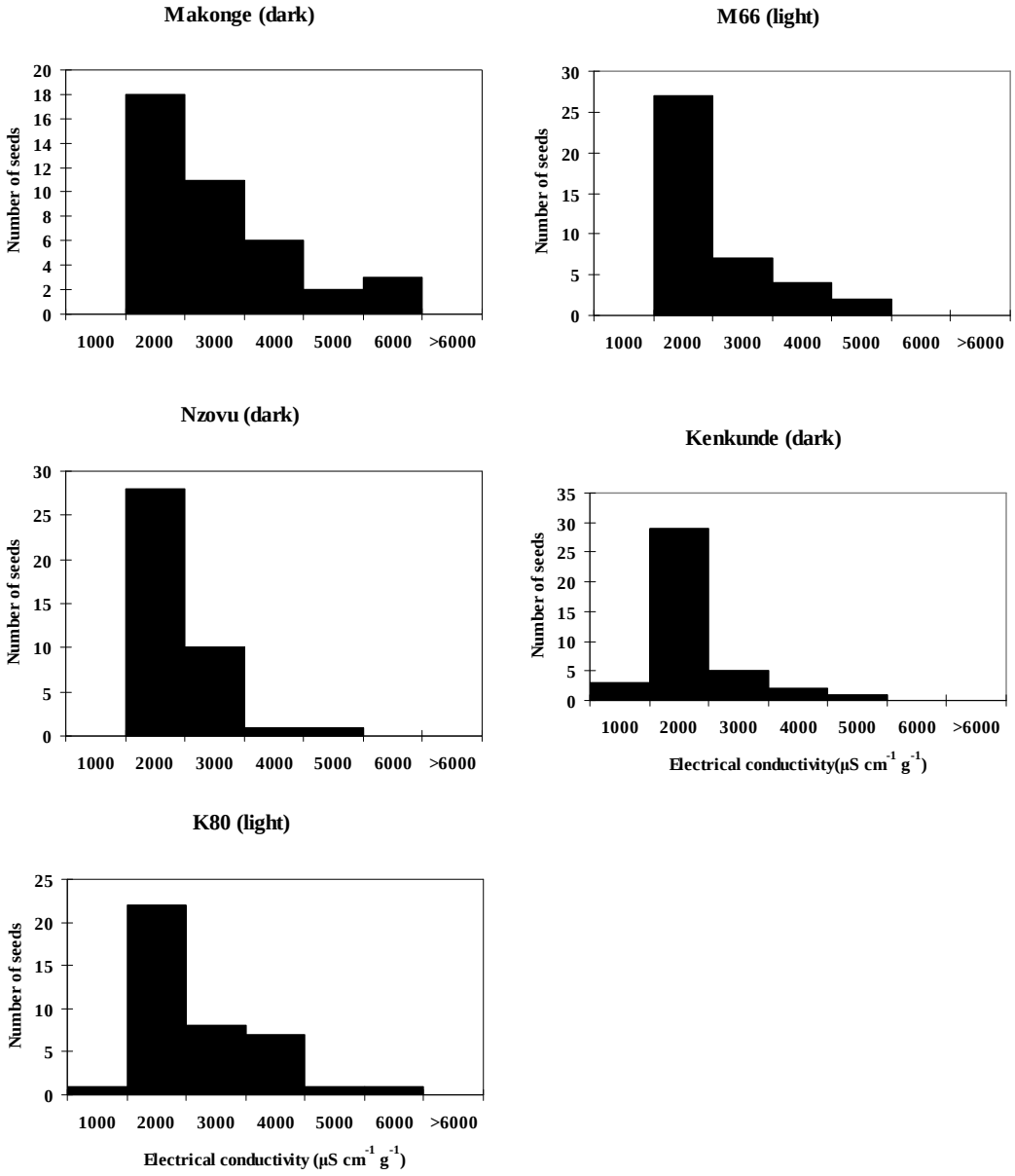

Figure 4-3 Individual seed electrical conductivity (µS cm^{-1} g^{-1}) distribution patterns of five cultivars of cowpea differing in seed coat pigmentation.

Box plots were used to illustrate the variation in individual electrical conductivity values between the 25th and 75th percentile which represent the inter-quartile range and contains 50 % of the data (Figure 4.4). There seemed to be a larger variability in individual electrical conductivity overall in seed samples from Ukulinga than those from Umbumbulu. Although samples from the two production sites showed a positive

skew and contained a high number of outliers and far outliers, the sample from Umbumbulu had a lower median and showed more symmetry than from Ukulinga. Similarly, the overall sample data set for unaged seeds seemed to follow a normal distribution. However, aged seeds were more variable, had a higher median than unaged and showed a pronounced skew to the right. Both samples of overall data sets for unaged and aged seeds showed quite a number of outliers and far outliers (Figure 4.4).

Variation in individual electrical conductivity in unaged seeds of all the cultivars from Ukulinga was generally low (Figure 4.4). Light coloured seeds of M66 and dark coloured seeds of Kenkunde showed reasonable symmetry; however, samples of Makonge (MKE), Nzovu and K80 showed skewed distributions. Unaged dark coloured seeds of the cultivar Nzovu (NZ) and Kenkunde (KK) produced at Ukulinga showed the largest variability. Unaged dark coloured seeds of Kenkunde (KK) had the lowest median followed by unaged light coloured seeds of M66. Dark coloured seeds (unaged) of Makonge (MK), and Nzovu (NZ) and light seeds of K80 showed almost similar median values. Samples of unaged seeds of Makonge (MKE), K80 and M66 contained a number of outliers. There was a general increase in variability in all samples after aging. However, aged dark-coloured seeds of Makonge (MK) appeared to be the most variable and had a similar median value to aged light-coloured seeds of K80, which were less variable. Similarly, seeds of M66 also showed a considerable increase in variability after AA. Aged dark coloured seeds of Nzovu and Kenkunde produced at Ukulinga showed less variability after AA and had the lowest median values. Samples of data sets of both aged light-coloured and dark-coloured seeds of all the cultivars from Ukulinga tended to show a positive skew and no outliers were identified.

Similar trends were observed in unaged light and dark coloured seeds of all the cultivars from Umbumbulu. Samples of the cultivar Nzovu (NZ), M66 and Kenkunde (KK) from Umbumbulu showed considerably less variability. Dark coloured seeds of Nzovu (NZ) and Kenkunde (KK) and light coloured seeds of M66 showed fairly reasonable symmetry with dark coloured seeds of Kenkunde showing the lowest median value. Unaged dark coloured seeds of the cultivar Makonge (MK) showed the largest variability, followed by K80. Although the median values for aged seeds from

Umbumbulu were evidently higher than those of the unaged, aged seeds of all cultivars from Umbumbulu were not highly variable with respect to individual electrical conductivity. Aged dark-coloured seeds of Kenkunde had the lowest median value, but showed a slightly skewed distribution with short tails. Aged dark coloured seeds of Nzovu and light coloured seeds of K80 had similar median values, but seeds of K80 were more variable. Aged light coloured seeds of M66 showed a negative skew and aged dark coloured seeds of the cultivar Makonge appeared to be the most variable and showed a positive skew. In addition, samples of aged seeds of Nzovu, K80, M66 and Kenkunde showed quite a number of outliers.

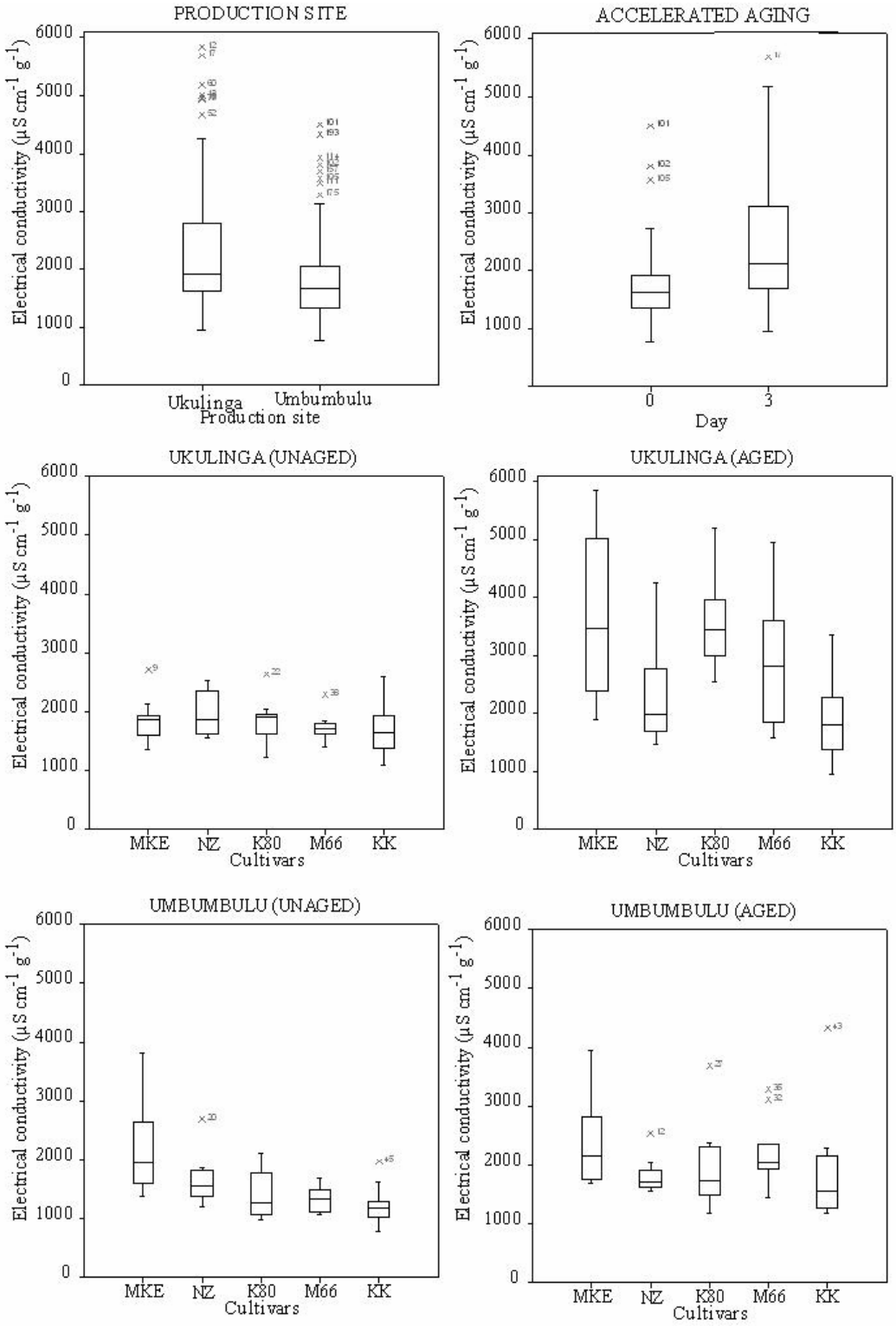

Figure 4-4 Box-whisker plots comparing the variability in individual seed electrical conductivity (µS cm^{-1} g^{-1}) in 5 cowpea cultivars at two production sites, before and after AA.

The relationship between the seed coat colour parameter hue and seed performance, with respect to electrical conductivity, germination capacity and germination index before and after AA was examined. Seeds were arranged from the darkest (Nzovu with the highest pixel value for hue) to the lightest (M66 with the lowest hue values) and compared for performance before and after AA. There were no clear associations between hue and electrical conductivity for unaged seeds of all cultivars produced at both sites (Figure 4.5). However, unaged dark coloured seeds of Makonge produced at Umbumbulu showed the highest conductivity values. Electrical conductivity for seeds of all cultivars produced from both sites increased after AA. Aged dark coloured seeds produced at both sites except for Makonge generally had lower conductivity than light coloured seeds. The differences with respect to electrical conductivity between aged light and dark coloured seeds were more evident in the seeds produced at Ukulinga than those produced at Umbumbulu.

No clear relationship between hue and germination capacity was observed for unaged seeds of all cultivars produced at both sites (Figure 4.6). Germination capacity for seeds of all cultivars produced at both sites decreased after AA. Aged dark-coloured seeds produced at both sites, with the exception of Makonge, generally displayed higher germination capacity than light coloured seeds (Figure 4.6). Again the differences with respect to germination capacity between aged light- and dark-coloured seeds were more pronounced in those produced at Ukulinga than those produced at Umbumbulu (Figure 4.6).

No clear relationship between hue and germination index were observed for unaged seeds of all cultivars produced at both sites (Figure 4.7). Germination index for seeds of all cultivars produced from both sites decreased after AA. Aged dark coloured seeds produced at both sites except for Makonge generally had higher germination index than light coloured seeds. Again the differences with respect to germination index between aged light and dark coloured seeds were clearer in those produced at Ukulinga than at Umbumbulu.

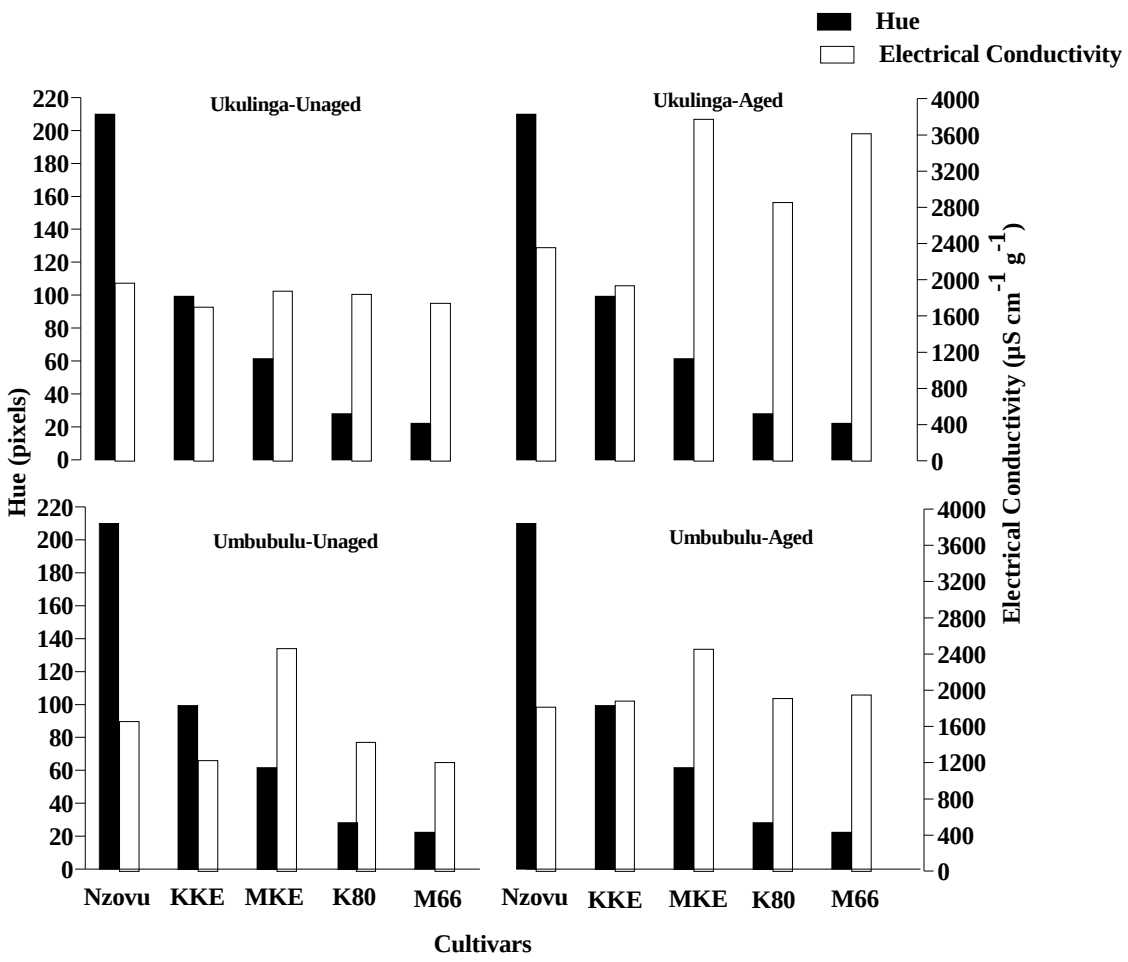

Figure 4-5 Relationship between seed coat colour as determined by hue, and seed performance with respect to electrical conductivity, for unaged and aged seeds of five cowpea cultivars produced at two different sites (Ukulinga and Umbumbulu) and ranging widely in colour. Seeds are arranged from the darkest (Nzovu) to the lightest (M66). Note KKE and MKE denotes Kenkunde and Makonge respectively.

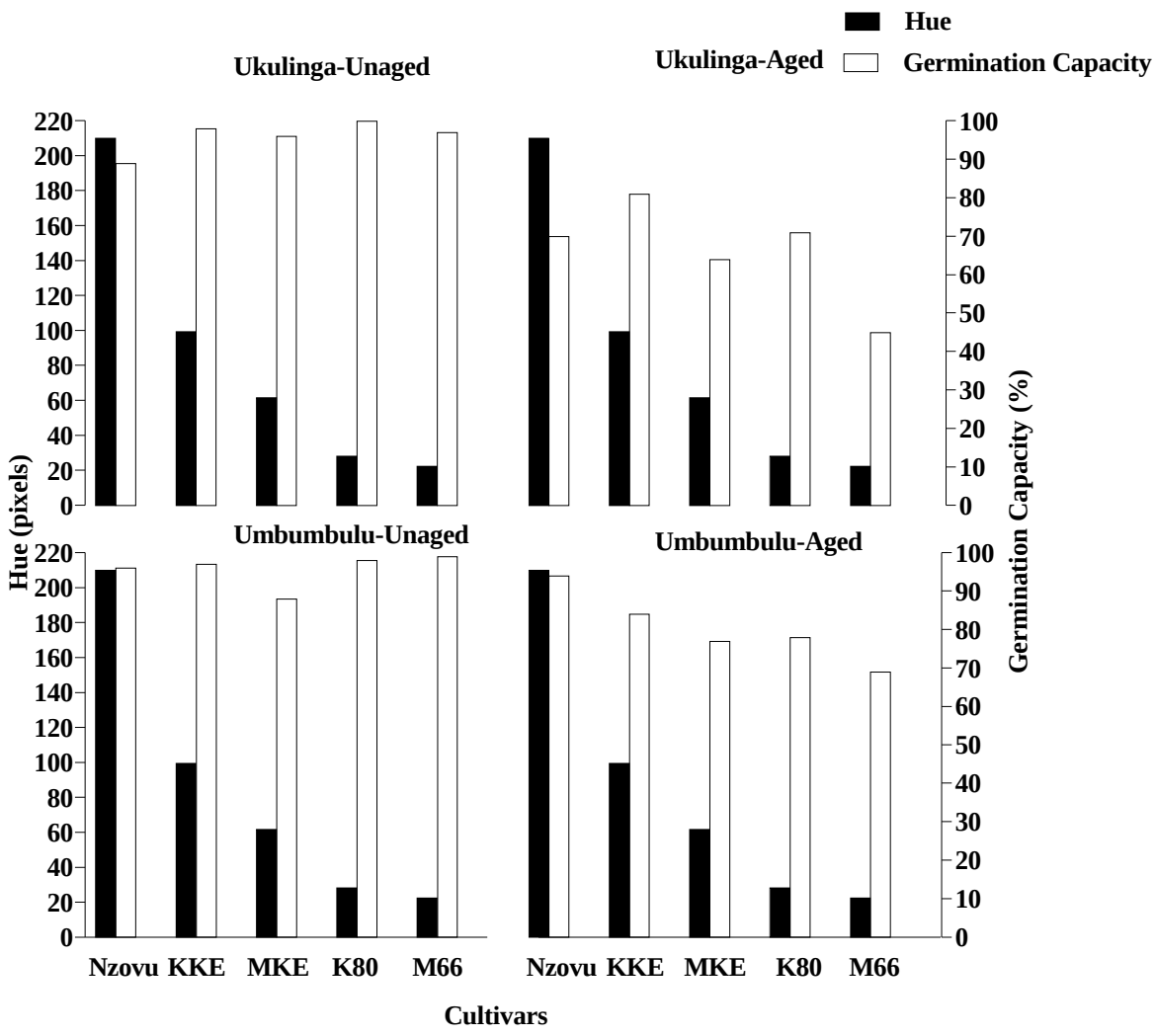

Figure 4-6 Relationship between seed coat colour as determined by hue, and seed performance with respect to germination capacity, for unaged and aged seeds of five cowpea cultivars produced at two different sites (Ukulinga and Umbumbulu) and ranging widely in colour. Seeds are arranged from the darkest (Nzovu) to the lightest (M66). Note KKE and MKE denotes Kenkunde and Makonge respectively.

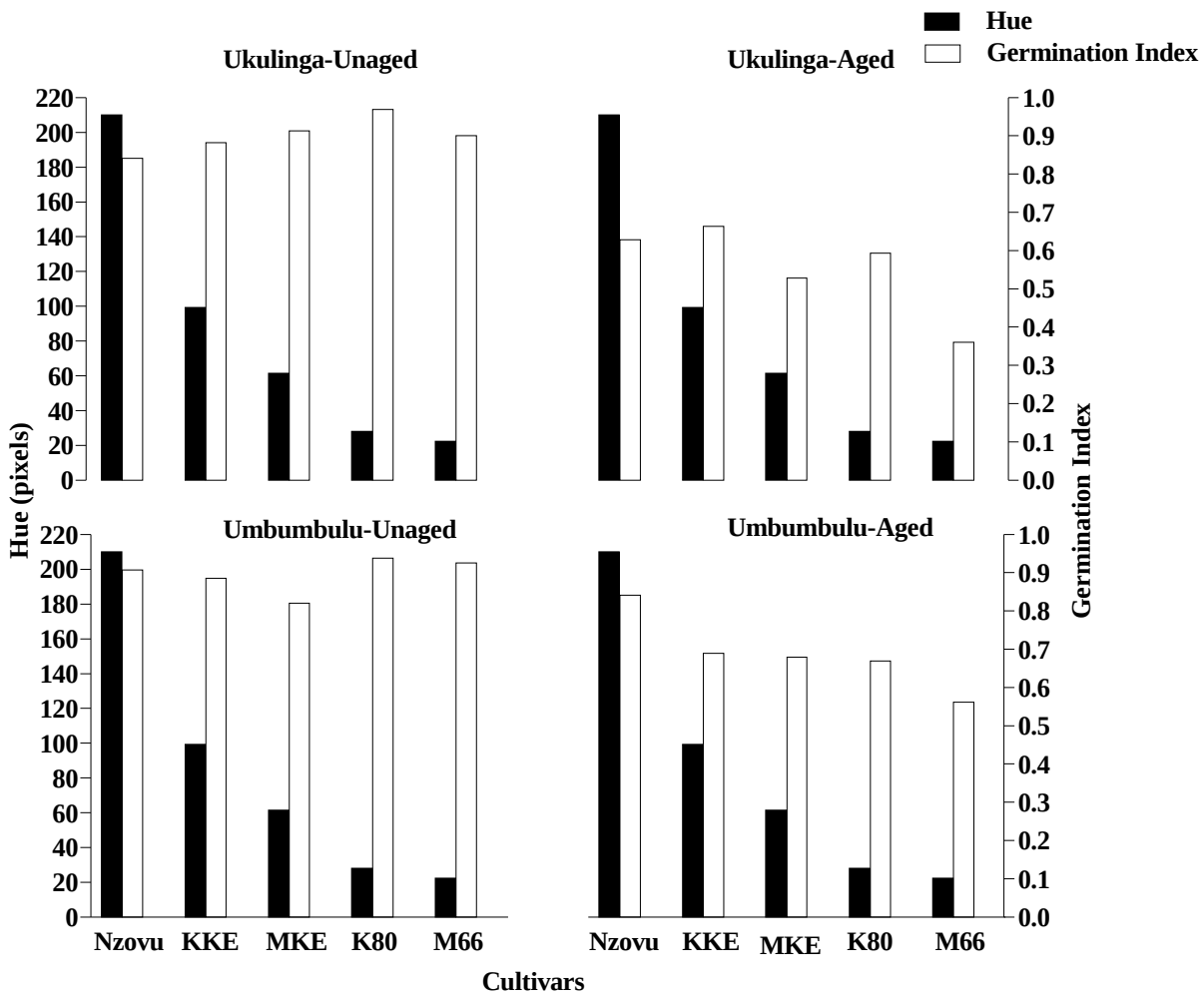

Figure 4-7 Relationship between seed coat colour as determined by hue, and seed performance with respect to germination index, for unaged and aged seeds of five cowpea cultivars produced at two different sites (Ukulinga and Umbumbulu) and ranging widely in colour. Seeds are arranged from the darkest (Nzovu) to the lightest (M66). Note KKE and MKE denotes Kenkunde and Makonge respectively.

The Aging Stress Differential Index (ASDI) was used to examine whether seed coat colour (hue) was associated with stress sensitivity in cowpea (Figure 4.8). Dark coloured seeds produced at both sites, except for seeds of Makonge, generally showed lower ASDI values (indicative of a higher degree of stress tolerance) for germination index, germination capacity and electrical conductivity (Figure 4.8) than light coloured seeds. The differences in ASDI values between dark and light coloured

seeds were larger and more evident in seeds produced at Ukulinga compared with seeds produced at Umbumbulu.

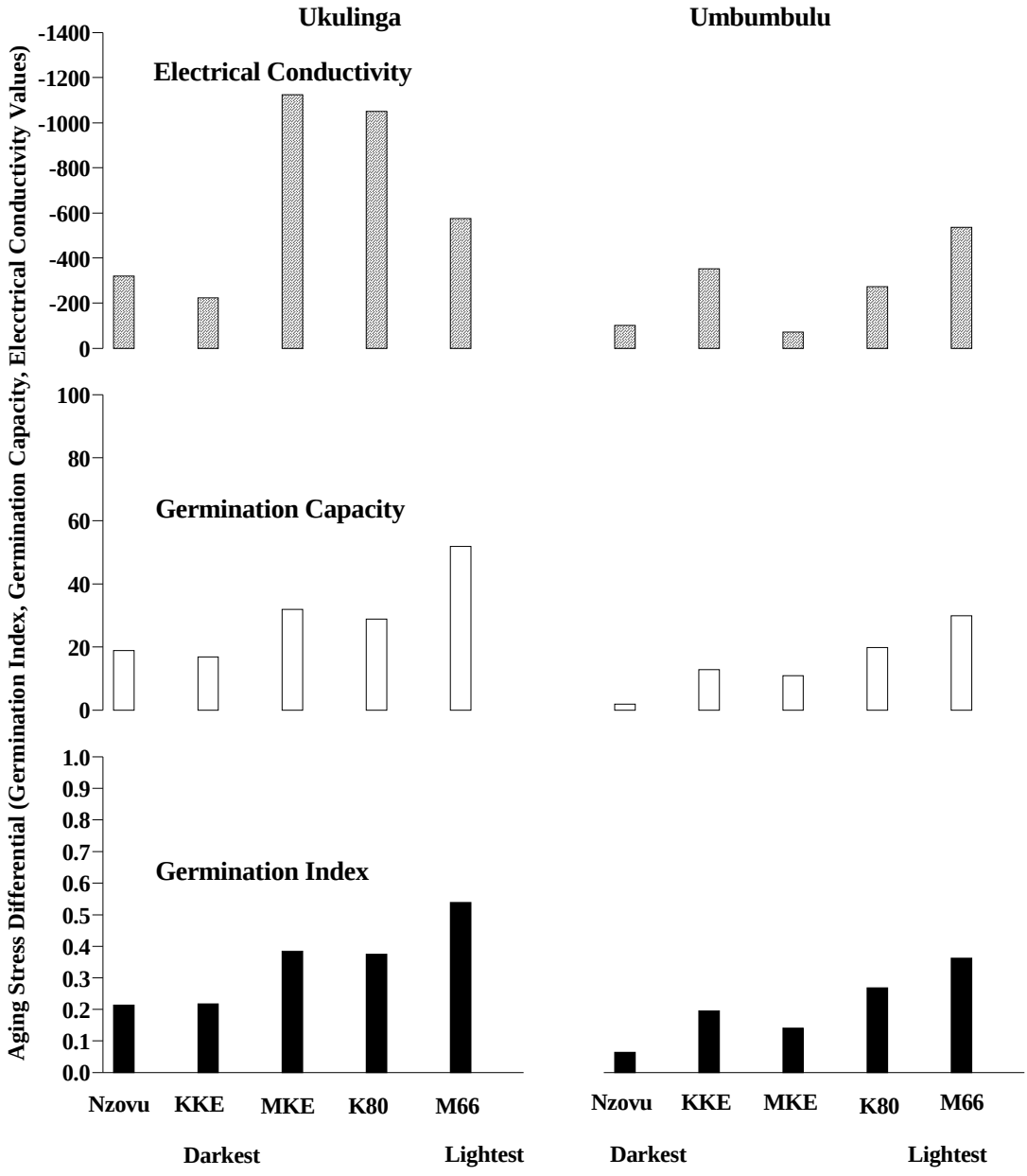

Figure 4-8 Aging stress differential for five cowpea cultivars produced at two different sites (Ukulinga and Umbumbulu) and ranging widely in colour. Seeds are arranged from the darkest (Nzovu) to the lightest (M66). Note KKE and MKE denotes Kenkunde and Makonge respectively.

Electrical conductivity values before and after AA were compared with other aspects of performance (germination capacity and germination index) (Table 4.1). Cultivars were ranked according to electrical conductivity values from the lowest (1) to the highest value (5). Highly significant ($P < 0.001$) site x cultivar x day interactions were observed with respect to electrical conductivity. Electrical conductivity which ranged from 895.6 – 908 $\mu S\ cm^{-1}g^{-1}$ for unaged light coloured seeds of M66, K80 and dark coloured seeds of Kenkunde produced at Ukulinga did not differ significantly. These were ranked first, second and third respectively. Unaged (0d) dark coloured seeds of Nzovu and Makonge produced at Ukulinga ranked fourth and fifth, respectively, were significantly different from the rest of the seeds and had the second highest (1009.9 $\mu S\ cm^{-1}g^{-1}$) and highest (1039 $\mu S\ cm^{-1}g^{-1}$) conductivity values. At Umbumbulu , light-coloured seeds of M66 ranked first had the lowest conductivity (634 $\mu S\ cm^{-1}g^{-1}$) which was not significantly different from dark coloured seeds of Kenkunde (678.5 $\mu S\ cm^{-1}g^{-1}$) ranked second but was significantly ($P < 0.001$) different from the third ranked seeds of K80 (742 $\mu S\ cm^{-1}g^{-1}$). Dark coloured seeds of Nzovu (914 $\mu S\ cm^{-1}g^{-1}$) produced at Umbumbulu ranked fourth were significantly ($P < 0.001$) different from K80. Again, unaged dark coloured seeds of Makonge produced at Umbumbulu showed the highest conductivity (1290 $\mu S\ cm^{-1}g^{-1}$) and were ranked fifth. For all cultivars there was a significant ($P<0.001$) increase in solute leakage for aged seeds produced at both sites. Electrical conductivity for aged seeds was influenced by site and cultivar differences. Aged dark coloured seeds of Kenkunde and Nzovu produced at Ukulinga showed the best performance with respect to electrical conductivity (1132.5 and 1332.3 $\mu S\ cm^{-1}g^{-1}$) and were ranked first and second, up from third and fourth before aging. Aged light coloured seeds of M66 and K80 were ranked third and fourth whereas dark coloured seeds of Makonge produced at Ukulinga again showed the highest conductivity (2165 $\mu S\ cm^{-1}g^{-1}$) after aging. At Umbumbulu although aged light coloured seeds of K80 (1015.8 $\mu S^{-1}cm^{-1}g^{-1}$), had the lowest electrical conductivity and were ranked first, they did not differ significantly from aged dark coloured seeds of Nzovu (1017.1 $\mu S\ cm^{-1}g^{-1}$) and Kenkunde (1031.1 $\mu S\ cm^{-1}g^{-1}$) ranked second and third respectively. M66 (1172 $\mu S\ cm^{-1}g^{-1}$) ranked first before aging dropped to position four after aging. Again, dark coloured seeds of Makonge consistently showed the highest conductivity (1362.9 $\mu S\ cm^{-1}g^{-1}$) and were ranked fifth as similarly observed for aged and unaged seeds at both sites.

Germination capacity for unaged seeds (0d) from both sites (Ukulinga and Umbumbulu) was generally high and ranged between 88-100 %. However, germination capacity for aged seeds produced at Ukulinga was highly variable and ranged from 45 -81%. Dark coloured aged seeds of Kenkunde produced at Ukulinga showed the best performance with respect to germination capacity (81 %) after AA. However, aged seeds of Nzovu (dark coloured) and K80 (light coloured) had similar germination (70 and 71 % respectively) after AA. Makonge (dark coloured) performed poorly (64 %) than light coloured seeds of K80. Aged light coloured seeds of M66 produced at Ukulinga had the lowest germination capacity (45 %) and performed poorest. Similar trends were observed for aged seeds produced at Umbumbulu. However, these seeds (produced at Umbumbulu) performed generally better than those produced at Ukulinga. Germination capacity for aged seeds produced at Umbumbulu was likewise highly variable and ranged from 69 – 94 %. Dark coloured seeds of Nzovu and Kenkunde, with germination capacities of 94 % and 84% respectively showed the best performance. However, light coloured seeds of K80, had a germination capacity of 78 % which did not differ significantly from those of Makonge (77 %), which are dark coloured. Again aged light coloured seeds of M66 produced at Umbumbulu with a germination capacity of 69 % showed the poorest performance.

Similar trends were observed in germination index. Unaged seeds (0 d) for all five cultivars produced at both sites had high vigour (> 0.82). However, large variations were observed in seed vigour after AA. Aged dark coloured seeds of Kenkunde and Nzovu produced at Ukulinga showed better performance with respect to germination index (0.66 and 0.63 respectively). Aged light coloured seeds of K80 (0.59) and dark coloured seeds of Makonge (0.53) produced at Ukulinga did not differ significantly with respect to germination index. However, aged light coloured seeds of M66, showed the poorest performance with respect to germination index (0.36). Similarly, aged seeds produced at Umbumbulu generally performed better than those at Ukulinga. Aged dark coloured seeds of Nzovu (0.84) showed better performance than K80 (0.67), Kenkunde (0.69) and Makonge (0.68). Likewise aged light coloured seeds of M66 showed poorer performance, which was consistent with observations made earlier for aged seeds of this cultivar produced at Ukulinga.

Table 4-1 Means of electrical conductivity ($\mu S^{-1}cm^{-1}g^{-1}$), germination capacity and germination index for seeds of five cowpea cultivars produced at two different sites (Ukulinga and Umbumbulu) and subjected to the Accelerated aging test. Conductivity values were ranked from the lowest (1) to the highest (5) based on the cultivars and compared with germination capacity and vigour between sites and before and after seed aging.

Site	Rank	Cultivar	Seed Coat Colour	0 days			Rank	Cultivar	3 days		
				Electrical Conductivity $\mu S^{-1}cm^{-1}g^{-1}$	Germination Capacity (%)	Germination Index			Electrical Conductivity $\mu S^{-1}cm^{-1}g^{-1}$	Germination Capacity (%)	Germination Index
Ukulinga	1	M66	Light Brown	895.6	97	0.90	1	Kenkunde	1132.5	81	0.66
	2	K80	Light Brown	908	100	0.97	2	Nzovu	1332.3	70	0.63
	3	Kenkunde	Dark Brown	908	98	0.88	3	M66	1472.6	45	0.36
	4	Nzovu	Black	1009.9	89	0.84	4	K80	1960	71	0.59
	5	Makonge	Variegated	1039	96	0.91	5	Makonge	2165	64	0.53
Umbumbulu	1	M66	Light Brown	634	99	0.93	1	K80	1015.8	78	0.67
	2	Kenkunde	Dark Brown	678.5	97	0.89	2	Nzovu	1017.1	94	0.84
	3	K80	Light Brown	742	98	0.94	3	Kenkunde	1031.1	84	0.69
	4	Nzovu	Black	914	96	0.91	4	M66	1172.3	69	0.56
	5	Makonge	Variegated	1290	88	0.82	5	Makonge	1362.9	77	0.68
LSD				82.2	8	0.08					
P				P<0.001	P<0.001	P<0.001					

4.4 Discussion

Image analysis was able to objectively discriminate seed coat colour variation in unaged and aged seeds of cowpea. The exact quality of a colour is determined by the hue, which measures wavelength (colour), the saturation (purity) and the intensity (brightness). The parameter hue can provide an objective means of determining seed coat colour variation in cowpea. Hue determines the colour, tint or shade and it is the feature of a colour that distinguishes it from another colour (Evans *et al.*, 1953). The lack of consistent evidence on seed coat colour as a factor influencing seed performance (Dalianis, 1980; Wien and Kueneman, 1980; Mugnisjah *et al.*, 1987; Asiedu and Powell, 1998; Pederson and Toy, 2001; Aiazzi *et al.*, 2006) may probably be partly attributed to methods used in seed coat colour determination. Seeds which may exhibit a wide range of coat colour are generally described visually as light or dark (Wien and Kueneman, 1980) pigmented or unpigmented (Asiedu and Powell, 1998), reddish (Aiazzi *et al.*, 2006) brown or yellow (Dalianis, 1980). This can be subjective, as was observed in the visual ranking of Brown, Kenkunde and Makonge. Image analysis revealed clear differences with respect to hue between seeds of these three cultivars. The cultivar Brown was ranked third before Kenkunde and Makonge which were ranked fourth and fifth, respectively by visual inspection. However, image analysis showed that Kenkunde and Makonge were more pigmented and should have been ranked third and fourth respectively followed by Brown as fifth with respect to hue. The determination of seed coat colour by visual inspection is likely to be inconsistent and may compromise the interpretation of results correlating seed performance and seed coat colour. Other methods that have been used to determine seed coat colour variation include the Hunter Lab Colorimeter which measures light reflectance on a scale of 100 = white and 0= black and uses the values to calculate a saturation index and hue angle (Modi, 1999). However, the Hunter Lab Colorimeter when compared to image analysis is clearly limited in scope. Image analysis can objectively discriminate seed coat colour variation in cowpea and provide a basis for the development of a simple, rapid and non-destructive method of assessing seed performance.

In this study seeds were planted at two sites (Ukulinga and Umbumbulu) distinguished by irrigated and dry land conditions to produce own fresh seed of

comparable quality. Clear differences with respect to electrical conductivity were observed in seeds produced at the two sites. The higher electrical conductivity values observed for unaged seeds produced at Ukulinga could be partly attributed to the effect of environmental conditions during maturation and drying. Seeds of different cultivars matured at different times; however, they were all harvested at the same time. This may have contributed to a larger extent of seed quality deterioration in early maturing cultivars because of delayed harvesting. In addition weather conditions during harvesting at Ukulinga were also wet and may have exacerbated the ageing process. On the other hand, the lower electrical conductivity observed for unaged seeds produced at Umbumbulu could be attributed to favourable conditions at harvesting which were dry and sunny. The seeds were also harvested separately according to the time taken to reach harvest maturity for each cultivar.

The differences in the methods of harvesting between the two sites may have had potential implications in the attempts to compare the two sites with regards to seed performance. However, it is important to note that despite these differences (in harvesting methods), germination percentage and germination index of the mature dry seeds (R9 development stage at harvest maturity) from both sites did not differ significantly (Chapter 2, Figure 2.6 and 2.7). Siddique and Wright (2004) reported that for pea (starchy seed) and flax (oily seed) neither differences in environmental conditions during development and maturation, nor concentrations of accumulated food reserves were able to provide a basis for vigour differences. In their study sowing dates (and presumably harvesting date) had relatively small effects on germination of non-aged seeds but much larger effects on emergence of non-aged seeds and on the germination and emergence of aged seeds. In the present study, differences in harvesting methods and environmental conditions during harvesting had a discernible effect on the vigour (electrical conductivity) of seeds harvested from Ukulinga.

The potential implications with regards to the statistical significance of these effects (harvesting methods) at Ukulinga on the comparison of seed performance between sites were investigated. Separate analysis of variance for the two sites showed that cultivar differences with respect to germination percentage and germination index at Ukulinga were not significantly different (Appendix 2.4a & c) whereas at

Umbumbulu where generally environmental conditions were characterised by inadequate moisture; germination percentage and germination index differed significantly between the cultivars (Appendix 2.4b & d). Separate analysis for the data from Ukulinga and Umbumbulu with respect to germination capacity and germination index also showed significant cultivar x stage interactions at Umbumbulu (for germination capacity). Similarly cultivar x stage interaction was significant for germination index at Ukulinga (Appendix 2.4c). Despite the differences in harvesting methods between the two sites, the tests for homogeneity of variances showed that both sites had a common variance (Appendix 2.4e). The analysis from the pooled data showed highly significant Site x Cultivar x Stage interactions.

Interestingly, dark coloured seed of Nzovu, produced at Umbumbulu who's harvesting was timely and were unlikely to have been subjected to adverse conditions at maturation showed the highest rate of solute leakage. This clearly further confounds the problem of higher electrical conductivity values which could also be attributable to differences in harvesting methods (Site effects). This may also suggest probably that this cultivar has a genetic predisposition to higher rates of moisture uptake, which influenced solute leakage. Light coloured seeds of K80 produced at Ukulinga, which showed the highest rate of solute leakage, may be genetically more predisposed to rapid deterioration. This is likely since at Umbumbulu where conditions were dry and sunny during harvesting, seeds of K80 did not differ much in the rate of solute leakage with other seeds both light and dark. The observation that light coloured seeds of Brown Mix produced at both sites showed the lowest rate of solute leakage could be due to the seed coat characteristics, which were not determined in this study (Wyatt, 1977).

The skewed distributions of individual electrical conductivity values may possibly raise concerns with respect to the suitability of using standard statistical analyses which compare means to analyse such data. This is because such data violates the statistical assumptions of normality, constant variance and independence of error (Steel and Torrie, 1980). In common bean, logarithmic transformations have been attempted on individual electrical conductivity data to reduce skewness; however, this did not result in significant gain since seed lots with high quality seeds tended to show higher ranges than those with poor quality (Muasya *et al.*, 2006). It may be possible

that the problems that have been reported in the literature with respect to the use of critical levels of individual electrical conductivity values to differentiate germinable and non-germinable seeds (Hepburn *et al.*, 1984; Thornton *et al.*, 1990) were related to the lack of normality in the distribution patterns of electrical conductivity values. This is because all data points, including extreme values, are used in the computation of the arithmetic mean commonly used to describe differences in single seed electrical conductivity. Variability in individual electrical conductivity and the presence of outliers and far outliers may explain why Hamman *et al.* (2001) could not establish a relationship between electrical conductivity of individual soybean seeds and their emergence performance; and also the observation made by these authors that seeds with low conductivities performed poorly while those with high values performed well.

Box-whisker plots showed that extreme values tended to be on the higher end as was evident from the number of outliers and far outliers observed when production sites were compared. The larger variability in individual electrical conductivity values and the presence of outliers observed at Ukulinga than Umbumbulu is consistent with the suggestion discussed earlier that solute leakage in seeds from Ukulinga may have been influenced by adverse weather conditions, causing seed quality deterioration. The box-whisker plots provided a good illustration of the increased variability in electrical conductivity of aged seeds which was indicative of a larger extent of seed quality deterioration. The usefulness of box-whisker plots in portraying the variation in individual electrical conductivity between cultivars was clearly illustrated by the side by side comparison of cultivars within production sites and between sites for both unaged and aged seeds. However, the variability observed in aged seeds could not be associated with seed coat colour, despite the observation that dark-coloured seeds from both sites were generally less variable and showed the lowest median values after AA. This is because aged dark-coloured seeds of Makonge showed the largest variability at both sites even though the variation was larger at Ukulinga than at Umbumbulu. Seeds of Makonge were described as dark; however the seeds showed a large variation in colour and were more variegated than uniformly dark. Three distinct components of seeds based on hue and intensity had been separated using image analysis (data not shown). The results observed in Makonge clearly show the problems that may be encountered in associating seed coat colour and performance in

mixed seed populations which may exhibit a high degree of genetic variation. The population may be described using the dominant colour; however, it is possible that different colour components may represent different genotypes with diverse physiological responses to stress factors that can influence single seed conductivity.

In addition, the electrical conductivity test measures only the leakage of electrolytes; however, other substances that may influence seed performance may possibly also leak from the seeds. As seeds age, the integrity of the cell membrane is destroyed and compartmentation within the cell is disrupted resulting in a mixing of enzymes and substrates (Jacobsen, 1984). This leads to the leaching of sugars, amino acids and electrolytes when the seed is soaked. It is feasible that seeds with low conductivity and poor performance may have leaked more sugars and enzymes essential for the germination process and vigour. Mathews and Bradnock (1968) observed a direct correlation between the quantity of carbohydrate exuded from seeds and seed rot in peas and French beans. Other substances that can influence solute leakage may include compounds such as phenols, tannins and /or lignin present in the seed coat. Resistance to pathogen infection in dark coloured seeds was attributed to phenols, tannins and/or lignin present in the seed coat (Aveling and Powell, 2005).

The general observation which agrees with the work of Asiedu and Powell (1998) however was that in cowpea, aged dark-coloured seeds produced at both sites tended to show lower conductivity, higher germination capacity and germination index than light coloured seeds. Aged dark coloured seeds were also more tolerant of stressful conditions than light coloured seeds as evident from the Aging Stress Differential Index (ASDI) (Figure 4.8). This may imply that seed colour may be linked to physiological responses during stress. Several reports in the literature cite examples where genes controlling seed colour may be linked to other physiological functions in the plant. For example, resistance to damping-off in peas was always associated with the A gene for anthocynin production (Kraft, 1974). Stasz *et al.,* (1980) reported that pea seeds coloured by anthocynins were resistant to soil-borne fungi and seedling diseases, whereas seeds with uncoloured coats were susceptible. Lokaprakash (1981) found that in cowpea, the gene Pda 1, which controls pedicel pigmentation is also involved in the control of seed coat colour and immature pod pigmentation; however, the role of this gene in stress resistance was not discussed. Probably the distinct

colour components of Makonge, which were identified using image analysis, may have responded differently to stress during the AA test. The possibility that genes controlling colour in cowpea are linked to genes that determine responses to stress may need further investigation.

The results of the AA test, with respect to electrical conductivity, germination capacity and germination index values were indicative of the deteriorative changes that may have occurred during seed ageing and reflected changes in seed vigour. However, ranked electrical conductivity values after AA did not consistently reflect differences in seed performance between cultivars and sites and did not correlate well with germination capacity and germination index. Interestingly, light coloured seeds tended to show lower conductivity values before AA than dark coloured seeds; however, this trend was reversed after AA where dark coloured seeds, with the exception of Makonge, showed lower conductivity. Generally lower conductivity values related well with higher germination and vigour in dark coloured seeds. However, lower conductivity values were not always associated with higher germination and vigour, as was evident in the performance of light coloured seeds of the cultivar K80 from Umbumbulu and M66 from Ukulinga. Furthermore there were no significant differences with respect to germination capacity and germination index in light coloured seeds of K80 and dark coloured seeds of Nzovu produced at Ukulinga despite the highly significant differences in electrical conductivity. These results showed the difficulties in attempts to establish cut off points between acceptable and unacceptable levels of seed vigour and suggest that cultivar differences may influence the extent to which conductivity values may reflect poor performance in cowpea. Similar observations have been reported in the literature for example, in field corn (Tao, 1980b) and peas (Bedford, 1974) where cultivar effects on the conductivity test have been shown to occur. More recently attempts have been made to develop prediction models for germination capacity in *Brassica* spp. using measurements on bulk electrical conductivity (Mirdad *et al.*, 2006). These prediction models based on electrical conductivity values after AA may be applicable only to seed lots within a particular cultivar and should be treated with circumspection when interpreting results of AA tested seeds of different cultivars. This is because low vigour seeds of a particular cultivar may have lower conductivity reading than the high vigour seeds of another cultivar, which may influence the prediction models. In

the present work it has been observed that whereas the results of the AA test reflect changes in seed vigour, electrical conductivity values after AA did not consistently reflect differences in seed performance between cultivars and sites and did not correlate well with other aspects of performance. This can have an influence on the results of an AA test, which is traditionally used to discriminate between high and low vigour legume seeds.

In conclusion, this study has demonstrated that image analysis can objectively discriminate seed coat colour variation in cowpea. Dark coloured seeds in general performed better than light coloured seeds; however seed coat colour was not always associated with performance. The skewed distribution patterns in individual electrical conductivity and the presence of extreme values may have implications with respect to the suitability of using standard statistical analyses which compare mean values to evaluate such data. In addition variation in individual electrical conductivity may also be influenced by cultivar differences and the chemical composition of the seed coat; therefore associations between seed coat colour and electrical conductivity as a measure of performance should be treated with caution. The AA test does reflect changes in seed vigour; however ranked electrical conductivity values after AA did not consistently reflect differences in seed performance between cultivars and sites and did not correlate well with other aspects of performance.

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5 RAFFINOSE FAMILY OF OLIGOSACCHARIDES ACCUMULATION IN RELATION TO WATER STRESS AND THE DEVELOPMENT OF SEED QUALITY IN COWPEA

5.1 Introduction

It is generally believed that environmental conditions acting on the mother plant during plant growth and interacting with seed developmental processes may influence the seeds physiological potential (Hilhorst and Toorop, 1997; Tekrony, 2003). It is possible that some of the interactions could cause impairment of physiological processes during seed development. For example, drought stress interacting with seed developmental processes may lead to intermediate levels of desiccation tolerance which may influence seed vigour (Vertucci and Farrant, 1995; Hilhorst and Toorop, 1997). Maximum desiccation tolerance (defined as survival of the seed after the complete removal of water and the maintenance of vigour in the desiccated state) is achieved upon the successful completion of maturation (Vertucci and Farrant, 1995). Maturation, which occurs after histodifferentiation involves: 1) accumulation of storage compounds (normally proteins, carbohydrates and lipids depending on species); 2) development of desiccation tolerance and 3) final loss of water when the seed becomes quiescent. It is likely that drought stress occurring during seed maturation can cause alterations in the quantities and composition of the accumulated storage compounds and lead to incomplete acquisition of desiccation tolerance; this may ultimately influence vigour. Drought and desiccation tolerance are correlated with the accumulation of substantial quantities of compounds such as soluble carbohydrates including: sucrose and the raffinose family of oligosaccharides (RFOs), specific proteins such as the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) (Hoekstra *et al.*, 2001). Reports cited in the literature have shown that a reduction in some of these compounds, specifically the RFOs may result in poor seed quality (Obendorf, 1997). For example, mutant soybean lines developed for high satiric, politic and oleic acids were reported to be of low vigour (Wang *et al.*, 2001). Similarly, mutant *mips* soybean lines with reduced phytate and raffinose were reported to have shown lower germination and vigour (Meis *et al.*, 2003).

The major soluble carbohydrates in legume seeds are sucrose and its galactosides raffinose, stachyose and verbascose (Ma *et al.*, 2005). In cowpea, sucrose and the

RFOs (raffinose, stachyose and verbascose) constitute approximately 8 % of the seed dry mass (defatted dry mass basis) (Kuo *et al.*, 1988). Sucrose content is estimated at about 32% of the total soluble carbohydrates, stachyose is the most predominant and contributes about 60%; raffinose and verbascose are found in lesser quantities about 5% (Kuo *et al.*, 1988). These sugars are observed to accumulate in orthodox seeds when desiccation tolerance is acquired and degraded when the tolerance is lost (Kermode, 1997; Hoekstra *et al.*, 2001; Hoekstra *et al.*, 2002). It is speculated that these sugars and specific proteins protect subcelluar surfaces and confer desiccation tolerance (Crowe *et al.*, 1987; Leopold *et al.*, 1994). The proposed mechanism of protection by these sugars is based on the hydroxyl groups forming hydrogen bonds with the phosphate group of the polar head group in phospholipids and with the carboxyl group of proteins (Crowe *et al.*, 1987), thus preventing phase transitions of lipids and denaturation of proteins. This is the basis of the so called water replacement hypothesis (Vertucci and Farrant, 1995) whereby sugars replace water molecules during drying, thus maintaining the structural and functional integrity of membranes, proteins and other macromolecules. A second hypothesis proposes that these oligosaccharides may promote the formation of a vitreous (glassy) state that protects macromolecular structures during desiccation (Leopold *et al.*, 1994; Buitink and LePrince, 2004).

During seed development, it is possible that drought stress may limit the production of assimilates required to synthesize the RFOs. This may have implications for the acquisition of maximum desiccation tolerance and ultimately influence germination and vigour since these compounds are also a source of energy that subsequently drives the germination process. Sucrose, which acts as a galactosyl acceptor in RFO biosynthesis, is the major carbohydrate imported in to the developing seed. The concentration of sucrose and myoinositol, which act as initial substrates in RFO synthesis and feed back loops can affect the final content of RFOs deposited in mature seeds (Peterbauer and Richter, 2001). During photosynthesis, the first step in sucrose synthesis is the formation of sucrose phosphate from UDP-glucose and fructose-6-phosphate. The reaction is catalysed by sucrose phosphate synthase. This is followed by hydrolysis with a specific sucrose phosphatase (Matheson, 1984). Temperature and water stress during seed development may alter metabolic pathways during photosynthesis that can result in alterations in the quantities and composition of the

accumulated food reserves. Lahuta *et al.,* (2000) reported that the composition of accumulated food reserves in field bean (*Vicia faba*) was altered by drought; seeds produced under drought conditions had less dry matter, contained less starch but more oligosaccharides, sucrose and reducing monosaccharides. In addition, alterations in metabolic processes may also lead to the synthesis of protective compounds such as polyols (Bohnert *et al.*, 1995). Polyols have been generally proposed to act as compatible solutes or osmoprotectants to allow osmotic adjustment of plant cells exposed to water deficit (Bray, 1993).

Polyols including straight chain metabolites such as mannitol and sorbitol (Bieleski, 1982) or cyclic polyols such as myoinositol (Loewus and Loewus, 1983) are known to accumulate in response to drought stress. Myoinositol is formed after cyclisation of glucose-6-phosphate catalysed by myoinositol-1-phosphatase. Myoinositol is also implicated in cell membrane biosynthesis and signal transduction mechanisms (Bohnert *et al.*, 1995). Significant changes observed in relative ratios of raffinose, stachyose and verbascose induced by water stress in field bean (Lahuta *et al*., 2000) and cucumber (Widders and Kwantes, 1995) could probably be linked to the accumulation of myoinositol. In mature seeds of pea lines with different RFO composition, the synthesis of galactinol in the line RRRbRb, with higher RFO content is probably supported by a higher myoinositol concentration during the initial phase of galactinol accumulation (Peterbauer *et al.*, 2001). Similarly, levels of galactinol and raffinose were reported to be much lower in tubers of transgenic potato (*Solanum tuberosum*) with a reduced content of myoinositol (Keller *et al.*, 1998).

The genetic regulation of myoinositol in relation to biochemical pathways during water stress has also been investigated. In *Xerophyta viscosa* transcript levels for XvGoIS, a gene encoding a galactinol synthase enzyme responsible for the first catalytic step in RFO biosynthesis, increased in leaf tissues exposed to water deficits (Peter *et al.*, 2007); however, galactinol synthase activity *in planta* could not be correlated with RFO accumulation, but a negative correlation was observed between RFO accumulation and myoinositol depletion, during water deficit stress. This correlation was reversed after rehydration, suggesting that during water deficit myoinositol is channelled into RFO synthesis, but during the rehydration process it is channelled to metabolic pathways related to the repair of desiccation-induced damage

(Peter *et al.,* 2007). No studies cited in the literature have examined the accumulation of myoinositol in developing seeds of cowpea produced under water tress in relation to RFO concentration and desiccation stress.

The effect of drought stress on soluble carbohydrate concentrations, including RFOs, and how this may relate to seed vigour has not been clearly explained in the literature. Siddique and Wright (2004) examined differences in environmental conditions experienced during development and maturation , the length of seed filling period, concentrations of fat, soluble carbohydrates , proteins and starch as potential causes of differences in seed vigour in peas (*Pisum sativum*) and flax and concluded that none of these variables could alone explain the observed variations in seed vigour. Black *et al.,* (1999) reported that wheat (*Triticum aestivum* L.) embryos that were induced into water stress tolerance by a 24-h water loss had no detectable raffinose; in addition the oligosaccharide was observed to accumulate at later times even in detached grains that had not become desiccation tolerant. The authors concluded that desiccation tolerance and the occurrence of raffinose are not correlated but did not relate this to seed vigour. Similarly, Sinniah *et al.,* (1998) observed that in *Brassica spp.* seeds, there were no significant differences in the final concentration of RFOs in plants that were subjected to water stress and the controls. In cucumber (*Cucumis sativus*), lupin and soybean seeds, maturation temperature had little effect on the concentration of RFOs (Obendorf *et al.,* 1998). In contrast, Thomas *et al.* (2003) observed that total non-structural carbohydrates (TNC) in soybean decreased as temperature increased, and the proportion of soluble sugars to starch decreased. Similar observations were made by Pattanagul and Madore (1999) who reported that in coleus (*Coleus blumei* Benth.), both RFO levels and galactinol synthase activity were depressed by drought stress. These authors suggested that for drought stress alone, RFOs may not play a direct role in inducing tolerance in vegetative tissues; however they (RFOs) may be important in allowing plant tissues to achieve the level of dessication required in a dry seed (Koster and Leopold, 1988). Although Lahuta *et al*., (2000) related changes in relative ratios of RFOs under conditions of water stress to a build up in desiccation tolerance in field beans, these authors did not extend their study to examine how this would relate to seed vigour.

An interesting suggestion has been made that the developing embryo or accumulated food reserves can be modified by interaction between the environment and the mother plant, so that effects carry over through a number of succeeding generations (Salisbury and Ross, 1991; Wulf, 1995). The observation by Highkin and Lang (1966) that each generation (up to about the fifth) of peas grown for several generations under adverse temperature conditions, grew more poorly than the previous one suggested that environmental effects on the mother plant influenced the vigour of the subsequent generations. However, similar studies on such maternal effects are few; in addition such studies may require multiple generations, separating environmental and genetic effects (Wulf, 1995). The mechanisms by which these effects are induced, maintained and transmitted sometimes along several generations are also not well understood.

Soluble carbohydrates have also been associated with seed germination and storability in relation to desiccation tolerance. In maize (*Zea mays*) seeds, examination of changes in sugars during accelerated aging indicated that the decline in germination and vigour is associated with a marked decline in monosaccharides and in raffinose (Bernal-Lugo and Leopold, 1992). These authors speculated that the decline of raffinose in germinating maize seeds would be expected to lead to a loss of desiccation tolerance. Loss of desiccation tolerance was observed to coincide with an increase in reducing monosaccharide content of embryonic axes in pea (*Pisum sativum*) and soybean (*Glycine max*) (Koster and Leopold, 1988). This has led to suggestion that the association between desiccation tolerance and seed viability and possibly vigour could be as a result of the accumulation of reducing sugars in a drying seed leading to the occurrence of the Maillard reaction, which may cause protein and nucleic acid damage; this may influence the viability and possibly vigour of the seed. Moreover, the suggestion that oligosaccharides are important in desiccation tolerance not because they serve as cellular protectants, *per se* but because they reduce the pool of monosaccharides (Koster and Leopold, 1988) is further supported by the observations made by Zhu and Chen, (2007) who reported that the ratio of non-reducing sugars/reducing sugars and the resistance to aging of moderately ultra-dried peanut seed was higher than those of control (non-ultra dried seeds) after accelerated aging. It can also be argued that the correlation between seed storability (an aspect of vigour) with total RFO content and the ratio of RFO to sucrose reported by

Horbowicz and Obendorf (1994) is perhaps related to the absence of reducing sugars and their possible role in Maillard reactions.

On the contrary, different views have been proposed that RFO are not involved directly or are at least not the only factors necessary for desiccation tolerance and storability. Still *et al.,* (1994) observed that sucrose and RFOs accumulated in desiccation-intolerant wild rice (*Zizania palustris* var *interior*) axes to about twice that found in mature rice (*Oryza sativa* L.) embryos which are desiccation tolerant and concluded that desiccation sensitivity in wild rice grains is not due to an inability to synthesize soluble carbohydrates. Similarly Bochicchio *et al.,* (1997) reported that desiccation tolerance can occur in the absence of the oligosaccharide raffinose in maize. Buitink *et al.,* (2000) used a spin probe technique to examine the suggested role of oligosaccharides in seed longevity via increased glass stability. The authors used the technique to measure the molecular mobility and transition temperature of the cytoplasm of impatiens (*Impatiens walleriana*) and bell pepper (*Capsicum annuum*) seeds that were osmo-primed to change oligosaccharide content and longevity; although osmo-priming of the seeds resulted in considerable decrease in longevity and oligosaccharide content, there were no differences in the glass transition temperature between the control and primed seeds at the same temperature and water content. Similarly, no differences were observed in the rotational motion of the spin probe in the cytoplasm between control and primed seeds. These authors concluded that there might be no specific role for oligosaccharides in seed longevity. There seems to be a lack of conclusive evidence in the literature on the role of RFOs in desiccation tolerance and seed vigour.

In chapter 3, an attempt was made to determine cowpea adaptation to simulated drought conditions. The RFOs accumulate during seed development and have been implicated in the acquisition of desiccation tolerance. However, despite the widespread phenomenon of desiccation tolerance in orthodox species and its importance to seed quality development, the mechanism(s) that lead to desiccation tolerance are poorly understood. In addition, there is no conclusive evidence in the literature on the role of RFOs in desiccation tolerance and specifically in relation to drought tolerant crops such as cowpea, and no studies have shown their occurrence in developing cowpea seeds under conditions of water stress. The objective of this study

was to determine the patterns of RFO accumulation in relation to water stress and the development of seed quality in cowpea. Attempts were made to investigate whether differences between the environmental conditions in the initial production environment could be associated with the pattern of RFO accumulation during subsequent growth and development under conditions of water stress by comparing unpaired samples of individual sugars using a t-test.

5.2 Materials and methods

5.2.1 Seed materials

5.2.1.1 Seeds at different developmental stages

Seeds of cowpea (cultivar Nzovu) were initially donated by Environmental Action Team (EAT), Kitale, Kenya. Cultivar M66 was originally purchased from the Basic Seed Unit of the Kenya Agricultural Research Institute (KARI), Machakos, Kenya and Kenkunde from the Kenya Seed Company, Kitale, Kenya. The seeds were initially planted at two different sites in South Africa (Ukulinga and Umbumbulu) under different production conditions in an earlier experiment and to produce own fresh seed with known quality characteristics (chapter 2). Mature seeds harvested from these two sites were planted in a pot experiment under simulated drought conditions (chapter 3).

Seeds were planted under simulated drought conditions in a tunnel at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The tunnel was enclosed in clear polythene sheeting. Temperature, solar radiation (PAR) and relative humidity (RH) were monitored electronically using HOBO 2K Loggers (Onset Computer Corporation, Bourne, USA). Soil from the Ukulinga production site, whose physical characteristics had been determined, was used in the pot experiment. The soil was a clay-loam with a bulk density of 1562 kg m^{-3}. The average field capacity (FC) was 27% by volume and the soil contained 19% water content (% vol) at a matric potential of -1500KPa corresponding to the permanent wilting point (PWP) as determined from soil water retentivity curves developed using a ceramic pressure plate.

Drought conditions were simulated based on two contrasting moisture environments typical of semi-arid tropics where cowpea is grown, and were defined as follows:

Intermittent stress, characteristic of the wet season in the monsoonal semi-arid tropics, when stress can occur at any time and with varying intensities between emergence and maturity; Terminal stress, where crops are grown relying only on stored moisture and where crops grow and mature progressively on depleted soil moisture profile (Ludlow and Muchow, 1990). Pots containing 20 kg air dry soil with a soil moisture content of ~ 3% were then placed individually on a weighing balance and a precise quantity of water was added until the required mass equivalent to 17.47% by mass the gravimetric water content at FC (no stress), corresponding to a matric potential of -33kPa was attained. Drought conditions were imposed by placing individual pots on a weighing balance and adding a precise quantity of water until the required mass equivalent to 12.26 % gravimetric water content at permanent wilting point (PWP), corresponding to a matric potential of -1500kPa was attained. Plant available water capacity (PAWC) from the -33 and -1500 kPa water contents was determined gravimetrically (mass) by calculating the difference between the % gravimetric water content at -33 matric potential and -1500 kPa. An additional quantity equivalent to 30% PAWC was calculated and added to each pot to attain the required soil mass at 30% FC. The pots were individually emptied into a concrete mixer and mixed thoroughly so as to achieve a uniform distribution of soil and water inside the pot and the weights were recorded and written on the pots. Intermittent stress was imposed at 30% FC during the vegetative stage of growth only, whereas terminal stress was imposed at 30% FC throughout development from emergence to maturity.

Five seeds were planted in each pot by placing four equidistantly and one at the centre. The seeds were planted at a depth of 10mm. A compound fertilizer 2:3:2 :(22) was applied during planting at the rate of 600 kg ha^{-1}. Soil water content in the pots was monitored gravimetrically by weighing individual pots periodically, and continuously using ThetaProbe soil water sensors (Lukangu *et al.,* 1999).

Pods were tagged at R4 stage of plant development (Fageria, 1992), and harvested during three developmental stages fortnightly until harvest maturity. The stages of reproductive growth at which seeds were harvested corresponded to R4, R6 and R8 (Fageria, 1992) (Figure 5.1). Seeds were removed from pods to determine dry mass and water content (% fresh mass). Dry mass was determined by lyophilizing surgically excised tissues (cotyledons, axes and seed coats) for 24h after freezing in

liquid N. Dry tissues were then stored at -20°C until determination of soluble carbohydrates.

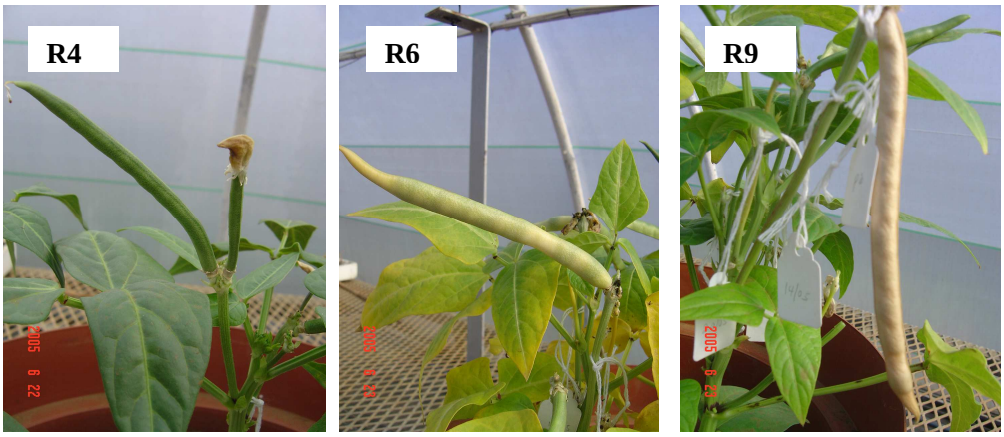

Figure 5-1 Stages of reproductive growth at which seeds were harvested corresponded to R4, R6 and R9 (Fageria, 1992).

5.2.1.2 Mature seeds harvested from water stress treatments

Seeds were harvested from the cultivar Nzovu only, because stressed plants of the other two cultivars (Kenkunde and M66) did not produce sufficient seeds for further analysis. Mature seeds from the cultivar Nzovu from the no stress and intermittent stress and terminal stress treatments were germinated in plastic Petri dishes (100 x 15mm) lined with three layers of Whatman No.1 filter paper moistened with dd H_2O. Twelve seeds were placed in each Petri dish and incubated in a controlled environment chamber at 25°C in darkness. Germination was scored at regular intervals: 8, 16 and 24h. Seeds were considered to have germinated when the emerged radicle had reached at least 2mm length. The germinated seeds were removed from the Petri dishes at each interval and surgically excised to separate the embryo, cotyledons and seed coat. The cotyledons were lyophilized after freezing in liquid N. Dry cotyledons were then stored at -20°C until determination of soluble carbohydrates.

5.2.2 Soluble carbohydrate extraction and analysis

5.2.2.1 Ethanol extraction

Two hundred- milligram samples of dry cotyledons were ground using a pestle and mortar and placed in 50 ml polyethylene centrifuge tubes and mixed with 5 ml of 80% (v/v) ethanol. Mixtures were agitated and placed in a shaker at room temperature for 1h. Soluble sugars were extracted using the procedure described by Black *et al.*, (1996) with some modifications. The agitated mixture was placed in an oven and heated for 15 minutes at 80^{o}C, and then centrifuged for 15 minutes at 11500 rpm at 25^{o}C in a Sorvall® RC 5C Plus Super Speed Centrifuge manufactured by Kendro Laboratory Products, Newtown, Connecticut, U.S.A and the supernatant transferred to a 20ml scintillation vial. The wet tissue was resuspended in 5 ml 80% ethanol and the extraction process repeated one more time. The supernatants were pooled and taken to dryness under vacuum using a Savant Speed Vacuum SC 200. Solids in the scintillation vials were dissolved in 2 ml of chloroform plus 4 ml double deionised water and stored at 4^{o}C for at least 48 h to allow settling of lipid and chlorophyll associated material (Streeter and Strimbu, 1998). The clear aqueous layer was then sampled for carbohydrate analysis; 200µl aliquots were placed in 2 ml reaction vials and taken to dryness under a stream of nitrogen gas in a heating block at 45^{o}C. Replicates consisted of replicate weighed sub-samples taken from the same large sample of dried cotyledons.

5.2.2.2 Derivatization and analysis of carbohydrates

Derivatization and analysis of carbohydrates was done using the procedure described by Streeter and Strimbu (1998) with some modifications. Solids were dissolved in 125µl pure pyridine and 125µl pyridine containing 5mg of an internal standard, phenyl β-D glucopyranoside. Two hundred-µl hexamethyldislazane (HMDS) and 20µl trifluoroacetic acid (TFA) were added, mixed and allowed to react for 60 minutes before analysis. Trimethyl silyl derivates of sugars were then separated and determined by gas chromatography using the conditions shown in Table 1.

Table 5-1 GC conditions for separation and determination of carbohydrates

Instrument			
GC	Varian CP-3800 (Varian Inc, Middelburg, Netherlands)		
Auto sampler	Varian CP-8400		
Column	DB-5MS (Agilent J&W, Agilent technologies)		
Dimensions	30 m x 0.25 mm 0.25 µm film	**Carrier Gas**	Nitrogen
		Flow rate	1.0 ml/min
Injector	Model 1177 split/splitless	**Column Oven**	
Temperature	250^{o}C	Initial temp	100^{o}C
Injection volume	1 µl	Initial hold	1 min
Injection mode	Split	Ramp rate	5^{o}C/min
Split ratio	20	Hold temp	220^{o}C
		Hold time	25 min
Detector	FID	Ramp rate	10^{o}C/min
Temperature	330^{o}C	Final temp	350^{o}C
Range	12	Final hold	10 min

Carbohydrates were identified by retention time; the retention times for the different carbohydrates were determined using derivatised samples of authentic standards.

5.2.2.3 Quantitation

For quantitation of the carbohydrates, an internal standard (β-phenylglucose) was used .The internal standard was added to all samples and standards. Standard solutions were prepared from fructose, pinitol, galactose, glucose, myoinositol, sucrose, raffinose, stachyose and verbascose (Sigma Chemical Company, St Louis, MO USA). Calibration curves were prepared using peak area ratios (analyte/internal standard) and were linear over the concentration range 0.16 – 2.5 mg ml^{-1}. The mean of three replicates at each of five concentration levels was used to construct the calibration curve.

Method validation was performed by spiking dried ground samples with an appropriate quantity of each of the analyte sugars. The spiked samples were then carried through the analytical procedure and the recovery of each sugar was determined.

5.3 Results

Figure 5.2 shows the mean soluble carbohydrate content in cowpea seeds from three cultivars (Nzovu, Kenkunde and M66) during development under well-watered conditions. The disaccharide sucrose and the oligosaccharide stachyose were relatively abundant in cowpea. The other members of the raffinose family of oligosaccharides, raffinose and verbascose were also detected although in lower quantities. In addition, relatively smaller quantities of other compounds, for example myoinositol, pinitol and reducing sugars such as glucose and fructose and galactose were also observed to be present in cowpea seeds.

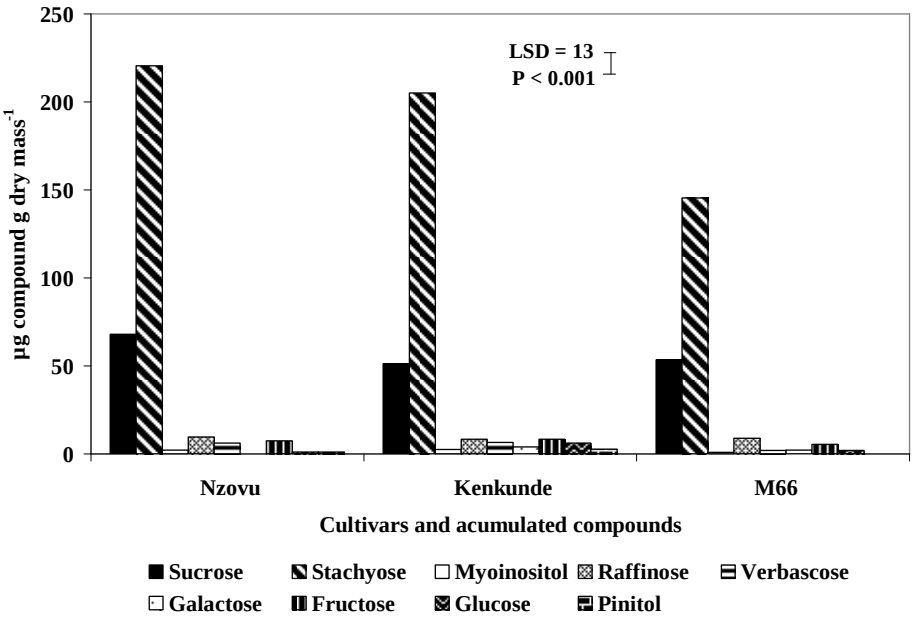

Figure 5-2 Mean soluble carbohydrates content in cowpea seeds from three cultivars (Nzovu, Kenkunde and M66) during development under well watered conditions.

Significant cultivar differences with respect to stachyose content (µg g dry mass^{-1}) were observed between cultivars (Figure 5.2). The cultivar Nzovu showed the highest

stachyose concentration, followed by Kenkunde. M66 showed the lowest stachyose content. Sucrose was present in more or less in equal amounts in the three cultivars.

The seeds that were used in the present experiment had been initially produced from two distinct sites (Ukulinga and Umbumbulu) characterised by different environmental conditions (Chapter 2 and 3). The results obtained from the two sites were analysed separately and could not be pooled because the variances were unequal. Similarly during development stachyose was the most predominant oligosaccharide observed from both sites (Figure 5.3 A & B). Stachyose content (µg g dry mass^{-1}) in seeds from both sites generally increased from development stage 2 to 6 weeks. The other oligosaccharides raffinose and verbascose followed a similar pattern of increase although verbascose accumulation appeared to be later and was only detected after 4 weeks of development which declined slightly after 6 weeks Figure (5.3 A & B). The sugar alcohol myoinositol, and the reducing sugars galactose , fructose and glucose showed the highest concentration at 2 weeks of development which declined after 4 weeks and could hardly be detected after 6 weeks (Figure 5.3 A and B). However, seeds which had been initially produced from Ukulinga showed relatively higher quantities of these compounds (myoinositol, fructose, galactose and glucose) at developmental stage 2 than those from Umbumbulu. The pattern of sucrose accumulation differed considerably between sites. Sucrose content was very high at Ukulinga at developmental stage 2 (266µg g dry mass^{-1}). This showed a rapid decline after 4 weeks and 6 weeks of development to approximately 70 µg g dry mass^{-1}. However, this was in sharp contrast to the results obtained from seeds which were initially produced from Umbumbulu. Sucrose accumulation ranged between 50-80µg g dry mass^{-1} throughout development.

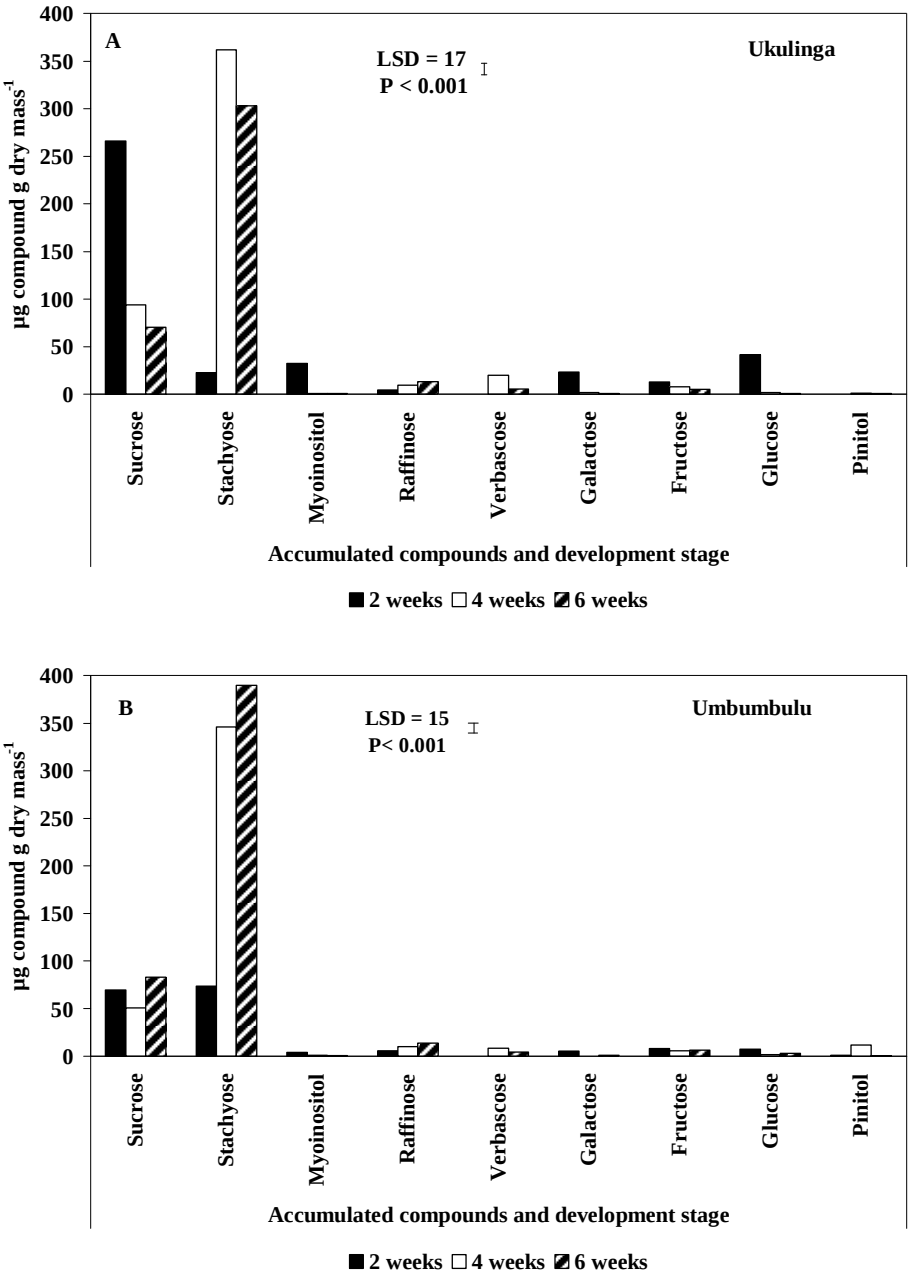

Figure 5-3 Pattern of accumulation of soluble carbohydrates during three developmental stages (2, 4 and 6 weeks) in cowpea. Seeds which initially originated from two different production sites (A-Ukulinga and B-Umbumbulu) are compared.

Significant developmental Stage x Water stress interactions were observed with respect to stachyose accumulation in seeds initially produced from both sites (Figure 5.4). Stachyose content (µg g dry mass^{-1}) increased during development under both

stress and well watered conditions. However, both intermittent stress and terminal stress significantly increased the rate of stachyose accumulation in seeds initially produced at Ukulinga ($P < 0.05$) and Umbumbulu ($P < 0.001$). Stachyose content (µg g dry mass^{-1}) during developmental stage 6 (Harvest maturity) was significantly higher in seeds harvested from plants that had been subjected to intermittent and terminal stress than those from well watered plants.

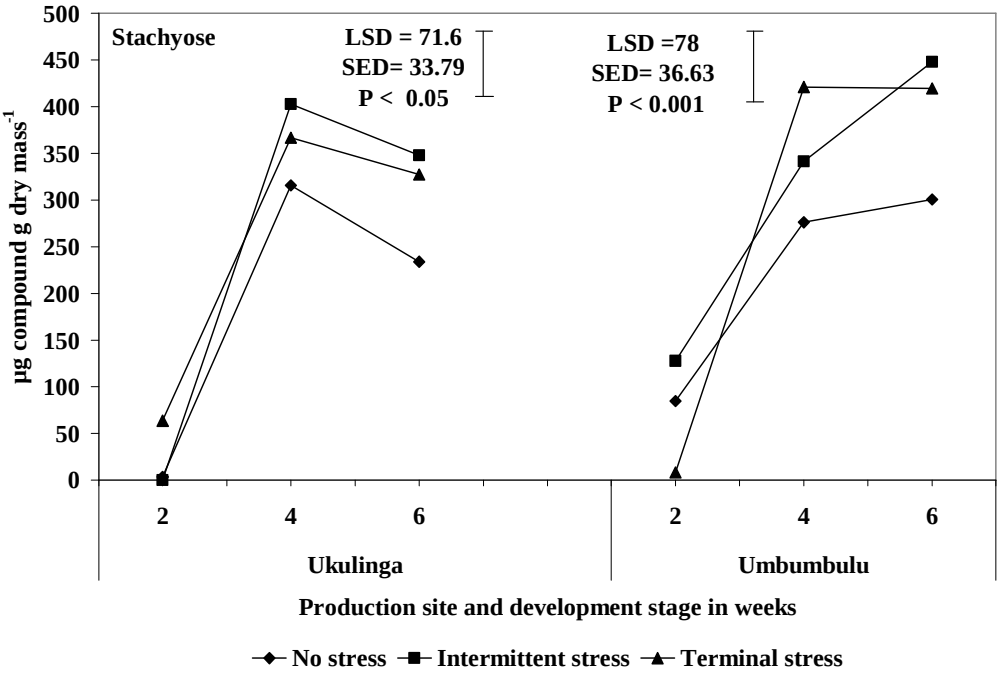

Figure 5-4 The pattern of stachyose accumulation in cowpea seeds during development under water stress conditions. Seeds that were used in the water stress experiment had been initially planted at two different production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions.

Similar trends in the pattern of raffinose accumulation were also observed. The interaction between developmental stage and water stress was similarly highly significant ($P < 0.001$) in seeds initially produced from both sites (Figure 5.5). Raffinose accumulation increased during development. Likewise the rate of accumulation was significantly higher in seeds from plants that were subjected to intermittent and terminal stress than those from well watered conditions. Raffinose

content was also significantly higher at HM in seeds from plants subjected to both intermittent and terminal stress than well watered conditions.

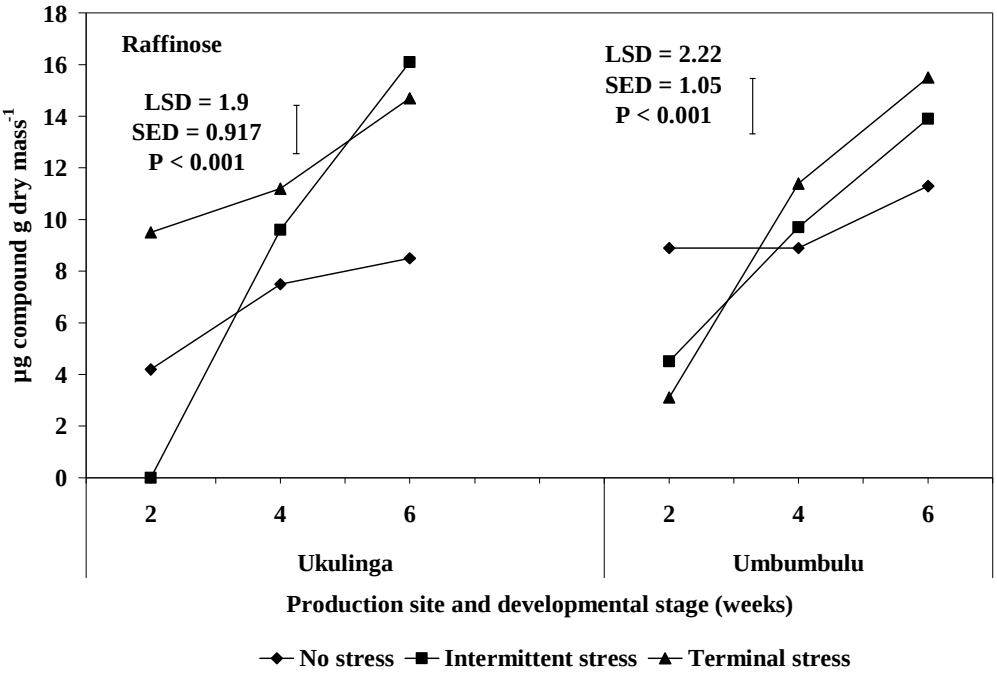

Figure 5-5 The pattern of raffinose accumulation in cowpea seeds during development under water stress conditions. Seeds that were used in the water stress experiment had been initially planted at two different production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions.

The oligosaccharide verbascose was not detected at developmental stage 2 (Figure 5.6). However verbascose appeared at 4 weeks of development in seeds initially produced from both sites. Differences in the pattern of verbascose accumulation under conditions of water stress were not clearly discernible from the data. A decline in verbascose content was observed at developmental stage 6 from both production sites.

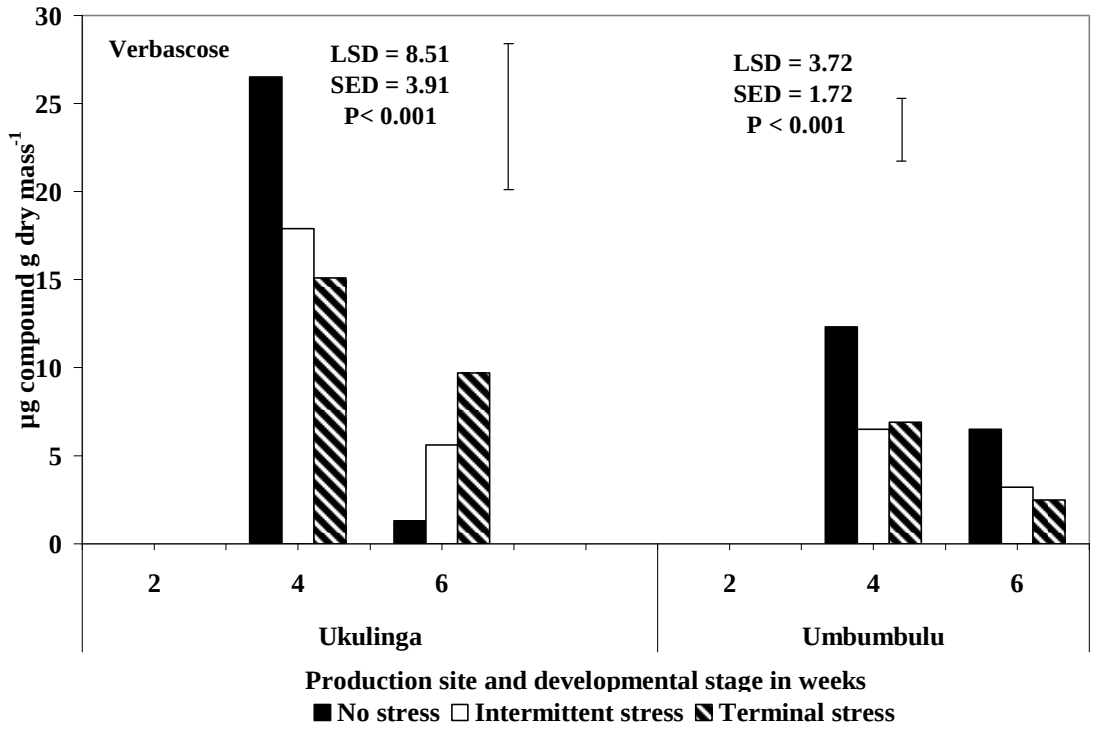

Figure 5-6 The pattern of verbascose accumulation in cowpea seeds during development under water stress conditions. Seeds that were used in the water stress experiment had been initially planted at two different production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions.

Sucrose content generally declined from developmental stage 2 to 6. Seeds from well watered plants from the Ukulinga site had considerably high sucrose content (420µg g dry mass^{-1}) at 2 weeks which differed significantly from seeds from intermittently and terminally stressed plants. Sucrose content during development in seeds from the well watered plants and intermittently stressed plants from Ukulinga decreased rapidly at 4 weeks to ~55µg g dry mass^{-1} and was significantly lower than that of seeds from terminally stressed plants (150µg g dry mass^{-1}). However, there were no significant differences in sucrose content at developmental stage 6 (HM).

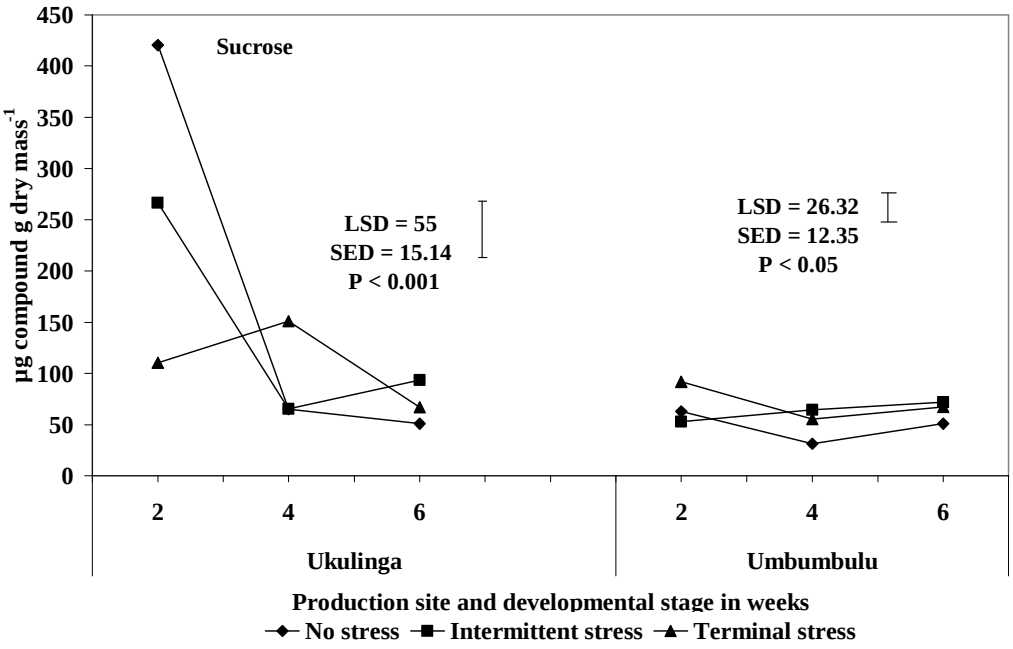

Figure 5-7 Change in sucrose content in cowpea seeds during development under water stress conditions. Seeds that were used in the water stress experiment had been initially planted at two different production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions.

Seeds that had been initially produced at Umbumbulu and subsequently used in the water stress experiment showed much lower sucrose content at 2 weeks of development than those initially from Ukulinga. However, seeds from terminally stressed plants from Umbumbulu showed a relatively higher sucrose content at 2 weeks than those from intermittently stressed and well watered plants; though there were no significant differences in sucrose content between the treatments at 4 and 6 weeks of development.

Myoinositol content was maximum at developmental stage 2 in seeds produced initially from both sites (Ukulinga and Umbumbulu) (Figure 5.8), and showed a sharp decrease at developmental stage 4. This trend in the decrease in myoinositol content progressed up to stage 6 and was not significantly different between seeds from well-watered plants and stressed plants from both sites. However, highly significant differences were observed in myoinositol content in seeds produced initially from

Ukulinga and Umbumbulu at developmental stage 2. Seeds harvested from well-watered plants whose origin was initially from Ukulinga showed a considerably higher myoinositol content at 2 weeks (71.1µg g dry mass^{-1}) than those originating from Umbumbulu (4.8µg g dry mass^{-1}). In addition these seeds from well-watered plants at Ukulinga had the highest myoinositol content followed by seeds from intermittently stressed plants. Terminally stressed plants from Ukulinga showed the lowest myoinositol content. In contrast in seeds originating from Umbumbulu seeds from terminally stressed plants showed significantly the highest myoinositol content at developmental stage 2 although the values were considerably lower than those from Ukulinga. This was followed by seeds from well watered plants. Seeds from intermittently stressed plants which originated from Umbumbulu showed the lowest myoinositol content.

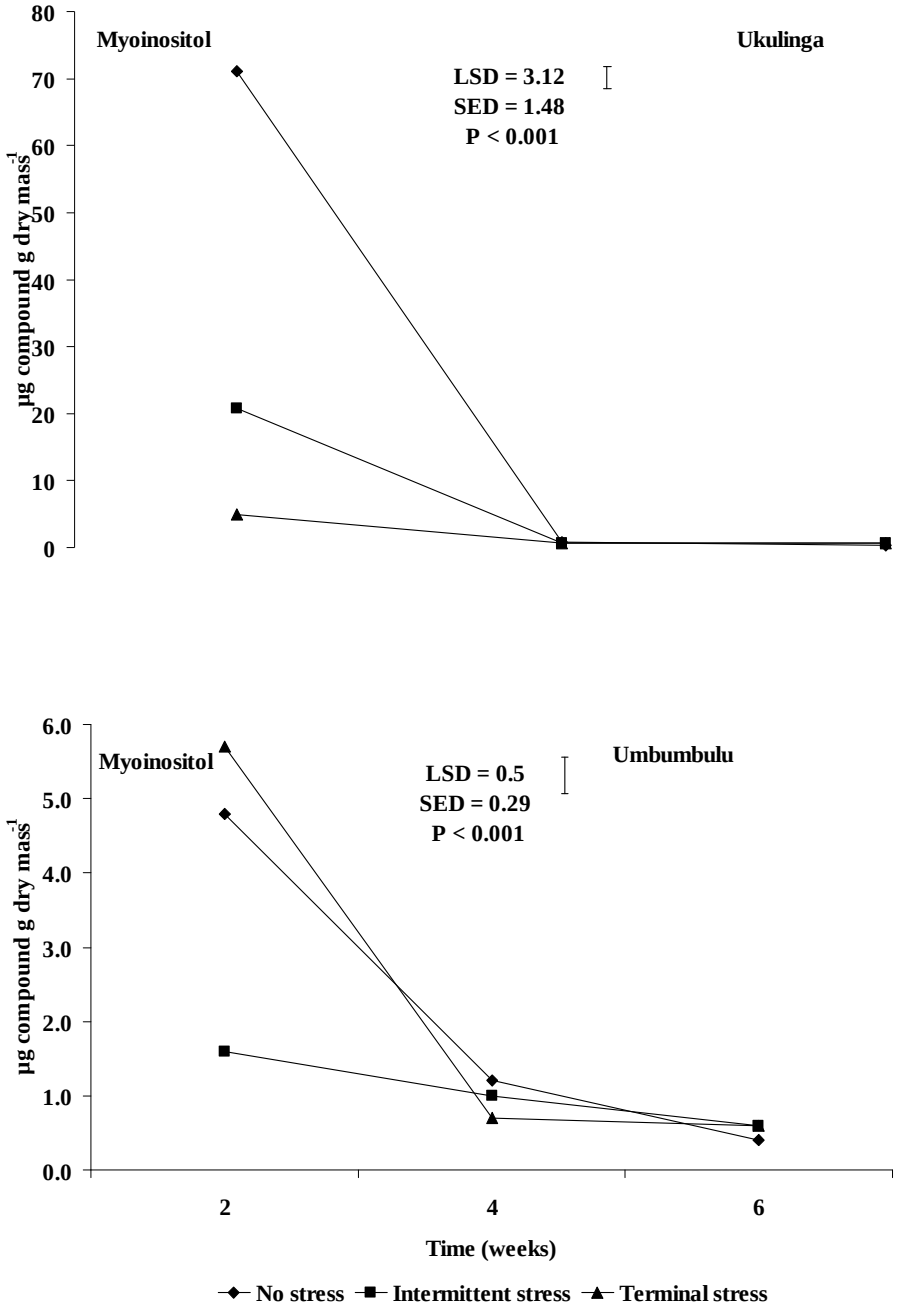

Figure 5-8 Change in myoinositol content in cowpea seeds during development under water stress conditions. Seeds that were used in the water stress experiment had been initially planted at two different production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions.

Cowpea seeds had been initially planted at two production sites (Ukulinga and Umbumbulu) characterised by different environmental conditions. Seeds from these two sites were harvested and subsequently planted under simulated drought conditions in a tunnel experiment. A two sample (unpaired) T-test was used to compare whether differences in

environmental conditions at the two initial production sites influenced the accumulation of soluble carbohydrates in harvested cowpea seeds subsequently planted under water stress conditions. The tests assumed that the variances were unequal and compared the two sites for each compound separately. There were no significant differences ($P > 0.05$) in the accumulation of stachyose, raffinose and verbascose between seeds that had been initially produced at both sites (Ukulinga and Umbumbulu) (Table 5-2). However, significant differences ($P < 0.01$) between the two sites were observed with respect to in sucrose accumulation. Seeds produced from Ukulinga had higher mean sucrose content (143.4µg g dry mass^{-1}) than those from Umbumbulu (62.3 4µg g dry mass^{-1}). Similarly myoinositol accumulation in seeds initially produced from Ukulinga was significantly different ($P < 0.05$) from those initially produced from Umbumbulu (11.2 and 1.74µg g dry mass^{-1}).

Table 5-2 The use of two sample (unpaired) t-test to compare whether differences in the initial seed production site (Ukulinga and Umbumbulu) influenced soluble carbohydrate accumulation in cowpea seeds subjected to water stress during development. Seeds had been initially planted at the two sites with contrasting environmental conditions and then harvested and subsequently planted under simulated drought conditions.

Sample	Stachyose		Sucrose		Myoinositol		Raffinose		Verbascose	
	Ukulinga	Umbumbulu	Ukulinga	Umbumbulu	Ukulinga	Umbumbulu	Ukulinga	Umbumbulu	Ukulinga	Umbumbulu
Sample Size	25	24	27	23	27	26	27	26	21	21
Mean	221.3	299.2	143.4	62.3	11.2	1.7	9.1	9.7	8.5	4.3
Variance	26668	19844	14823	439	508.3	3.5	23.38	16.46	111.93	22.67
Standard Deviation	163.3	140.9	121.75	20.96	22.55	1.86	4.835	4.058	10.58	4.762
Standard Error of Mean	32.66	28.75	23.43	4.37	4.339	0.365	0.9305	0.7958	2.309	1.039
Difference of the means		-77.92		81.04		9.45		-0.671		4.199
Standard Error Differences		43.65		23.84		4.354		1.228		2.532
Test statistic t		-1.79		3.4		2.17		-0.55		1.66
*Probability		0.081		0.002		0.039		0.588		0.108

* Test of the null hypothesis that mean of Ukulinga is equal to mean of Umbumbulu

5.4 Discussion

The results obtained in the present study showed that stachyose is the predominant soluble carbohydrate found in cowpea and concur with the work of Kuo *et al.,* (1988). The presence of sucrose in relatively higher quantities is also consistent with the observations made by other workers (Kuo *et al.*, 1988; Horbowicz and Obendorf, 1994; Ellis *et al.*, 2000; Lahuta *et al.*, 2000) and supports the suggestion that both these compounds (sucrose and stachyose) may have a physiological role in seeds. The significant difference in stachyose content observed between the three cultivars is probably due to genetic differences. Interestingly, the cultivar Nzovu which showed the highest stachyose content matured earliest and was able to produce sufficient seed under water stress.

The seeds that were used in this experiment had been initially produced at two distinct sites (Ukulinga and Umbumbulu) characterised by different environmental conditions (see Chapters 2 and 3). Preliminary data analysis revealed that the variances between the two sites were unequal; this could not allow a pooled analysis. The differences in variances between the two sites may probably be accounted for by the observation that sucrose and myoinositol content in seeds from Ukulinga was considerably greater than those from Umbumbulu. The reasons for these large differences are not clear; nonetheless the results obtained from the two sites were generally consistent and clearly showed the pattern of RFO accumulation in developing cowpea seeds.

The relatively low level of raffinose accumulation during the three stages of development was consistent with the biochemical processes implicated in RFO synthesis. Raffinose provides the substrate for the subsequent synthesis of stachyose and verbascose (Kandler and Hopf, 1982; Peterbauer and Richter, 2001). Presumably, cowpea seed chemical composition and the predominance of stachyose as the major soluble carbohydrate is determined by genetic and environmental variables. The role of genetic factors in the determination of seed chemical composition has also been demonstrated in pea lines with contrasting RFO content; differences in RFO accumulation were associated with the expression of specific genes (Peterbauer *et al.*, 2001). Genetic regulation of RFO biosynthesis may explain why verbascose appeared

at later stages of development and could not be detected at two weeks of development.

Under drought stress (both terminal and intermittent stress), stachyose and raffinose accumulation was faster during maturation of the cowpea seeds on the maternal plants and their concentration at 6 weeks was higher than controls (no stress water stress and agrees with the work of Lahuta *et al.,* (2000). In contrast, Sinniah *et al.,* (1998) observed). This implies that the synthesis of these compounds was intensified under that in *Brassica spp.* seeds, water stress considerably affected RFO accumulation within the developing and maturing seeds; however, there were no significant differences in the final concentration of RFOs in plants that were subjected to water stress and the controls. Probably in the work by Sinniah *et al.,* (1998) RFOs accumulated faster in the seeds from stressed plants due to accelerated ontogeny, but this did not have an effect on final RFO concentration. However, in the report by Lahuta *et al.,* (2000) metabolic processes were probably altered by drought stress. These alterations were probably mediated by changes in gene expression triggered by drought stress. Similar thoughts have been expressed by Thomas et *al.,* (2003) who reported that in soybean, transcripts of a gene that is down-regulated by auxin (ADR12) were dramatically down-regulated by elevated temperature, possibly reflecting the altered course of seed development under environmental stress. Both groups (Sinniah *et al.,* 1998; Lahuta *et al.,* 2000), however, concurred that RFOs may be implicated in the acquisition of desiccation tolerance and potential longevity in Brassica. In the present study, it is possible that specific genes may have been down regulated or up regulated by drought stress thus altering the course of seed development hence the higher accumulation of stachyose and raffinose that was observed. The effect of the increased RFO content on a build up to desiccation stress tolerance as suggested by Lahuta *et al.,* (2000) may have occurred but is at most speculative. Sinniah *et al.,* (1998) and Ellis *et al.,* (2000) in Brassica reported a positive correlation between RFO accumulation and potential longevity which is can be taken as evidence that desiccation tolerance and seed vigour are correlated. However, similar evidence to support this suggestion was difficult to find in the present study because no significant differences were observed in seed quality (percent germination and vigour) between seeds harvested from well watered (also high quality) and from stressed plants. A build up in desiccation stress tolerance may

have occurred in seeds from stressed plants; however, this is probably a mechanism to enable the plant cells to survive the combined effect of water loss during maturation and drought stress. Apparently the effect that this build up in desiccation tolerance may have on the germination and vigour of seeds in a drought tolerant species such as cowpea is at most minimal.

It can be argued that the reason why seeds from plants that were subjected to drought stress, which accumulated more soluble sugars but did not differ with respect to germination and vigour from seeds harvested from non-stressed plants, is because the latter had attained a minimum level of desiccation tolerance required for optimal vigour. Vertucci and Farrant (1995) noted that the essence of true desiccation tolerance is possibly the ability to maintain sufficient structural integrity to repair damage when water is available once again. Thus, cowpea seeds that were grown under well-watered conditions were able to develop sufficient structural integrity, which provided a minimal level of desiccation tolerance adequate for optimal germination and vigour. However, in seeds that were subjected to water stress, the accumulation of RFOs may have enhanced the structural integrity, albeit with minimal effects on percent germination and seed vigour. Studies on the effect of premature harvest on seed vigour and viability suggest that the point of maximum desiccation tolerance is achieved only after complete maturation and rapid drying (Vertucci and Farrant, 1995). It has also been shown that for many seeds, the timing of maximum dry matter accumulation and the acquisition of desiccation tolerance of the embryonic axis are within days (Berry and Bewley, 1991). In addition, several orthodox seeds acquire maximum vigour and longevity in dry storage a time after maximum dry matter accumulation. For example, Hilhorst and Toorop (1997) reported that maximum seed quality expressed as potential longevity was attained after the end of seed filling in bean, wheat and barley; similarly seed quality (percent germination and vigour) in tomato seeds increased when further ripening on the mother plant was allowed. In the present study perhaps it can be argued that in cowpea which is drought tolerant, maximum vigour does not necessarily imply the acquisition of maximum desiccation tolerance; rather there is a minimum level of desiccation tolerance that is required for the development of optimal seed vigour.

Sucrose content in seeds harvested from plants subjected to terminal stress from the Umbumbulu site was markedly higher than from the well-watered and intermittent stress treatments. Similarly terminal stress induced a higher synthesis of myoinositol as was evident by the cowpea seeds harvested from stressed plants from the Umbumbulu site. It has been suggested that the concentration of sucrose and myoinositol, which act as initial substrates in RFO synthesis and feed back loops can affect the final content of RFOs deposited in mature seeds (Peterbauer and Richter, 2001). In the present study the higher concentrations of sucrose and myoinositol were positively correlated with the higher accumulation of raffinose and stachyose in seeds from the stressed plants. Similarly, it may well be possible that in field bean (Lahuta *et al.,* 2000) and cucumber (Widders and Kwantes, 1995) significant changes observed in relative ratios of raffinose, stachyose and verbascose induced by water stress could probably be linked to the accumulation of myoinositol. However, as has already been discussed earlier a higher accumulation of initial substrates and subsequently increased RFO accumulation may not necessarily have an effect on the acquisition of desiccation tolerance and seed quality. Furthermore the initial substrates may be channelled to other biochemical pathways depending on species and environmental variables. For example, the decrease in total non-structural carbohydrates in response to elevated temperatures in soybean observed by Thomas *et al.,* (2003) is inconsistent with other reports which showed that soluble carbohydrates increased or showed no differences in concentration. It may be possible that the initial substrates were channelled to other biochemical pathways and this resulted in to a depletion of the total non-structural carbohydrates in developing soybean seeds. The channelling of initial substrates to other biochemical pathways may also explain the observations by Pattanagul and Madore (1999) working on coleus who reported that drought stress depressed RFO levels and galactinol synthase activity in leaf tissue. Galactinol synthase is the enzyme responsible for the first catalytic step in RFO biosynthesis. In *Xerophyta viscose*, transcript levels for a gene encoding for galactinol synthase activity increased when leaf tissues were exposed to water deficit (Peter *et al.*, 2007); however, galactinol synthase activity *in planta* could not be correlated with RFO accumulation. Apparently myoinositol was channelled to other biochemical processes as was noted by the authors who observed a negative correlation between RFO accumulation and myoinositol depletion, during water deficit stress which was reversed after rehydration. This implied that during water deficit myoinositol is

channelled into RFO synthesis, but during the rehydration process it is channelled to metabolic pathways related to the repair of desiccation-induced damage (Peter *et al.*, 2007).

The observation that cowpea seeds on the maternal plants that were subjected to intermittent stress (imposed during the vegetative stage only) also showed higher accumulation of stachyose and raffinose during seed maturation implies that stress during the vegetative growth stage may influence metabolic processes during the reproductive growth and development stages. Thus, drought stress during vegetative growth may have elicited responses that influenced the RFO biosynthesis at later stages during reproductive development. Most probably, the expression or suppression of specific genes and their effect on the regulation of biochemical processes are implicated. For example, a certain class of plant genes are known to be responsive to both developmental and environmental cues and under stress conditions; these genes can be activated in tissues or organs where they would normally not be expressed under the control of their normal developmental programmes (Bray, 1993; Kermode, 1995). Contrastingly, other genes may also be down-regulated by stress conditions. For example, transcripts of ß-glucosidase, a gene expressed during normal soybean seed development, were detected in seed grown at lower temperatures conducive to soybean seed development; however these transcripts were not detected at elevated temperatures suggesting that normal programs affecting seed composition were perturbed by elevated temperature (Thomas *et al.*, 2003). On the other hand, RFOs have also been shown to accumulate in leaf tissues (Pattanagul and Madore, 1999; Peter *et al.*, 2007). It is possible that drought stress during the vegetative growth stage altered metabolic processes and caused an increase in RFO synthesis in the leaves; this may have later been translocated to the developing seeds together with sucrose.

A two sample (unpaired) t-test was used to investigate whether differences between the environmental conditions in the initial production site could be associated with the pattern of RFO accumulation during subsequent growth and development under conditions of water stress. Studies on maternal effects on the seed quality and performance of subsequent generation often require multiple generations to separate environmental and genetic effects (Highkin and Lang, 1966; Wulf, 1995). However,

this was not possible in the present study, since only one generation was planted and harvested. The unpaired t-test was used as a novel way of trying to determine statistically whether conditions during growth and development of the maternal plant can influence the performance of the subsequent generation. Genetic variability was minimised by using one cultivar. However, the results obtained showed generally seeds from the two sites did not differ with respect to soluble carbohydrate accumulation during development under water stress conditions and do not seemingly support the observations made by other workers (Highkin and Lang, 1966; Salisbury and Ross, 1991; Wulf, 1995). Clearly there could be limitations in the use of a t-test to compare the different sites. Perhaps the major limitation can be attributed to the observation that vigour in itself is complex response to environmental variables; different vigour tests measure responses which are determined by different underlying physiological mechanisms which can be influenced by interactions between physiological, morphological and environmental factors during plant growth, seed development and maturation. Hence attempts to relate maternal environmental conditions and biochemical processes such as food reserve accumulation must take into cognisance all these factors and the inherent variability therein. However, the use of the unpaired t-test was able to describe the variability between and within the sites for individual sugar compounds and may be useful in studies that attempt to relate maternal environmental conditions and seed vigour.

In conclusion, stachyose is evidently the predominant member of the raffinose family of oligosaccharides in cowpea. The rate of RFO accumulation and final concentration increased in response to water stress. The relationship between RFO concentration and the acquisition of desiccation remains as a matter of speculation in the present study and is still generally inconclusive; however there was no evidence to suggest the acquisition of maximum desiccation tolerance is associated with maximum seed vigour. Perhaps it can be argued that in cowpea which is drought tolerant, maximum vigour does not necessarily imply the acquisition of maximum desiccation tolerance; rather there is a minimum level of desiccation tolerance that is required for the development of optimal seed vigour.

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6 DETERMINATION OF DEHYDRIN-LIKE PROTEINS AND RESPONSE TO EXOGENOUS ABA IN SEEDS OF WATER-STRESSED COWPEA PLANTS

6.1 Introduction

The late embryogenesis abundant (LEA) proteins were first discovered in the cotton plant (*Gossypium hirsutum*) (Galau *et al.*, 1986; Dure, 1993) and as the name suggests, are expressed at high levels during the later stages of seed development (Galau *at al.*, 1986; Blackman *et al*., 1995; Oliver and Bewley, 1997). They are highly hydrophilic proteins with characteristic sequence motifs, which are largely unstructured in the hydrated state (Tunnacliffe and Wise, 2007). Based on amino acid/mRNA sequences and expression patterns, LEA proteins were initially classified into at least five groups (Cuming, 1999; Wise, 2003). However, using a newly developed bioinformatics tool known as peptide profile (POPP) analysis, these proteins are now classified in 3 groups only: Group 1 proteins are characterised by a hydrophilic 20 amino- acid motif (Cuming, 1999); Group 2 have at least two of three distinct sequence motifs named Y, S and K (Close, 1997); and Group 3 contain multiple copies of an 11-amino-acid motif (Cuming, 1999). Groups 4 and 5 have been eliminated when LEAs were classified using the POPP analysis and redistributed to groups 2 and 3 (Tunnacliffe and Wise, 2007). LEA proteins have been described in a range of different plants and plant tissues (Galau *et al.*, 1991; Dure, 1993). Homologues of group 1 and 3 have also been found in bacteria and in certain invertebrate (Brian *et al.*, 2004). However, it is speculated that group 2 are likely to be specific to plants (Tunnacliffe and Wise, 2007).

Group 2 LEA proteins which are widely known as dehydrins (dehydration-induced proteins) (Bray, 1993; Close, 1997) can be split into two subsets using POPP analysis; 2a and 2b, corresponding to the super-families 1/10 and 3 respectively (Tunnacliffe and Wise, 2007). Members of group 2a are expressed late in embryogenesis while those of group 2b are associated with cold tolerance (Wise and Tunnacliffe, 2004). These proteins (most likely group 2a LEA) and sucrose and the raffinose family of oligosaccharides are thought to participate in the acquisition of desiccation tolerance during the later stages of embryo development when seeds undergo extreme water loss (Crowe and Crowe, 1992; Dure, 1993; Leopold *et al.*, 1994; Close, 1997;

Kermode, 1997; Crowe *et al.*, 1998). The proteins might act as hydration buffers, slowing down the rate of water loss during dehydration (Cuming, 1999). This may allow sufficient water activity for proteins to retain their functions (Tunnacliffe and Wise, 2007).

LEA proteins, including group 2 have been implicated in a molecular shield function by protecting sensitive enzymes such as LDH (Kazuoka and Oeda, 1994) and protein stabilisation by preventing aggregation of dehydration sensitive proteins (Goyal *et al.*, 2005). However, this is not a unique property to LEA proteins because other hydrophilic proteins which cannot be assigned to LEA protein groups have also been reported to possess similar activity (Reyes *et al.*, 2005).

A role for group 2 LEA proteins has also been suggested in relation to ion binding and antioxidant function (Tunnacliffe and Wise, 2007). Because of their many charged amino acid residues, LEA proteins might act to reduce oxidative stress in dehydrating cells both by scavenging ROS and indirectly by sequestering metal ions that generate Reactive Oxygen Species (ROS) (Dure, 1993; Danyluk *et al.*, 1998). Dehydrins (group 2 LEA) from maize embryos (Egerton-Warburton *et al.*, 1997) and wheat (Danyluk *et al.*, 1998) were found to be associated with cytoplasmic endomembrane and plasma membrane, respectively, suggesting that they might be involved in the protection of these membranes against desiccation.

LEA proteins have also been implicated in membrane association, folding and stabilisation. The acidic group 2 LEA protein accumulates at the plasma membrane during cold acclimation, particularly in tissues more sensitive to freeze damage (Danyluk *et al.*, 1998). Membrane structures are also particularly susceptible to damage induced by water deficit stress (Crowe and Crowe, 1992; Oliver and Bewley, 1997). Membrane modifications and changes in the pattern of protein synthesis and/or expression can also occur as a result of drought stress (Bray, 1993; Bohnert *et al.*, 1995).

Membrane integrity is also an important component of seed vigour (ISTA, 1995) and loss of membrane integrity is known to be a fundamental cause of loss of vigour (Bewley and Black, 1994). Free radicals, which increase during desiccation, can cause

extensive peroxidation and de-esterification of membrane lipids, thus decreasing the fluidity of the membranes (Senaratna and Mackersie, 1983; Tetteroo *et al.*, 1996). Peroxidation can lead to loss of cell membrane integrity and subsequent extensive leakage of cytoplasmic components upon imbibition and influence the development of seed quality.

However, the role of LEA proteins on the improvement of seed quality during development has been contentious (Blackman *et al.*, 1991; Wechsberg *et al.*, 1994). The observation that strains of transgenic tobacco (*Nicotiana tabacum*) constitutively expressing a group 2 protein (citrus dehydrin) showed improved germination and growth than controls at low temperature (15^{o}C) (Hara *et al.*, 2003) is suggestive of a relationship between group 2 LEA protein and the development of seed quality. Ellis *et al.*, (2000) examined changes in soluble carbohydrates and heat-stable proteins in relation to desiccation tolerance and developed a model which suggested that these sugars and proteins are equally likely to be required for seed quality development. In soybean, the level of LEA proteins was closely correlated with desiccation tolerance; the authors concluded that although LEA proteins may contribute to desiccation tolerance of soybean seeds, they may not be sufficient to induce tolerance by themselves (Blackman *et al.*, 1991). However, group 2 LEA dehydrins have also been detected in seeds that remain desiccation-sensitive at shedding (Kermode, 1997). Black *et al.*, (1999) observed that detachment of wheat grains induced the appearance of dehydrins at an earlier stage of development, even in embryos that had not been made desiccation- tolerant by incipient drying. These authors suggested that possibly there is a maternal suppression of dehydrin accumulation by embryos which is relieved by detachment from the mother plant (Black *et al.*, 1999) and concluded that dehydrin accumulation is not regulated by factors that specifically control the induction of desiccation tolerance.

It has been suggested that there are at least two LEA protein gene sets; one with a seed-specific expression, and another which is solely expressed in vegetative tissues (Boudet *et al.*, 2006). Illing *et al.*, (2005) tested the hypotheses that desiccation-tolerance in vegetative tissues of angiosperms could be due to 1) appropriation of the seed-specific program of gene expression that protects orthodox seeds against desiccation, and/or 2) a sustainable version of the abiotic stress response. These

authors compared molecular and physiological data from the development of orthodox seeds, the response of desiccation-sensitive plants to abiotic stress, and the response of desiccation-tolerant plants to extreme water loss. The result was identification of LEA protein gene sets that could be grouped into those with seed-specific expression and those expressed in vegetative tissues. Furthermore, the observation that two proteins (LEA6 and 1-cys-peroxiredoxin) were not expressed in vegetative tissues in *Arabidopsis thaliana* but had orthologues that were specifically activated in desiccating leaves of the resurrection plant *Xerophyta humilis* was found to be consistent with the hypothesis that resurrection plants acquired systemic desiccation tolerance by reprogramming seed-specific gene sets (Illing *et al.*, 2005). However, this presumed that LEA proteins normally expressed in seeds are more potent than those expressed in vegetative tissue (Tunnacliffe and Wise, 2007). Boudet *et al*. (2006) investigated whether proteins in *Medicago truncatula* can be identified that are specifically linked to desiccation tolerance. These authors found several isoforms of dehydrins that were linked to drought-tolerance rather than desiccation-tolerance and proposed that LEA proteins can be divided into two groups. The first group containing LEAs that seem to be seed specific and are induced only in tissues that are desiccation tolerant, whereas the second group contains proteins including dehydrin that are expressed in vegetative tissues (Boudet *et al*., 2006).

The presence of dehydrin in recalcitrant seeds from temperate climates (Kermode, 1997), the absence of correlation between their amounts and seed longevity (Wechsberg *et al.*, 1994), together with the observation that dehydrins protect enzyme activities only at water potentials above -3 MPa (Reyes *et al.*, 2005) has led to the suggestion that dehydrins might play a protective role at high hydration levels (> 0.8 g g^{-1}) (Boudet *et al.*, 2006). However, LEA proteins that may be specifically linked to desiccation tolerance might play a role at lower moisture levels corresponding to 0.3 g g^{-1} (Boudet *et al.*, 2006). At higher moisture levels, desiccation tolerant-linked LEA proteins may act as compatible solutes that preferentially exclude reactive substances from the surface of macromolecules (Liu and Zheng, 2005; Reyes *et al.*, 2005). Similarly at water contents less than 0.3 g g^{-1} they might exert their protective effects by replacing water molecules by hydrogen bonding and /or forming a glass which stabilizes the system in the dry state (Hoekstra *et al.*, 2001).

Examination of proteins in *Medicago truncatula* revealed that several LEA proteins including Group 1 LEA proteins as being seed specific (Boudet *et al.*, 2006). Group 1 LEA proteins have been proposed to function *in planta* to protect against desiccation stress, by possibly functioning as water binding and /or replacement molecules (Gilles *et al.*, 2007). Gilles (2007) confirmed the existence of a PII-like structure that could be significant with respect to the proposed role of Group I LEA proteins as water replacement molecules during the acquisition of desiccation tolerance in seed embryos. Analysis of Circular Dichroism (CD) spectra of a recombinant Group I LEA revealed the presence of an isodichroic point indicating a transition between two structural states (Soulages *et al.*, 2002): an extended helical or poly (Pro) II (PII) structure in the recombinant Group I LEA and little, ‹-helix. PII-like structures have been repeatedly implicated in protein-protein and protein-peptide interactions (Anderson and Gorski, 2005). The presence of this structural element in Group I LEA and the abundance of these proteins in seed embryos suggest that these proteins may be important in the amelioration of desiccation related stresses (Gilles *et al.*, 2007).

Most of the studies on LEA proteins are done *in vitro* using mutant plants such as *Arabidopsis* and transgenic plants of species such as tobacco (Hara *et al.*, 2003) and rice (Rohila *et al.*, 2002). Tunnacliffe and Wise (2007) observe that in *vitro* data may not accurately reflect *in vivo* function. Therefore, more *in vivo* studies are imperative. In their recent review covering over 20 years of work done on LEA proteins, these authors noted that the role of LEA proteins should not be considered in isolation, because cells dealing with water loss launch a whole series of defence or repair mechanisms besides LEA proteins. In addition LEA proteins are not the only hydrophilic proteins involved in the response to dehydration. Furthermore interaction between LEA and other hydrophilic proteins and sugars and other compatible solutes associated with water stress tolerance in many organisms and other components of desiccation tolerance are not well understood.

Such interaction may involve the role of the plant growth regulator abscisic acid (ABA). Group 2 LEA proteins (dehydrins) have received particular attention because they share extensive amino acid sequence with proteins synthesized in response to ABA and drought/water stress (Bradford and Chandler, 1992; Farrant *et al.*, 1992; Finch-Savage and Blake, 1994). For example, Blackman *et al.* (1995) observed that

when abscisic acid was used to activate LEA gene expression prematurely in immature soybean seeds, a corresponding improvement in cell integrity was noted after desiccation stress. An improvement in cell integrity may possibly imply an effect on seed vigour. However, in developing wheat embryos, embryonic ABA prevented the germination of the embryo, blocked the expression of germination-specific enzymes and promoted embryo development (Walker-Simmons, 1987).

Abscisic acid has also been applied in many studies aimed at understanding physiological response mechanisms in plants. Although Finkelstein (2002) questioned the biological relevance of exogenous ABA application on the inhibition of germination, its application can still be a convenient quantitative assay for ABA sensitivity that can be used to study response mechanisms. ABA has been applied exogenously to mature mustard seeds (*Sinapis alba* L.) to determine whether the primary action of ABA in inhibiting seed germination is through the control of water uptake of the embryo tissues or rather the control of DNA, RNA or protein synthesis (Schopfer *et al.*, 1979). Exogenous application of ABA induces desiccation tolerance to developing embryos (Bartels *et al.*, 1988; Anandarajah and Mackersie, 1990) and prolongs the desiccation tolerant phase in mature embryos (Blackman, 1991). However, the mechanism by which exogenous ABA may prolong desiccation tolerance is not clear (Walker-Simmons, 1987)

In summing up, different groups of the late embryogenesis abundant proteins have been proposed to contribute in various ways to protection from desiccation during embryo maturation. These proteins are also degraded during germination, implying that they have a role in seed development and germination. However, in seeds, direct in *vivo* evidence for the role of LEA proteins in tolerance to complete water loss is not well understood. It is possible that LEA expression may be influenced by complex interactions involving seed developmental processes, plant growth regulators, environmental factors and other compatible solutes such as soluble sugars. The expression of LEA proteins, including dehydrins, in vegetative tissues in response to drought and temperate conditions differs with crop species and has led to the speculation that there are LEA proteins that are specifically linked to desiccation stress rather than drought stress. Thus it can be hypothesized that these proteins might play protective roles at different hydration levels, and that those that may be

specifically linked to desiccation tolerance may have a protective function at lower hydration levels (~ 0.3g g^{-1}). The objectives of this study was 1) to investigate the effect of water stress on the accumulation of stress LEA proteins in developing cowpea seeds and 2) to study the effect of exogenous application of ABA on LEA protein expression and the RFO metabolism in germinating cowpea seeds.

6.2 Materials and methods

6.2.1 Seed materials

6.2.1.1 Seeds at different stages of development

Seeds of cowpea (cultivar Nzovu) were initially donated by the Environmental Action Team (EAT), Kitale, Kenya. Cultivar M66 was originally purchased from the Basic Seed Unit of the Kenya Agricultural Research Institute (KARI), Machakos, Kenya and Kenkunde was originally purchased from the Kenya Seed Company, Kitale, Kenya. The seeds were initially planted at two different sites in South Africa (Ukulinga and Umbumbulu) under different production conditions in an earlier experiment to produce fresh seed with known quality characteristics (Chapter 2). Mature seeds harvested from these two sites were planted in a pot experiment under simulated drought conditions (Chapter 3).

Plants were grown under simulated drought conditions in a tunnel at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The tunnel was enclosed in clear polythene sheeting. Temperature, solar radiation (PAR) and relative humidity (RH) were monitored electronically using HOBO 2K Loggers (Onset Computer Corporation, Bourne, USA). Soil from the Ukulinga production site, whose physical characteristics had been determined, was used for the pot experiment. The soil -loam with a bulk density of 1562 kg m^{-3}. The average field capacity (FC) was 27% by volume and the soil contained 19% water content (% vol) at a matric potential of -1500KPa corresponding to the permanent wilting point (PWP) as determined from soil water retentivity curves developed using a ceramic pressure plate (see Chapter 3).

Drought conditions were simulated based on two contrasting moisture environments typical of semi-arid tropics where cowpea is grown, and were defined as follows: Intermittent stress, characteristic of the wet season in the monsoonal semiarid tropics

, when stress can occur at any time and with varying intensities between emergence and maturity; Terminal stress, where crops are grown relying only on stored moisture and where crops grow and mature progressively on depleted soil moisture profile (Ludlow and Muchow, 1990). Pots containing 20 kg air dry soil with a soil moisture content of ~ 3 % were then placed individually on a weighing balance and a precise quantity of water was added until the required mass equivalent to 17.47 % the gravimetric water content at FC (no stress), corresponding to a matric potential of -33 kPa was attained. Drought conditions were imposed by placing individual pots on a weighing balance and adding a precise quantity of water until the required mass equivalent to 12.26 % gravimetric water content at permanent wilting point (PWP), corresponding to a matric potential of -1500 kPa was attained. Plant available water capacity (PAWC) from the -33 and -1500 kPa water contents was determined gravimetrically (mass) by calculating the difference between the % gravimetric water content at -33 matric potential and -1500 kPa. An additional quantity equivalent to 30 % PAWC was calculated and added to each pot to attain the required soil mass at 30 % FC. The pots were individually emptied in to a concrete mixer and mixed thoroughly so as to achieve a uniform distribution of soil and water inside the pot and the weights were recorded and written on the pots. Intermittent stress was imposed at 30 % FC during the vegetative stage of growth only, whereas terminal stress was imposed at 30 % FC throughout development from emergence to maturity.

Five seeds were planted in each pot by placing four equidistantly and one at the centre. The seeds were planted at a depth of 10 mm. A compound fertilizer 2:3:2 (22) was applied during planting at the rate of 600 kg ha^{-1}. Soil water content in the pots was monitored gravimetrically by weighing individual pots periodically, and continuously using ThetaProbe soil water sensors (Lukangu *et al.,* 1999).

Pods were tagged at R4 stage of plant development (Fageria, 1992), and harvested during three developmental stages fortnightly until harvest maturity. The stages of reproductive growth at which seeds were harvested corresponded to R4, R6 and R8 (Chapter 5). Seeds were removed from pods to determine dry mass and water content (% fresh mass). Dry mass was determined by lyophilizing surgically excised tissues

(cotyledons, axes and seed coats) for 24 h after freezing in liquid N. Dry tissues were stored at -20^{o}C until determination proteins using gel electrophoresis.

6.2.1.2 Mature seeds treated with ABA

Samples, each containing twelve seeds harvested from the cultivar Nzovu were prepared and placed in separate 10 ml beakers. A 10 ml stock solution of 0.01M ABA was made in methanol and used to make 10, 100 and 1000 µM concentrations. Each concentration was made up to a volume of 25 ml. For each concentration, seed samples were imbibed in 5 ml ABA solution for 4h. The wet control seeds were imbibed in deionised water for 4h and the dry control seeds were not subjected to any treatment. In another experiment, acetone was used to apply ABA to the dry cowpea seeds. Acetone has been used to introduce chemicals to dry lettuce (*Lactuca sativa*) and cucumber (*Cucumis sativus*) seeds (Tao and Khan, 1974). Seeds were imbibed in 10, 100 and 1000 µM concentrations of ABA using acetone as a solvent. Both the ABA in water and the acetone treated seeds were then germinated using moistened paper towels according to ISTA (1999). Germination was recorded at regular intervals: 8. 16, 24, 48 and 72h. Seeds were considered to have germinated after radicle protrusion. Samples were prepared for gel electrophoresis by lyophilizing surgically excised tissues (cotyledons, axes and seed coats) for 24 h after freezing in liquid N. Dry tissues were then stored at -20^{o}C until determination of proteins using gel electrophoresis.

6.2.1.3 Protein analyses (Western blots)

Proteins were extracted on duplicate samples of 250 mg tissue using a buffer of TRIS (0.1M), EDTA (0.1M), NaCl (20 mM) and PMSF (1nM) after grinding in liquid nitrogen. Two ml extraction buffer was added to the ground sample, and shaken at 4^{o}C for 15 min. Samples were then centrifuged at 10,000 g for 15 min at 4^{o}C and the supernatants transferred to new Eppendorf tubes and kept under ice. Protein concentration of the extracts was determined using the Bradford Assay (1976) Reagent Kit with bovine serum albumin as a standard according to the manufacturer's instructions. A volume containing 10µg protein was measured in to fresh Eppendorf tubes and an equal volume of SDS-PAGE sample application buffer (TRIS 250mM, 10% SDS, 40% glycerol, 0.004 bromophenol blue and 10% v/v β-mercaptoethanol) added. The sample was made up to 20 µl by adding the extraction buffer and heated

on an Omeg HB/01 Digital Control Heating Block manufactured by Omeg Company, Cape Town, South Africa at 100^{o}C for 3 minutes before loading.

Proteins (10µg total protein) were separated using on a 12% SDS-PAGE (Laemmli, 1970) at 80V for 3 h. The gels were stained overnight with Coomassie Blue (0.25 g Coomassie blue, 10 ml acetic acid, 50 ml ethanol and 50 ml water) and then de-stained with Destaining solution (50ml acetic acid, 25 ml methanol and 425 ml water) until the gel background became clear.

Proteins were electro-transferred (3 h at 400 mA) to a nitrocellulose membrane using a Trans-blot semi dry system (BioRad). Membranes were fixed with 10% skim milk and incubated overnight in a cold room at 4^{o}C on a shaker. After washing three times for 10min each, twice with TBS- Twin /Triton buffer and once with TBS buffer at room temperature on a shaker, the membranes were incubated for 1h with anti-dehydrin antibody solution in blocking buffer (skim milk) at concentration of 1:500. The anti-dehydrin antibody was kindly donated by Professor Timothy Close from the University of California. The removal of the blocking buffer after the incubation period was done by washing the membrane using TBS-Twin/Triton and TBS buffers as described above. The secondary antibody used was 2 µl anti-rabbit 1gG peroxidase conjugate which was dissolved in 20 ml skim milk (blocking buffer at a concentration of 1:500). The solution was mixed well and incubated for 90minutes at room temperature on a shaker, then washed 4 times for 10 minutes each time in TBS-Twin/Triton buffer. Membranes were then incubated for 10-30min in 2ml stable perodixase solution (a chemiluminescent substrate which enhances detection) on a shaker. The membrane was then placed on a photographic film and sealed in a hyper cassette and the film developed in a dark room. The procedure was repeated and the membranes incubated using an anti-group 1 LEA as the primary antibody. The anti-group 1 LEA antibody had been kindly donated by Dr William R Marcotte from Clemson University.

6.3 Results

In the electrophoresis analysis of the total proteins, the pattern of expression of two bands of M_r ~25 and ~50 was clearly discernible (Figure 6.1). These bands were absent at 2 weeks of development in the control treatment (Lane 1) and terminal stress

(Lane 7) and appeared at 4 and 6 weeks (Lanes 2 and 3 for control and lanes 8 and 9 for the terminal stress). In the intermittent stress treatment, a faint band of M_r ~ 50kDa and ~25 kDa were observed at 2 weeks and increased in intensity at 4 and 6 weeks.

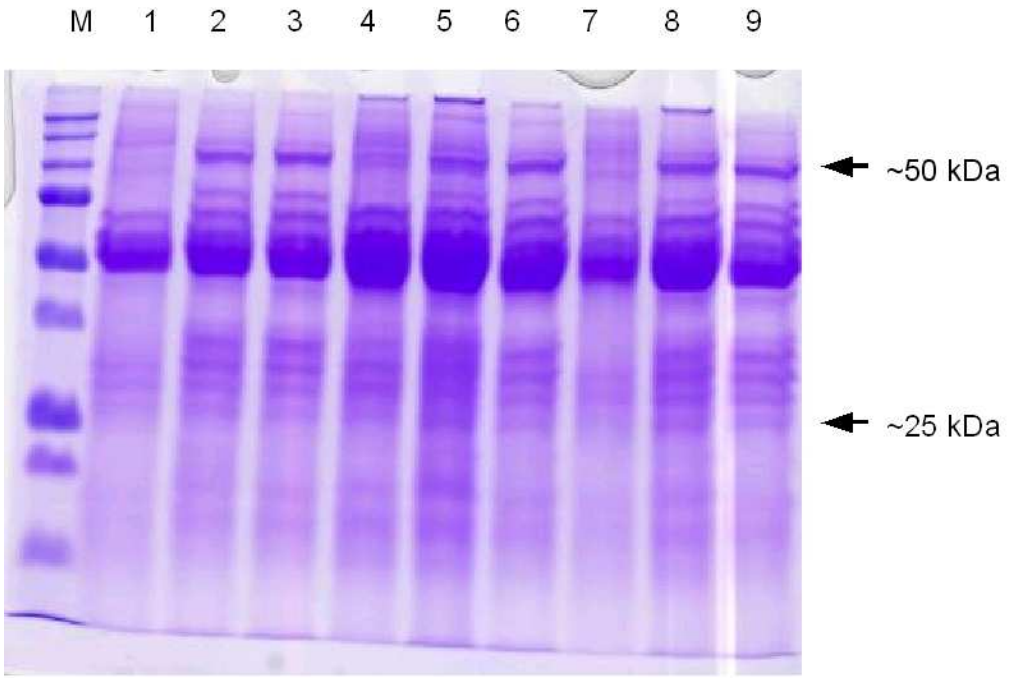

Figure 6-1 Protein gel electrophoresis showing differences in protein bands for cowpea seeds during development under water stress. M denotes the molecular marker, Lane 1, 2 and 3 (no stress), Lane 4, 5 and 6 (intermittent stress), Lane 7, 8 and 9 (terminal stress). For each treatment samples were analysed at 2, 4 and 6 weeks after podding (WAP)

The results of the Western Blot analysis identified the 25 kDa protein to be a dehydrin (Figures 6.2). Under well-watered conditions, no dehydrin protein was expressed at 2 weeks; however clear bands were observed at 4 and 6 weeks of development. For the intermittent stress treatment dehydrin protein was expressed much earlier at 2 weeks although the level was low. This showed an increase in concentration at 4 and 6 weeks. Similarly, there was no dehydrin expression at 2 weeks of development for the terminal stress treatment. Terminal stress caused an increased accumulation and expression of the group 2 LEA proteins (dehydrins) compared with intermittent stress and well-watered conditions.

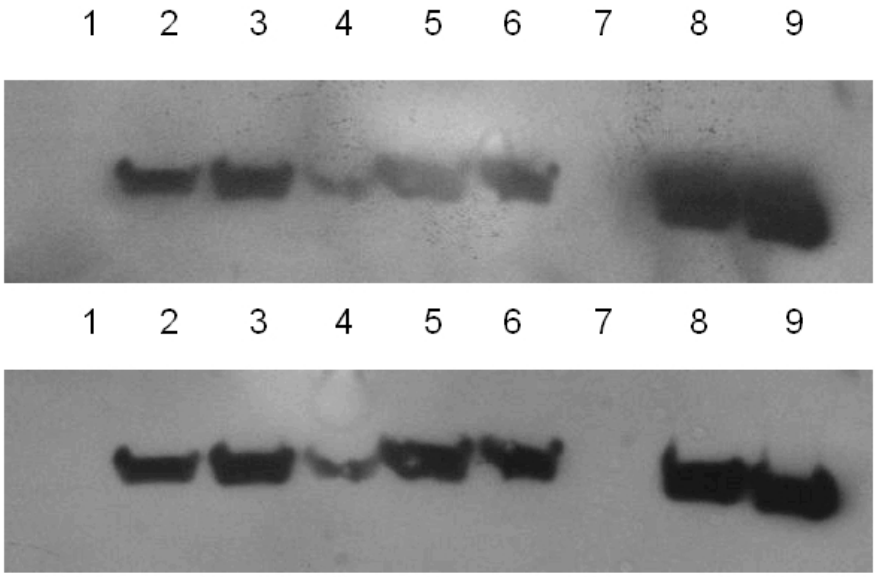

Figure 6-2 Western blot results showing group 2 LEA proteins (dehydrin) bands at ~ 25kDa, Lane 1, 2 and 3 (no stress), Lane 4, 5 and 6 (intermittent stress), Lane 7, 8 and 9 (terminal stress) (top). For each treatment samples were analysed at 2, 4 and 6 weeks after podding (WAP). Protein in Lane 4 which is 2 weeks after podding for the intermittent stress is expressed. No bands were seen at 2 weeks after podding for the no stress (Lane 1) and the terminal stress treatment (Lane 7. A similar pattern of expression was observed (bottom) after the experiment was repeated.

Western Blot analysis was able to identify the 50 kDa protein to be group 1 LEA protein (Figure 6.3). However, the blots were not clearly separated although it was clearly evident that terminal stress caused an increase in the expression of group 1 LEA. The concentration of the group 1 LEA antibody used 1:500 which was probably too high and therefore resulted in the poor separation.

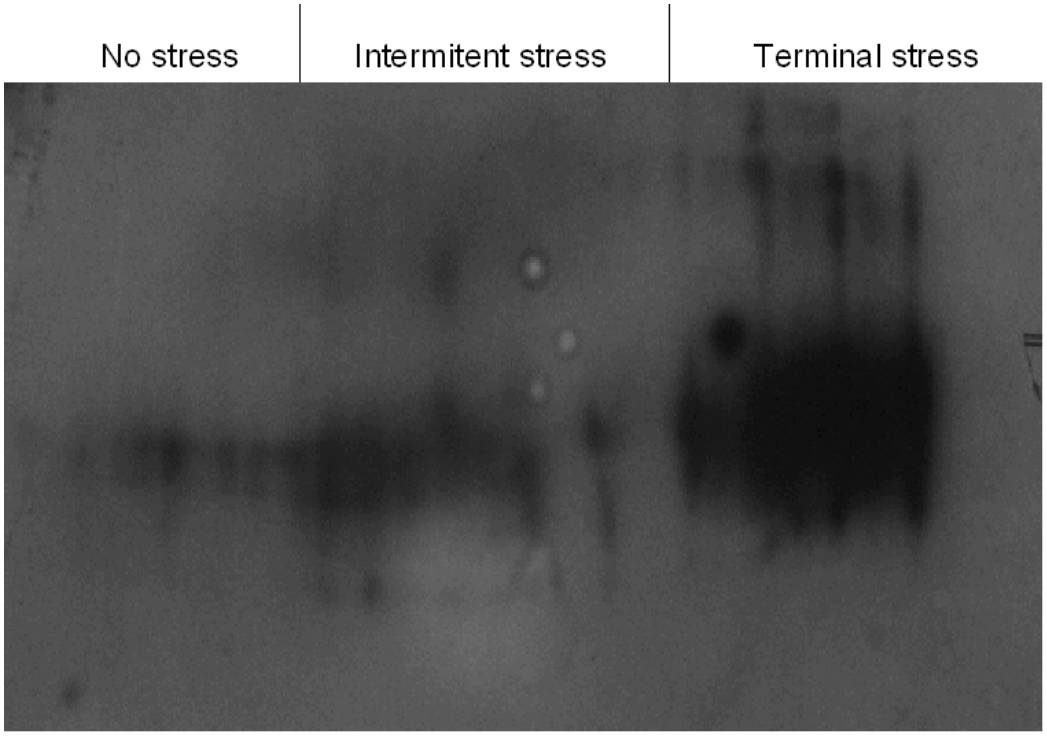

Figure 6-3 Western blot results showing group 1 LEA bands at ~ 50 kDa for well-watered controls, intermittent stress and terminal stress.

The experiment was repeated using the same antibody for group 1 LEA at a concentration of 1:1000 (Figure 6.4). However in this case it seemed as if there was incomplete oligomerization.

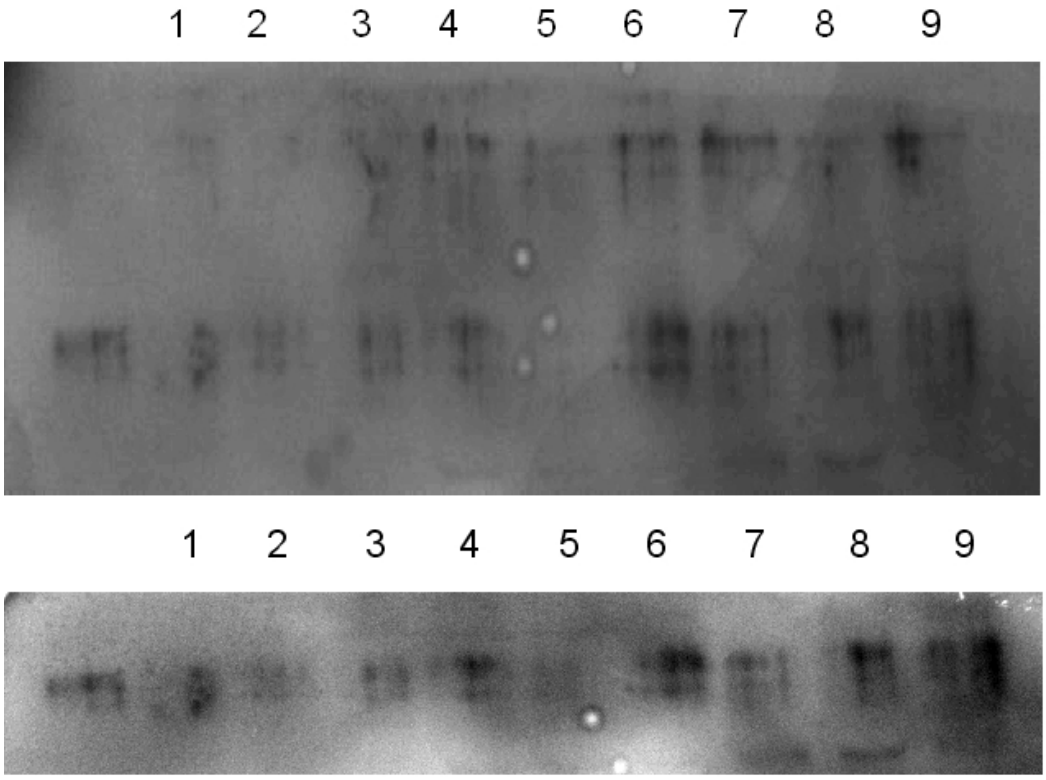

Figure 6-4 Western blot results for group 1 LEA showing more bands than expected possibly due to incomplete reduction of oligomers. The lanes 1, 2 and 3 (no stress), Lanes 4, 5 and 6 (intermittent stress), Lanes 7, 8 and 9 (terminal stress). For each treatment samples were analysed at 2, 4 and 6 weeks after podding (WAP).

The effect of different concentrations of exogenously applied ABA on germination of mature cowpea seeds was investigated. Seeds that were imbibed in water (4h) as a wet control germinated rapidly compared with the dry control seeds. However both controls germinated faster and did not differ significantly from the 10 µM concentration treatment after 24 h. The 100 µM concentrations of exogenous ABA significantly ($P < 0.001$) delayed germination compared with the controls (Figure 6.5). Both 1000 and 100 µM ABA concentrations resulted in significantly lower germination capacity after 72 h. However, the inhibition of germination after the application of 1000 µM ABA was delayed for 24 h, and was considerably lower after 72h (Figure 6.5).

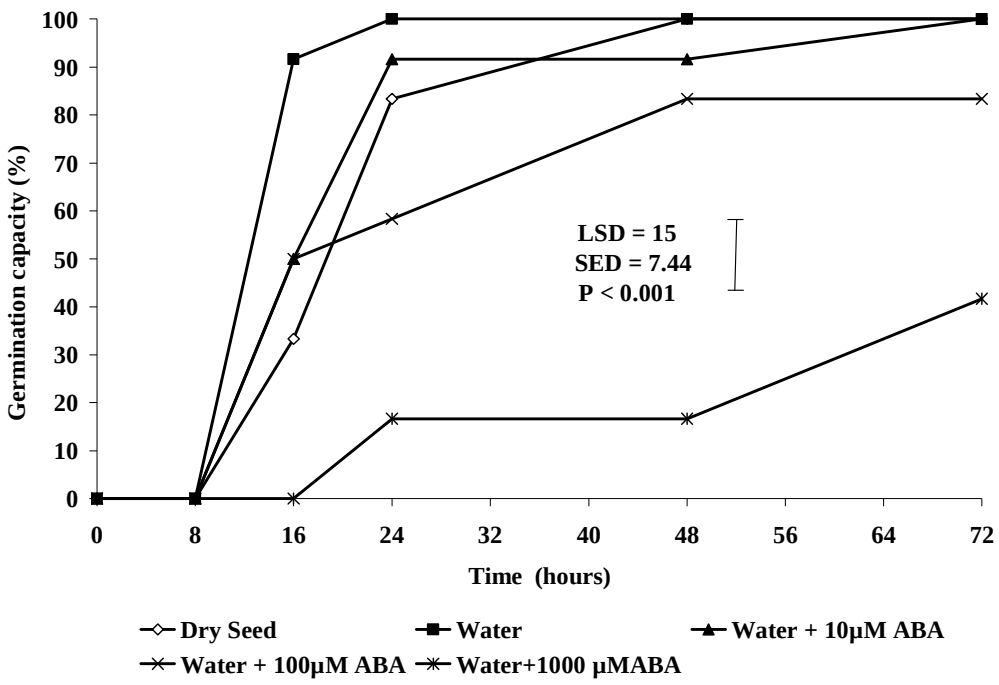

Figure 6-5 Effect of exogenous application of ABA on the germination of cowpea seeds

The pattern of dehydrin expression and/or accumulation after the exogenous application of 10, 100 and 1000µM ABA concentrations was investigated (Figure 6.6 and 6.7). Dehydrin was present in the control treatment and all the three ABA concentrations after 8h germination. Differences became evident after 24h and 72h between the dry control and the ABA concentrations. The dry control treatment showed a progressive decrease in dehydrin content with increase in germination time, but was still present after 72h. This differed from the wet control in which dehydrin breakdown occurred more rapidly and had disappeared completely after 72h (Figure 6.7). There was no dehydrin present after 72h in the 10µM ABA treatment. Both 100µM and 1000 µM ABA clearly suppressed dehydrin degradation.

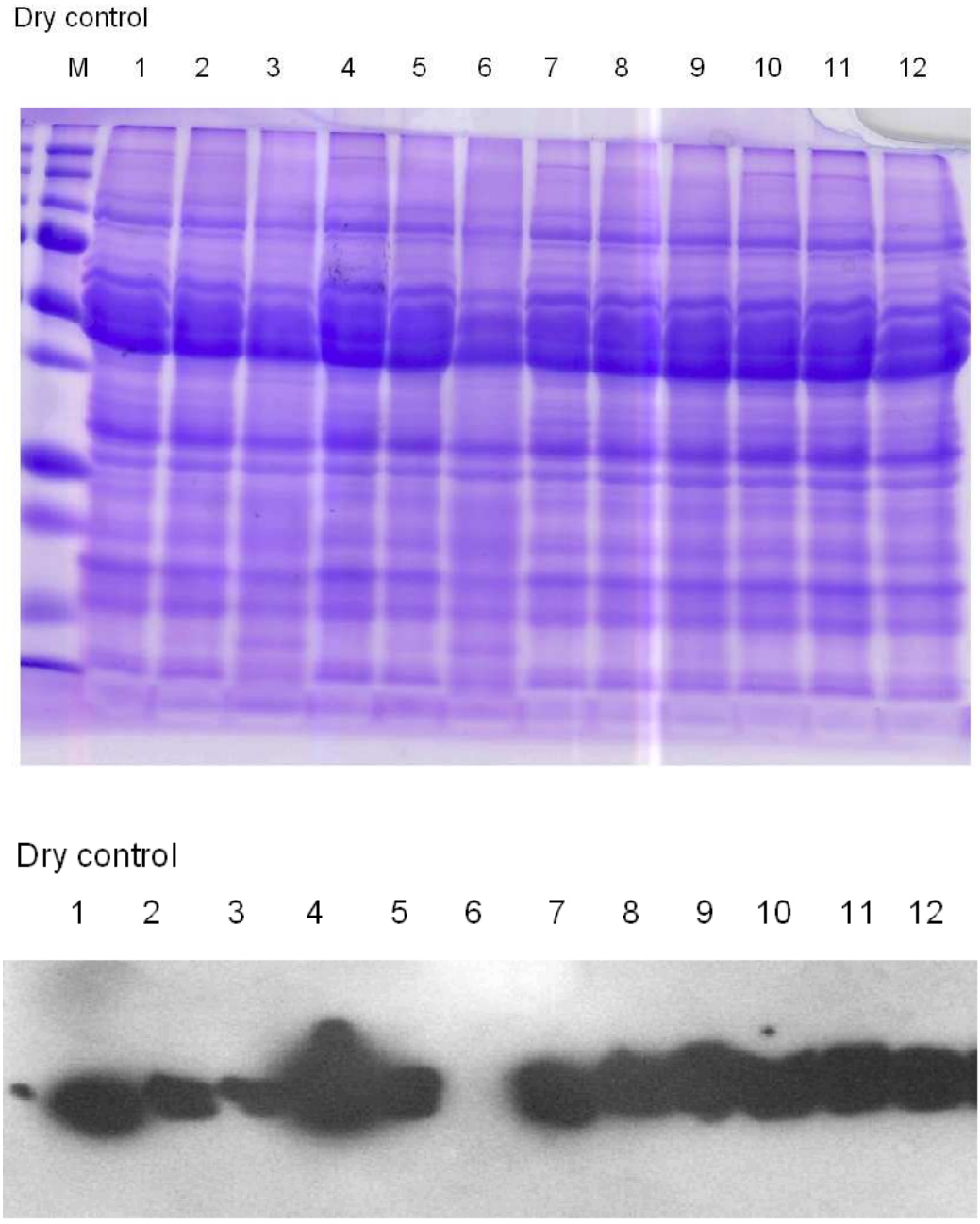

Figure 6-6 Protein gel electrophoresis and western blot for cowpea seeds after exogenous application of ABA. Lane 1, 2 and 3, dry seeds were germinated for 8, 24 and 72h. Lane 4, 5 and 6, germination for 8, 24 and 72h after 10µM ABA treatment .Lanes 7, 8, and 9, germination for 8, 24 and 72h after100 µM ABA treatment. Lane 10, 11 and 12 germination for 8, 24 and 72h after1000 µM ABA treatment.

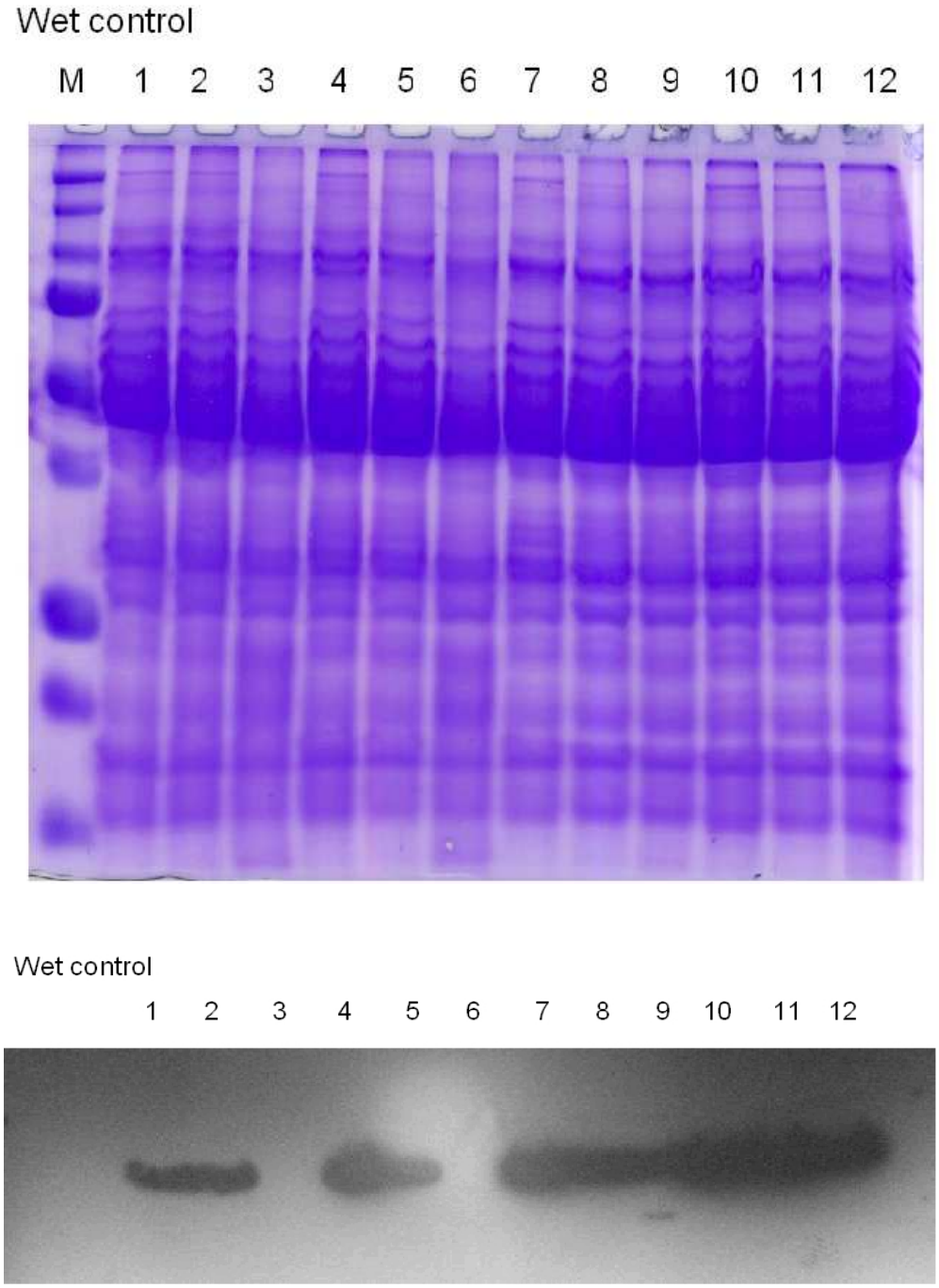

Figure 6-7 Protein gel electrophoresis and western blots for cowpea seeds after the exogenous application of ABA. Lane 1, 2 and 3, seeds were soaked for 4h then germinated for 8, 24 and 72h. Lane 4, 5 and 6, germination for 8, 24 and 72h after 10µM ABA treatment .Lanes 7, 8, and 9, germination for 8, 24 and 72h after100 µM ABA treatment. Lane 10, 11 and 12 germination for 8, 24 and 72h after1000 µM ABA treatment.

Mean squares for soluble carbohydrates in germinating cowpea seeds treated with 1000, 100 and 10µM concentrations of exogenous ABA were compared (Table 6.1). The results of the analysis showed that exogenous ABA application on soluble carbohydrate content in germinating cowpea seeds was highly significant. The control treatments differed significant with the ABA treatments with respect to sucrose, raffinose and myoinositol content. Highly significant differences were also observed between the ABA concentrations. In addition a highly significant interaction was observed between germination stage and ABA treatments (Table 6.1). Sucrose content in all the treatments including controls ranged between 66.6 and 76.7µg g dry mass^{-1}. The increase in sucrose content was rapid in the wet and dry controls compared to the ABA treatments after 16h germination. Sucrose content in the wet and dry controls were 260.1 and 385 7µg g dry mass^{-1} respectively after 16h germination and were significantly ($P < 0.001$) higher than in the 1000, 100 and 10 µM ABA concentrations which were 92.2, 137.1 and 129 7µg g dry mass^{-1} respectively. Sucrose metabolism was inhibited by the application of 100 µM ABA up to 24hh as was evident from the content (163.3µg g dry mass^{-1}) which was significantly lower than the controls (257 and 321 µg g dry mass^{-1} for the wet and dry controls respectively). However, 1000µM ABA severely inhibited sucrose metabolism up to 72h as was evident in the significantly lower content (160.7µg g dry mass^{-1}) compared to the wet and dry control (275.8 and 286.3 µg g dry mass^{-1}) and the 100 and 10 µM concentrations (247 and 253 µg g dry mass^{-1} respectively).

Table 6-1 Mean squares for soluble carbohydrates in germinating cowpea seeds treated with 1000, 100 and 10µM concentrations of exogenous ABA

Source of variation	d.f.	Sucrose	Stachyose	Raffinose	Verbascose	Myoinositol
Treatments	4	54481***	194191***	548.21***	14649.7***	3.7504***
Controls vs. all ABA treatments	1	147144***	8.0	454.59***	431.8	8.0692***
1000µM ABA vs. 100µM ABA	1	34338***	559217***	1178.44***	43430.4***	0.1537
1000µM ABA vs. 10µM ABA	1	36931***	601484***	1407.34***	37357.5***	5.9258***
100µM ABA vs. 10µM ABA	1	47	770	10.15	228.6	4.1709***
Germination stage	5	78760***	401862***	321.06***	10377.5***	7.8653***
Treatments x Germination Stage	20	9008***	33404***	134.8***	2972.5***	0.9246***
(Control vs. all ABA treatments) x .Stage	5	26353***	91885***	252.37***	5780.5***	1.3229***
(1000µM vs. 100µM) x Germination Stage	5	4680	8208**	143.83***	1347.2**	0.3985
(1000µM vs. 10µM) x Germination Stage	5	3131	15682****	131.8***	1827.2***	1.0995***
(100µM vs. 10µM) x Germination Stage	5	2506	7576**	127.51***	336.8	2.0025***
Residual	58	2367	2848	22.72	305.6	0.177

* denotes the level of significance:* at 0.05, ** at 0.01 and *** at 0.001

Table 6-2 Two-way interaction between 1000, 100 and 10 µM ABA treatments and the stage of germination (hours) with respect to changes in soluble carbohydrate content (µg g dry mass^{-1}) in cowpea seeds during germination for 72h.

Treatment	Stage (hours)	Soluble carbohydrate (µg g dry mass^{-1})				
		Sucrose	Stachyose	Raffinose	Verbascose	Myoinositol
Wet control	0	66.6	605.4	20.1	183.9	0.8
	8	52.9	473.3	23.0	98.3	0.9
	16	260.1	203.7	24.3	38.7	2.2
	24	257.0	187.9	15.9	24.5	1.8
	48	258.8	155.6	1.6	43.5	3.0
	72	275.8	16.6	1.7	6.7	3.5
Dry control	0	73.8	594.5	20.5	73.1	0.8
	8	101.7	672.6	23.1	110.8	0.8
	16	385.8	152.9	19.5	34.6	2.3
	24	321.4	113.2	16.4	32.9	1.7
	48	307.1	4.8	6.1	15.4	2.9
	72	286.3	5.6	6.5	0.0	3.2
1000µM ABA	0	74.7	524.6	23.3	91.7	0.5
	8	75.3	442.5	21.7	69.8	0.8
	16	92.2	474.4	26.5	111.9	1.2
	24	76.5	431.9	28.1	95.2	0.8
	48	97.3	420.3	37.5	106.7	1.4
	72	160.7	311.1	27.7	96.9	1.7
100µM ABA	0	76.7	345.6	20.3	40.1	1.1
	8	79.1	263.0	17.3	45.5	0.9
	16	137.1	226.9	20.6	44.2	1.2
	24	163.3	189.2	18.3	25.9	1.3
	48	243.4	39.8	8.9	7.0	1.8
	72	247.7	44.7	10.7	0.0	0.9
10µM ABA	0	72.2	356.7	17.3	60.0	0.6
	8	125.5	348.8	21.3	59.8	1.3
	16	129.3	157.2	16.5	28.3	1.1
	24	204.0	93.6	10.3	15.0	2.4
	48	176.6	92.0	23.8	22.6	2.3
	72	253.4	5.6	0.6	0.0	3.7
SED		39.7	43.6	3.9	14.3	0.3
LSD		79.5	87.5	7.8	28.7	0.7
P		<0.001	<0.001	<0.001	<0.001	<0.001

Stachyose content (µg g dry mass^{-1}) at 0h germination was highly variable and ranged between 605.4 and 345.6 µg g dry mass^{-1} in all the treatments (Table 6.2). The oligosaccharide decreased rapidly during germination in both controls (wet and dry) and did not differ significantly from the 100 and 10µM ABA concentrations after 72h. Stachyose metabolism in germinating cowpea seeds was severely inhibited by the application of 1000µM ABA. Stachyose content after 72h in the 1000µM ABA treatment was 311 µg g dry mass^{-1} which was significantly much higher compared to the content in the wet and dry controls (16.6 and 5.6 µg g dry mass^{-1} respectively) and in the 100 and 10 µM ABA treatments (44.7 and 5.6 µg g dry mass^{-1} respectively).

Similar trends were observed with respect to raffinose metabolism in response to the application of exogenous ABA (Table 6.2). Raffinose content (µg g dry mass^{-1}) in all the treatments at 0h germination ranged between 17.26 and 23.32 µg g dry mass^{-1}. Raffinose content decreased sharply in both the wet and dry controls after 48h and was significantly different from the content after the application of the ABA treatments. However, 1000µM ABA concentration significantly inhibited raffinose metabolism compared to the 100 and 10µM ABA concentrations after 48 and 72h germination period.

Verbascose content was variable at 0h germination and ranged between 183.9 and 40.1 µg g dry mass^{-1} for all the treatments and controls (Table 6.2). However, changes in verbascose content followed a more or less a similar trend to that of stachyose and raffinose. There was a general decline during germination in both controls and the 100 and 10µM ABA concentrations which declined to 6.7 µg g dry mass^{-1} in the wet control and was completely used up after 72h in the 100 and 10µM ABA concentrations and dry control. Similarly the 1000µM ABA concentration had a highly significant ($P < 0.001$) effect on verbascose metabolism.

The sugar alcohol myoinositol was generally found in low concentrations in mature cowpea seeds at 0h germination. This ranged between 0.814 and 1.112 µg g dry mass^{-1} in all the treatments including control. Myoinositol content increased during germination to 3.541, 3.213 and 3.715 µg g dry mass^{-1} for the wet and dry control and 10µM ABA concentration respectively at 72h. However, the 1000 and 100µM ABA

concentrations had a highly significant ($P < 0.001$) effect on myoinositol accumulation. The content after 72h in seeds treated with 1000 and 100µM ABA concentrations was 1.718 and 0.941 µg g dry mass^{-1} which was significantly lower than the controls and the 10µM ABA concentration.

6.4 Discussion

This study compared the effect of water stress on the accumulation and expression of two LEA proteins: group 1 LEA protein which has been suggested to be seed specific (Boudet *et al.,* 2006; Gilles *et al.,* 2007); and group 2 LEA (dehydrins) which has been found in both maturing plant seed and vegetative tissues and is speculated to be specific to plants (Tunnacliffe and Wise, 2007). The appearance of group 2 LEA proteins after 4 weeks of development under well watered and terminal stress conditions, and their increased expression and accumulation under terminal stress is consistent with information in the literature that these proteins accumulate during the later stages of embryogenesis and increase in concentration in response to water stress (Galau *et al.*, 1991; Cuming, 1999). These proteins are likely to be classified as members of group 2a according to POPP analysis (Tunnacliffe and Wise, 2007).The observation that intermittent stress imposed during the vegetative growth phase induced an earlier synthesis of dehydrin protein in seeds at 2 weeks is comparable to the work of Black *et al.,* (1999) who reported that detachment of wheat grains induced the appearance of dehydrin at an earlier stage of development. This was attributed by the authors to the possibility of a maternal suppression of dehydrin accumulation by embryos which may be relieved by detachment from the mother plant. However, in the present study the results imply that there may be a maternal influence on LEA protein gene expression which may induce dehydrin accumulation during the earlier stages of seed development. Probably ABA may be implicated in the earlier induction of dehydrins. ABA has been used to activate LEA protein gene expression prematurely in soybean seeds (Blackman *et al.,* 1995) and the plant hormone is also known to accumulate in response to water stress (Salisbury and Ross, 1991). It is not clear why terminal stress did not induce early dehydrin synthesis at 2 weeks of development. Evidently there were differences with respect to LEA protein gene expression and dehydrin accumulation between the two treatments (terminal and intermittent stress) at 2 weeks of development. The observations made in the present study support the conclusions made by Black *et al.,* (1999) that dehydrins

accumulation is not regulated by factors that specifically control the induction of desiccation tolerance.

The abundance of group 1 LEA proteins in seeds was evident in the higher accumulation and /or expression under terminal stress of this protein compared to dehydrins. An initial concentration of 1:500 was used for both group 1 and group 2 LEA proteins; however, the blot for group 1 LEA at the concentration of 1:500 was very large compared to the group 2 LEA (dehydrins). This clearly shows that group 1 LEA proteins are more abundant in seeds than group 2 (dehydrins) and is in agreement with the observations made by Boudet *et al.,* (2006) and Gilles *et al.,* (2007). The analysis of group 1 LEA was repeated using a concentration of 1:1000 and the western blots results showed more bands than expected. Probably there was an incomplete reduction of oligomers, hence the higher number of protein bands observed rather than the expected size. The several bands that were also detected in all the lanes suggests that group 1 LEA was present in all the three developmental stages and for the stress and the non-stressed treatments and supports the statement by Gilles (2007) who noted the abundance of these proteins in seed embryos and suggested that group 1 LEA proteins may be important in the amelioration of desiccation related stresses.. The increased expression of both group1 and group 2 LEA genes sets and the higher abundance of group 1 LEA in cowpea seeds subjected to water stress suggest that these two groups may play different roles in desiccating cells in maturing plant seeds.

In Chapter 3 the effect of water stress on the germination and vigour on cowpea seeds was investigated. It was observed that water stress resulted in to the production of fewer seeds but of high quality. However changes may have occurred in the mechanisms at the cellular level which may have modified the embryo and/or accumulated food reserves to influence the development of seed germination and vigour under water stress. This chapter attempts to relate changes in the expression and /or accumulation of group 1 and 2 LEA proteins at the cellular level with seed germination and vigour.

There were no significant difference in germination and vigour of cowpea seeds subjected to water stress and the controls (no stress) and although there is evidence of

an increase in the accumulation of LEA proteins in response to water stress; the evidence does not suggest any association or correlation between germination and vigour and an increase in the expression and/or accumulation of LEA proteins. This is contrary to the observations made by Hara *et al.,* (2003) relating expression of a group 2 protein (citrus dehydrin) to improved germination and growth in tobacco; thus suggesting a relationship between group 2 LEA protein and the development of seed quality.

However, the role of LEA proteins on the improvement of seed quality during development has been contentious (Blackman *et al.,* 1991; Wechsberg *et al.,* 1994). Part of the reasons for these contentious reports could be because most of the studies on LEA proteins are done *in vitro* using mutant and transgenic plants (Rohila *et al.*, 2002; Hara *et al.,* 2003). Such studies may not reflect the complex interactions between plant response mechanisms, environmental and genetic factors. Probably in the present study in which a drought tolerant crop species was used to investigate whether there is an association between LEA protein function and seed quality development under water stress; plant response mechanisms to water stress allowed the cowpea seeds to restore normal plant function and continue with normal metabolism (Bohnert *et al.*, 1995). Hence an increased expression of LEA proteins may be related more to their role as hydration buffers which slow down the rate of water loss during dehydration (Cuming, 1999). The proteins may have also acted as enzyme protectants (Kazuoka and Oeda, 1994) and antioxidants (Tunnacliffe and Wise, 2007). In addition these proteins may have also played a role in membrane stabilisation under water stress (Tunnacliffe and Wise, 2007). Ultimately as the seeds lost water as part of the normal development programme during maturation and as result of water stress, LEA proteins may function to minimise the impact of stress factors on the desiccating cells. LEA proteins have also been proposed to act with specific sugars in the conferment of desiccation tolerance in seeds and plant tissues (Blackman *et al.*, 1991).

The delay in germination in response to the exogenous application of 100 and 1000µM ABA is suggestive of a delay in the loss of desiccation tolerance. This is similar to the observation by Blackman *et al.,* (1991) who observed that in mature soybean seeds the desiccation tolerant phase was prolonged by the application of

exogenous ABA. Loss of desiccation tolerance in germinating seeds has been associated with the disappearance of LEA proteins (Blackman *et al.*, 1991) and the degradation of the RFOs (Bewley and Black, 1994). Attempts have been made to correlate the delay in the acquisition of desiccation tolerance and LEA protein degradation in barley embryos (Bartels *et al.*, 1988) and mature soybean seeds (Blackman *et al.*, 1991). In the present study the delayed loss of desiccation tolerance, could be associated with the inhibition of LEA protein breakdown. In addition the exogenous application of ABA also coincided with the inhibition of breakdown in stachyose, raffinose and verbascose and suppressed sucrose and myoinositol accumulation.

Although the biological relevance of the application of exogenous ABA on the inhibition of germination has been questioned (Finkelstein *et al.*, 2002); the results of the present study may provide some useful insights on the mechanisms by which exogenous ABA may prolong desiccation tolerance. It has been suggested that the primary action for ABA in inhibiting seed germination is the through the control of water uptake of the metabolically active embryo tissues rather than the control of DNA, RNA or protein synthesis (Schopfer *et al.*, 1979). In the present study the delay in the disappearance of the dehydrin proteins and the coincidence between prevention of soluble sugars breakdown is probably due to the effect that high concentrations of exogenous ABA may have had on the reduction of water uptake in the seeds. Imbibition or water up take occurs during the first phase of germination; and a reduction in the rate of water up take may delay the time taken for radicle protrusion. This delay in radicle protrusion is often assumed to imply a delay in the loss of desiccation tolerance.

In conclusion, firstly an *in vivo* study of LEA function in cowpea was able to accurately compare two different groups of LEA proteins in developing cowpea seeds under conditions of water stress. Second, in cowpea seeds both group 1 LEA and group 2 LEA (dehydrin) increased in concentration in response to water stress. Third, group 1 LEA protein has been demonstrated to be relatively abundant in cowpea seeds and may have a role that is seed- specific. Fourth, there may be a maternal influence on LEA protein gene expression under conditions of water stress which may induce dehydrin accumulation during the earlier stages of seed development. Fifth,

the delay in the loss of desiccation tolerance (radicle protrusion) in cowpea seeds in response to exogenous application of ABA may indeed, be associated with a delay in the disappearance of LEA proteins; however, it is more likely to be related to a delay in the germination process as a result of reduced water uptake.

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7 GENERAL DISCUSSION AND CONCLUSIONS

The studies presented in this thesis were based on the hypothesis that environmental and genetic factors which influence seed development and maturation can determine the seed's viability and vigour (Tekrony, 2003). A hypothetical model was developed to try and explain the effect of simulated drought conditions on metabolic processes during development and how this may ultimately influence seed germination and vigour (Figure 7.1). The model proposed that an interaction between drought stress and desiccation stress can alter metabolic processes and modify the composition and quantities of accumulated compounds in the seed. These compounds (sugars and proteins) are implicated in the stabilisation of the macromolecular structure of the cell membranes during the acquisition of desiccation tolerance. Hence alterations in their quantities and composition may result into membrane modifications. This may lead to incomplete or poor acquisition of desiccation tolerance. Membrane integrity is also a fundamental component of seed vigour. Presumably, poor or incomplete acquisition of desiccation tolerance may limit the maximum acquisition of germination and vigour. The hypothetical model was tested by firstly, examining the effect of simulated drought stress on cowpea seed quality with respect to germination capacity and vigour. Secondly, determining the patterns of sucrose, myoinositol, and raffinose family of oligosaccharides (RFOs) and the late embryogenesis abundant (LEA) proteins accumulation during seed development to mature dry stage and using this to physiologically relate seed quality to simulated drought stress.

The observed model (Figure 7.1) shows that myoinositol, stachyose and LEA proteins increased in concentration in response to simulated drought stress. However, simulated drought stress had no effect on the mature seed germination and vigour.

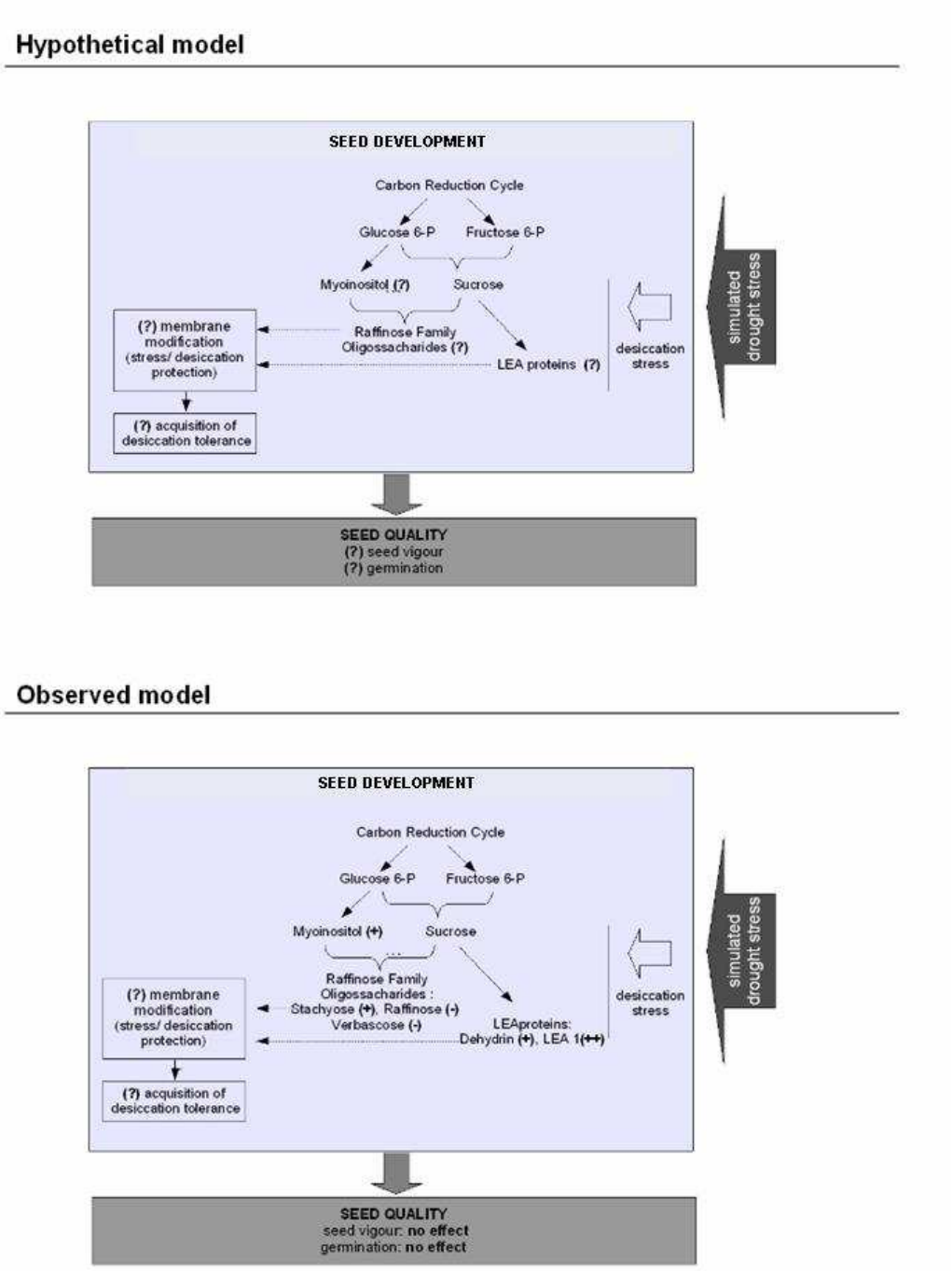

Figure 7-1 Hypothetical and observed (representing the activities of the present study) models showing the relationship between pre-maturation events during development and seed germination and vigour as influenced by water stress. Note: (+) denotes an increase and (-) a decrease.

Simulated drought under controlled environmental conditions resulted in production of fewer seeds per pod, but the seeds were of a high germination and vigour (Table 3.5). These results demonstrate the complex and diverse nature of plant response mechanisms to drought stress, which is often reflected in the apparently contradictory conclusions on the effects of water stress on seed quality. In the literature, reports are found which show that drought stress improved seed quality (Benech -Arnold *et al.*, 1991; Ellis *et al.*, 2000), decreased germination (Dornbos, 1995) or had no effect on germination and vigour (Vieira *et al.*, 1992). Cowpea as a drought-tolerant species has developed drought avoidance strategies at the whole plant and tissue levels (Chapter 3) which were evidently effective in enabling the species to survive simulated drought stress and produce few but high quality seeds.

A positive correlation between the increase in myoinositol and stachyose concentration in response to simulated drought stress is depicted in the observed model (Figure 7.1). In field bean (Lahuta *et al.*, 2000) and cucumber (Widders and Kwantes, 1995) significant changes, which were induced by water stress, observed in relative ratios of raffinose, stachyose and verbascose were linked to the accumulation of myoinositol. It is plausible that this positive increase is associated with a build up in desiccation tolerance in response to the combined effect of desiccation and simulated drought stress. In field bean, Lahuta *et al.* (2000) related changes in the relative ratios of RFOs to a build up in desiccation tolerance.

In the observed model, both group 1 (LEA 1) and 2 LEA (dehydrins) proteins are shown to increase in response to the combined effect of desiccation and drought stress. In the present study, dehydrin-like proteins were detected after two weeks of development in seeds harvested from plants that had been subjected to stress during the vegetative growth phase only (chapter 6). This is suggestive of a maternal influence on LEA protein gene expression, which may have induced dehydrin accumulation during the earlier stages of seed development. Similarly the possibility of a maternal suppression of dehydrin accumulation was alluded to by Black *et al.* (1999), who observed that detachment of wheat grains from the mother plant induced the appearance of dehydrin at an earlier stage of development. The observation that group 2 LEA dehydrin have also been detected in seeds that remain desiccation-sensitive at shedding (Kermode, 1997) casts some doubt on their specific role in the

acquisition of desiccation tolerance in orthodox seeds. Probably, as concluded by Black *et al,* (1999) dehydrin accumulation is not regulated by factors that specifically control the induction of desiccation tolerance in orthodox seeds.

In the observed model (Figure 7.1) LEA 1 protein was more abundant in the seeds than dehydrin. This may possibly support the argument that group 1 LEAs are seed specific as has been suggested by Boudet *et al.* (2006) and Giles *et al* .(2007). The increased concentration of LEA1 may be related to protection against desiccation and drought stress injury in cowpea seeds. These proteins may function to reduce oxidative stress in desiccating cells subjected to simulated drought stress by scavenging Reactive Oxygen Species (ROS) and indirectly by sequestering metal ions that generate (ROS) (Dure, 1993; Danyluk *et al.*, 1998). The proteins might also act as hydration buffers, slowing down the rate of water loss during dehydration (Cuming, 1999) thus allowing sufficient water activity for proteins to retain their functions.

In the observed model, direct evidence showing that membranes were modified is not presented because no tests were done to investigate changes in membrane structure. However since this was an *in vivo* study it can be can be speculated that the increase in stachyose and both LEA1 and dehydrin protein is indicative of modifications in cell membrane structure. The possible effect of such modifications on the acquisition of desiccation tolerance is also speculative. Thus it may be possible that an increase in stachyose and LEA proteins may have modified cell membranes and led to a build up in desiccation tolerance as suggested by Lahuta *et al.* (2000). On the other hand, membrane modifications may have led to poor or incomplete acquisition of desiccation tolerance. Alternatively the primary function of an increased accumulation of these compounds was probably protection against desiccation and drought stress injury. Consequently there was no effect on the acquisition of desiccation tolerance because the cells could continue with their normal functions.

The electrical conductivity test may have provided an indirect way of examining changes in the membrane structure and function (chapter 4). Notwithstanding the findings presented in chapter 4, a limitation of this study was that the observed model was not able to explain whether membranes were modified or not and whether these

modifications had an effect on the acquisition of desiccation tolerance. The results discussed in Chapter 4 demonstrate the ability of the conductivity test to detect differences in solute leakage in seeds produced between at different sites and subjected to accelerated aging stress. Significant differences in solute leakage between aged and unaged seeds may have been indicative of loss of membrane integrity.

In the hypothetical model (Figure 7.1), the relationship between acquisition of desiccation tolerance and seed germination and vigour is not shown. The observed model does not explain this relationship; however it (observed model) shows that the demonstrated increase in both sugars and proteins in response to simulated drought had no effect on seed germination and vigour. This is contrary to the report by Hara *et al.* (2003) who associated LEA protein expression with improved germination in tobacco. Apparently the role of LEA proteins and sugars on the improvement of seed quality during development is contentious (Blackman *et al.,* 1991; Wechsberg *et al.,* 1994). Probably the explanation is that optimal germination and vigour require a minimum level of desiccation tolerance.

However, it can be proposed that the increased accumulation of stachyose could be used as an indicator of stress tolerance in cowpea. In chapter 3 differences in stress tolerance/sensitivity of cowpea cultivars was shown to be considerable (Figure 3.7). The cultivar Kenkunde showed extreme sensitivity to stress. Nzovu which was able to produce adequate seed under all conditions also showed the highest stachyose concentration (Figure 5.2).

Similarly, based on the newly developed Aging Stress Differential Index (ASDI) (Figure 4.8) this study has demonstrated a link between seed coat colour and sensitivity to water stress. The ASDI correlated well with the observations in chapter 3 relating stress tolerance to stachyose accumulation. This is because light coloured seeds of M66 which showed the highest sensitivity to water stress also had the lowest stachyose content (Figure 5.2). The cultivar M66 which was developed as a drought tolerant cultivar clearly showed that plant drought tolerance is not associated with

seed quality (Table 4.1). This was evident in the poor performance of M66 seeds after the AA test (Table 4.1).

Image analysis was examined as a method that can be used to objectively determine seed coat colour variation in cowpea (Chapter 4). The study demonstrated that image analysis, and more specifically the colour parameter hue, can be used to objectively discriminate seed coat colour variation in cowpea. The use of image analysis could in future provide a basis for the development of a rapid and non-destructive method of seed vigour assessment.

This study explored the use of box-whisker plots in quantifying variability in individual seed electrical conductivity (Chapter 4). Box-whisker plots provided a good illustration of the variability in electrical conductivity particularly in aged seeds (Figure 4.4). The usefulness of box-whisker plots in portraying the variation in individual electrical conductivity between cultivars was clearly illustrated by the side by side comparison of cultivars within production sites and between sites for both unaged and aged seeds. The study has demonstrated that distribution patterns in individual electrical conductivity can be skewed. The skewness and the presence of extreme values may have implications with respect to the suitability of using standard statistical analyses which compare mean values to evaluate such data. The poor correlation between ranked electrical conductivity values and germination capacity and vigour (Table 4.1) was probably due to the lack of normality in the distribution of individual electrical conductivity values. Variation in individual electrical conductivity values was also not correlated with seed coat colour (Figure 4.3).

In many studies of water stress, methods of quantifying the intensity and duration of stress are often time consuming and laborious. Differences in the methods of quantifying stress intensity may also contribute to the contradictory conclusions often found in the literature about water stress effects on seed quality. In the present study, the Thetaprobe water sensor was compared with direct gravimetric methods and found to be a reliable approach of monitoring water stress in pot experiments. It is recommended that more studies should be conducted on the use of water sensors in monitoring water stress in pot experiments.

From the findings of the study, it is recommended that future studies should use a proteomic approach in investigating protein structures and functions in cowpea seeds in relation to environmental stress conditions and the development of seed quality. The use of tools such as Mass Spectrometry and FTIR spectroscopy in a proteomic analysis may provide a much better understanding of the interactions between different proteins and the environmental variables in relation to seed quality development.

A major limitation of this study was the differences in harvesting methods between the two sites (Chapter 2). These differences occurred as a result of certain mitigating factors beyond the control of the author. However, the potential implications of these differences are comprehensively discussed in Chapter 2 and 4 and did not generally have significant effects on the aims and objectives of study.

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Notwithstanding the limitations of the research performed during this study, it can be concluded that cultivar differences in relation to stress tolerance/sensitivity in cowpea are considerable. Changes in seed moisture content during development are associated with seed quality in cowpea. Cowpea seed vigour in response to water stress can be explained by changes in soluble carbohydrates concentrations and expression of late embryogenesis abundant proteins.

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APPENDICES

Note: Appendices are numbered according to the relevant chapters.

Appendix 2.1 Field layout showing treatments in RCBD with 3 replications

Ukulinga

Rep 1

1 C5	2 C4	3 C2	4 C6	5 C3	6 C1

Rep 2

12 C6	11 C2	10 C5	9 C3	8 C1	7 C4

Rep 3

13 C2	14 C5	15 C3	16 C4	17 C6	18 C1

Field Layout – Umbumbulu

Rep 1

1 C4	2 C5	3 C6	4 C2	5 C3	6 C1

Rep 2

12 C1	11 C4	10 C3	9 C5	8 C2	7 C6

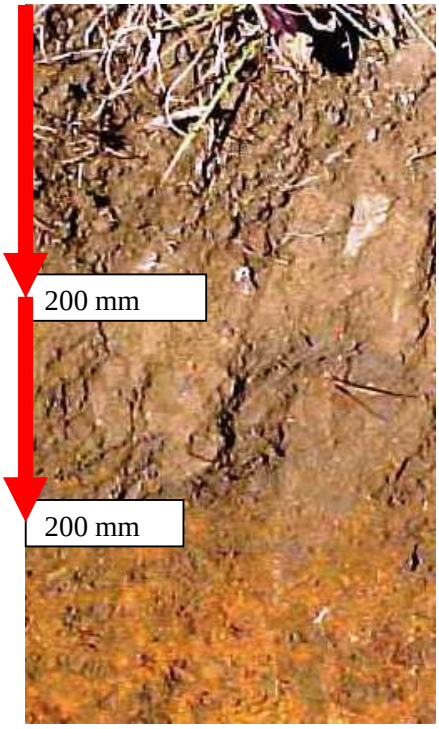
Rep 3

13 C3	14 C4	15 C2	16 C5	17 C1	18 C6

Plot size = 3 x 4m
Plant spacing 0.75 x 0.20m
Treatments = 6 cowpea cultivars: C1= Brown Mix, C2= Makonge, C3 = Nzovu, C4 = K80, C5 = M66, C6 = Kenkunde
Note that paths between blocks = inter row spacing

Appendix 2.2 Soil classification and analysis data

(a) Ukulinga – Avalon Mafikeng

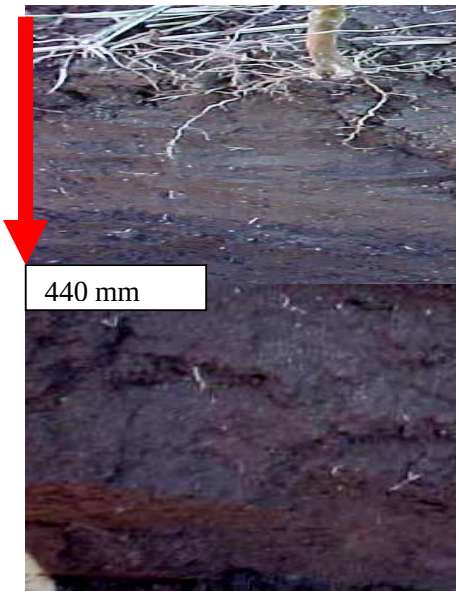


A – horizon: Orthic
Very dark greyish brown with blocky structure. Many fine to medium roots, self mulching properties with friable consistence

B1 – horizon: Yellow brown apedal
Very greyish brown soil colour containing very few fine roots with hard consistence. Has more clay content than B and has few yellowish and brownish congruent. The transition from A to B1 is not abrupt

B2 – horizon: Soft plinthic
Dark brown soil colour. The transition from B1 to B2 is not abrupt..

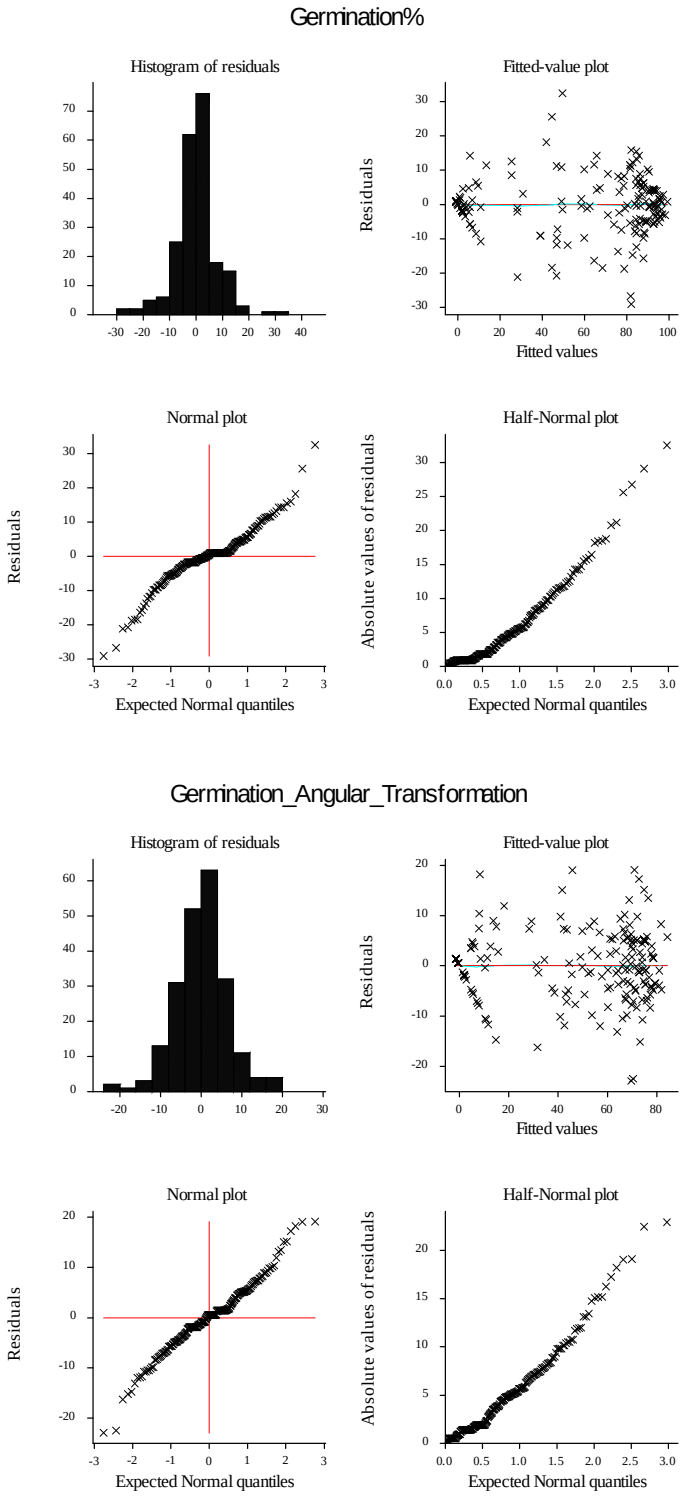
(b) Umbumbulu- Magwa Nstubane



A – horizon : melanic
Very dark brown soil with many fine and medium size roots and friable consistence and has a weak massive structure

B – horizon: Yellow brown apedal
Friable Massive structure with very fine and medium size roots. More clay content than A horizon. Well drained consistence.

Appendix 2.3 Testing assumptions underlying the use of analysis of variance



Appendix 2.4 ANOVA TABLES (CHAPTER 2)
2.4 (a) Analysis of variance (transformed germination data for Ukulinga)

```
 1019 “General Analysis of Variance."
 1020  BLOCK Rep
 1021  TREATMENTS Cultivar*Stage
 1022  COVARIATE "No Covariate"
 1023  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 1024   PSE=diff,lsd; LSDLEVEL=5] Germination_Angular_transformation
```

Variate: Germination_Angular_transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	596.43	298.22	3.78	
Rep.*Units* stratum					
Cultivar	5	497.20	99.44	1.26	0.291
Stage	5	97272.89	19454.58	246.71	<.001
Cultivar. Stage	25	3182.01	127.28	1.61	0.061
Residual	70	5519.96	78.86		
Total	107	107068.49			

Standard errors of differences of means

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
s.e.d.	2.960	2.960	7.251

Least significant differences of means (5% level)

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
l.s.d.	5.904	5.904	14.461

Stratum standard errors and coefficients of variation

Variate: Germination_Angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	2	2.878	6.2
Rep.*Units*	70	8.880	19.1

2.4 (b) Analysis of variance (transformed germination data for Umbumbulu)

```
1097 “General Analysis of Variance."
 1098  BLOCK Rep
 1099  TREATMENTS Cultivar*Stage
 1100  COVARIATE "No Covariate"
 1101  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 1102   PSE=diff,lsd; LSDLEVEL=5] Germination_angular_transformation
```

Variate: Germination_angular_transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	20.95	10.48	0.22	
Rep.*Units* stratum					
Cultivar	5	2556.12	511.22	10.53	<.001
Stage	5	100996.92	20199.38	416.10	<.001
Cultivar. Stage	25	6772.54	270.90	5.58	<.001
Residual	70	3398.13	48.54		
Total	107	113744.65			

Standard errors of differences of means

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
s.e.d.	2.322	2.322	5.689

Least significant differences of means (5% level)

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
l.s.d.	4.632	4.632	11.346

Stratum standard errors and coefficients of variation

Variate: Germination_angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	2	0.539	1.5
Rep.*Units*	70	6.967	19.1

2.4 (c) Analysis of variance (transformed germination index data for Ukulinga)

```
 9367 “General Analysis of Variance."
 9368  BLOCK Rep
 9369  TREATMENTS Cultivar*Stage
 9370  COVARIATE "No Covariate"
 9371  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 9372   PSE=diff,lsd; LSDLEVEL=5] GI_transformed
```

Variate: GI_transformed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.3594	0.1797	1.36	
Rep.*Units* stratum					
Cultivar	5	1.0671	0.2134	1.61	0.168
Stage	5	609.7037	121.9407	920.63	<.001
Cultivar. Stage	25	10.5353	0.4214	3.18	<.001
Residual	70	9.2718	0.1325		
Total	107	630.9373			

Standard errors of differences of means

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
s.e.d.	0.1213	0.1213	0.2972

Least significant differences of means (5% level)

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
l.s.d.	0.2420	0.2420	0.5927

Stratum standard errors and coefficients of variation

Variate: GI_transformed

Stratum	d.f.	s.e.	cv%
Rep	2	0.0707	2.0
Rep.*Units*	70	0.3639	10.1

2.4 (d) Analysis of variance (transformed germination index data for Umbumbulu)

```
9520 “General Analysis of Variance."
 9521  BLOCK Rep
 9522  TREATMENTS Cultivar*Stage
 9523  COVARIATE "No Covariate"
 9524  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 9525   PSE=diff,lsd; LSDLEVEL=5] GI_transformed
```

Variate: GI_transformed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.1722	0.0861	0.34	
Rep.*Units* stratum					
Cultivar	5	5.3245	1.0649	4.24	0.002
Stage	5	585.9468	117.1894	466.15	<.001
Cultivar. Stage	25	8.9952	0.3598	1.43	0.122
Residual	70	17.5978	0.2514		
Total	107	618.0365			

Standard errors of differences of means

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
s.e.d.	0.1671	0.1671	0.4094

Least significant differences of means (5% level)

Table	Cultivar	Stage	Cultivar Stage
rep.	18	18	3
d.f.	70	70	70
l.s.d.	0.3333	0.3333	0.8165

Stratum standard errors and coefficients of variation

Variate: GI_transformed

Stratum	d.f.	s.e.	cv%
Rep	2	0.0489	1.5
Rep.*Units*	70	0.5014	15.7

2.4 (e) Testing for homogeneity of variances between Ukulinga and Umbumbulu sites

Germination percentage

F statistic at 70 df = 1.62
Critical F-value at 70 df for both numerator and denominator at the 1% level of significance = 1.89

Germination index

F statistic at 70 df = 1.89
Critical F-value at 70 df for both numerator and denominator at 1% level of significance = 1.89

Comment: The ANOVA for the separate analysis for both sites (Ukulinga and Umbumbulu) are consistent with those before transformation. The F-calculated is not greater than F-tabulated, we therefore fail to reject the Null Hypothesis and conclude that there is a common variance. Hence data from both sites were pooled for subsequent analysis.

2.4 (f) Analysis of variance (transformed pooled germination data for Ukulinga and Umbumbulu)

```
97 “General Analysis of Variance."
 98  BLOCK Rep
 99  TREATMENTS Site*Cultivar*Stage
100  COVARIATE "No Covariate"
101  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
102   PSE=diff,lsd; LSDLEVEL=5] Germination_Angular_Transformation
```

Variate: Germination_Angular_Transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	420.48	210.24	3.28	
Rep.*Units* stratum					
Site	1	5301.02	5301.02	82.58	<.001
Cultivar	5	2604.07	520.81	8.11	<.001
Stage	5	189116.32	37823.26	589.24	<.001
Site. Cultivar	5	449.25	89.85	1.40	0.228
Site. Stage	5	9153.49	1830.70	28.52	<.001
Cultivar. Stage	25	5882.41	235.30	3.67	<.001
Site.Cultivar.Stage	25	4072.14	162.89	2.54	<.001
Residual	142	9114.99	64.19		
Total	215	226114.16			

Standard errors of differences of means

Table	Site	Cultivar	Stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
s.e.d.	1.090	1.888	1.888	2.671

Table	Site Stage	Cultivar Stage	Site Cultivar Stage
rep.	18	6	3
d.f.	142	142	142
s.e.d.	2.671	4.626	6.542

Least significant differences of means (5% level)

Table	Site	Cultivar	Stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
l.s.d.	2.155	3.733	3.733	5.279

Table	Site Stage	Cultivar Stage	Site Cultivar Stage
rep.	18	6	3
d.f.	142	142	142
l.s.d.	5.279	9.144	12.932

Stratum standard errors and coefficients of variation

Variate: Germination_Angular_Transformation

Stratum	d.f.	s.e.	cv%
Rep	2	1.709	4.1
Rep.*Units*	142	8.012	19.3

Comment: The ANOVA is consistent with that before transformation. The original data was used in the presentation and discussion of the results.

2.4 (g) Analysis of variance (transformed pooled germination index data for Ukulinga and Umbumbulu)

```
 733 “General Analysis of Variance."
 734  BLOCK Replicate
 735  TREATMENTS Site*Cultivar*Podding_stage
 736  COVARIATE "No Covariate"
 737  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 738   PSE=diff,lsd; LSDLEVEL=5] Germination_index_transformed
```

Variate: Germination_index_transformed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	2	0.3757	0.1879	0.99	
Replicate.*Units* stratum					
Site	1	9.9303	9.9303	52.18	<.001
Cultivar	5	5.1347	1.0269	5.40	<.001
Podding_stage	5	1156.0044	231.2009	1214.80	<.001
Site. Cultivar	5	1.2570	0.2514	1.32	0.259
Site.Podding_stage	5	39.6461	7.9292	41.66	<.001
Cultivar.Podding_stage	25	10.9264	0.4371	2.30	0.001
Site.Cultivar.Podding_stage	25	8.6040	0.3442	1.81	0.017
Residual	142	27.0255	0.1903		
Total	215	1258.9041			

Standard errors of differences of means

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
s.e.d.	0.0594	0.1028	0.1028	0.1454

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	18	6	3
d.f.	142	142	142
s.e.d.	0.1454	0.2519	0.3562

Least significant differences of means (5% level)

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
l.s.d.	0.1174	0.2033	0.2033	0.2875

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	18	6	3
d.f.	142	142	142
l.s.d.	0.2875	0.4979	0.7041

Stratum standard errors and coefficients of variation

Variate: Germination_index_transformed

Stratum	d.f.	s.e.	cv%
Replicate	2	0.0511	1.5
Replicate.*Units*	142	0.4363	12.8

Comment: The ANOVA is consistent with that before transformation. The original data was used in the presentation and discussion of the results.

2.4 (h) Analysis of seed water content (g water g^{-1} seed) (pooled data for Ukulinga and Umbumbulu sites)

1360 “General Analysis of Variance."
1361 BLOCK Rep
1362 TREATMENTS Site*Cultivar*Podding_stage
1363 COVARIATE "No Covariate"
1364 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
1365 PSE=diff,lsd; LSDLEVEL=5] grams_water_per_g_seed_FWB

Variate: grams_water_per_g_seed_FWB

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.004548	0.002274	0.97	
Rep.*Units* stratum					
Site	1	0.607253	0.607253	258.03	<.001
Cultivar	5	0.274852	0.054970	23.36	<.001
Podding_stage	5	17.487708	3.497542	1486.14	<.001
Site. Cultivar	5	0.067202	0.013440	5.71	<.001
Site.Podding_stage	5	0.212998	0.042600	18.10	<.001
Cultivar.Podding_stage	25	0.275250	0.011010	4.68	<.001
Site.Cultivar.Podding_stage	25	0.347757	0.013910	5.91	<.001
Residual	142	0.334189	0.002353		
Total	215	19.611756			

Standard errors of differences of means

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
s.e.d.	0.00660	0.01143	0.01143	0.01617

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	18	6	3
d.f.	142	142	142
s.e.d.	0.01617	0.02801	0.03961

Least significant differences of means (5% level)

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	108	36	36	18
d.f.	142	142	142	142
l.s.d.	0.01305	0.02260	0.02260	0.03197

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	18	6	3
d.f.	142	142	142
l.s.d.	0.03197	0.05537	0.07830

Stratum standard errors and coefficients of variation

Variate: grams_water_per_g_seed_FWB

Stratum	d.f.	s.e.	cv%
Rep	2	0.00562	1.2
Rep.*Units*	142	0.04851	10.6

2.4 (i) Analysis of dry matter accumulation (g) (pooled data for Ukulinga and Umbumbulu sites)

```
1623 “General Analysis of Variance."
 1624  BLOCK Rep
 1625  TREATMENTS Site*Cultivar*Podding_stage
 1626  COVARIATE "No Covariate"
 1627  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 1628   PSE=diff,lsd; LSDLEVEL=5] Dry_matter
```

Variate: Dry_matter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.000508	0.000254	0.03	
Rep.*Units* stratum					
Site	1	0.528720	0.528720	71.41	<.001
Cultivar	3	0.657628	0.219209	29.61	<.001
Podding_stage	3	25.638955	8.546318	1154.36	<.001
Site. Cultivar	3	0.070349	0.023450	3.17	0.031
Site.Podding_stage	3	1.432197	0.477399	64.48	<.001
Cultivar.Podding_stage	9	0.735525	0.081725	11.04	<.001
Site.Cultivar.Podding_stage	9	0.092936	0.010326	1.39	0.210
Residual	62	0.459017	0.007403		
Total	95	29.615835			

Standard errors of differences of means

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	48	24	24	12
d.f.	62	62	62	62
s.e.d.	0.01756	0.02484	0.02484	0.03513

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	12	6	3
d.f.	62	62	62
s.e.d.	0.03513	0.04968	0.07025

Least significant differences of means (5% level)

Table	Site	Cultivar	Podding_stage	Site Cultivar
rep.	48	24	24	12
d.f.	62	62	62	62
l.s.d.	0.03511	0.04965	0.04965	0.07022

Table	Site Podding_stage	Cultivar Podding_stage	Site Cultivar Podding_stage
rep.	12	6	3
d.f.	62	62	62
l.s.d.	0.07022	0.09930	0.14044

Stratum standard errors and coefficients of variation

Variate: Dry_matter

Stratum	d.f.	s.e.	cv%
Rep	2	0.00282	0.4
Rep.*Units*	62	0.08604	13.5

2.4 (j) Analysis of transformed field emergence data (%) (Pooled data for Ukulinga and Umbumbulu sites)

```
1712 “General Analysis of Variance."
 1713  BLOCK Rep
 1714  TREATMENTS Site*Cultivar
 1715  COVARIATE "No Covariate"
 1716  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 1717   PSE=diff,lsd; LSDLEVEL=5] Emergence_Angular_transformation
```

Variate: Emergence_Angular_transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	173.72	86.86	1.59	
Rep.*Units* stratum					
Site	1	925.28	925.28	16.89	<.001
Cultivar	5	276.34	55.27	1.01	0.436
Site. Cultivar	5	39.33	7.87	0.14	0.980
Residual	22	1205.11	54.78		
Total	35	2619.78			

Standard errors of differences of means

Table	Site	Cultivar	Site Cultivar
rep.	18	6	3
d.f.	22	22	22
s.e.d.	2.47	4.27	6.04

Least significant differences of means (5% level)

Table	Site	Cultivar	Site Cultivar
rep.	18	6	3
d.f.	22	22	22
l.s.d.	5.12	8.86	12.53

Stratum standard errors and coefficients of variation

Variate: Emergence_Angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	2	2.69	4.1
Rep.*Units*	22	7.40	11.2

Appendix 3.1 ANOVA TABLES (CHAPTER 3)
3.1 (a) Analysis of variance for germination percentage (transformed data)

1889 “General Analysis of Variance."
1890 BLOCK Rep
1891 TREATMENTS Site*Cultivar*Stress
1892 COVARIATE "No Covariate"
1893 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
1894 PSE=diff,lsd; LSDLEVEL=5] Germination_angular_transformation

Variate: Germination_angular_transformation

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Rep stratum	4		2425.41	606.35	7.69	
Rep.*Units* stratum						
Site	1		1494.05	1494.05	18.95	<.001
Cultivar	2		456.63	228.31	2.90	0.067
Stress	2		694.41	347.20	4.40	0.019
Site. Cultivar	2		1039.68	519.84	6.60	0.003
Site. Stress	2		1105.23	552.62	7.01	0.002
Cultivar. Stress	3	(1)	394.90	131.63	1.67	0.189
Site.Cultivar.Stress	3	(1)	1170.18	390.06	4.95	0.005
Residual	40	(28)	3152.94	78.82		
Total	59	(30)	6617.11			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
s.e.d.	1.872	2.292	2.292	3.242

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
s.e.d.	3.242	3.970	5.615

(Not adjusted for missing values)

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
l.s.d.	3.783	4.633	4.633	6.552

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
l.s.d.	6.552	8.025	11.349

(Not adjusted for missing values)

Stratum standard errors and coefficients of variation

Variate: Germination_angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	4	5.804	7.0
Rep.*Units*	40	8.878	10.7

3.1 (b) Analysis of variance for germination index (transformed data)

2091 “General Analysis of Variance."
2092 BLOCK Rep
2093 TREATMENTS Site*Cultivar*Stress
2094 COVARIATE "No Covariate"
2095 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
2096 PSE=diff,lsd; LSDLEVEL=5] GI_tansformed

Variate: GI_tansformed

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Rep stratum	4		0.014787	0.003697	1.65	
Rep.*Units* stratum						
Site	1		0.097829	0.097829	43.65	<.001
Cultivar	2		0.310939	0.155470	69.37	<.001
Stress	2		0.053391	0.026695	11.91	<.001
Site. Cultivar	2		0.146736	0.073368	32.74	<.001
Site. Stress	2		0.131417	0.065709	29.32	<.001
Cultivar. Stress	3	(1)	0.011386	0.003795	1.69	0.184
Site.Cultivar.Stress	3	(1)	0.088714	0.029571	13.19	<.001
Residual	40	(28)	0.089646	0.002241		
Total	59	(30)	0.473522			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
s.e.d.	0.00998	0.01222	0.01222	0.01729

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
s.e.d.	0.01729	0.02117	0.02994

(Not adjusted for missing values)

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
l.s.d.	0.02017	0.02470	0.02470	0.03494

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
l.s.d.	0.03494	0.04279	0.06051

(Not adjusted for missing values)

Stratum standard errors and coefficients of variation

Variate: GI_tansformed

Stratum	d.f.	s.e.	cv%
Rep	4	0.01433	0.3
Rep.*Units*	40	0.04734	0.9

3.1 (c) Analysis of variance for mean germination time (days)

```
2201 “General Analysis of Variance."
 2202  BLOCK Rep
 2203  TREATMENTS Site*Cultivar*Stress
 2204  COVARIATE "No Covariate"
 2205  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 2206   PSE=diff,lsd; LSDLEVEL=5] MGT
```

Variate: MGT

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Rep stratum	4		0.09901	0.02475	1.72	
Rep.*Units* stratum						
Site	1		0.78253	0.78253	54.48	<.001
Cultivar	2		2.87003	1.43502	99.90	<.001
Stress	2		0.23769	0.11885	8.27	<.001
Site. Cultivar	2		0.90703	0.45351	31.57	<.001
Site. Stress	2		0.76278	0.38139	26.55	<.001
Cultivar. Stress	3	(1)	0.15128	0.05043	3.51	0.024
Site.Cultivar.Stress	3	(1)	0.71143	0.23714	16.51	<.001
Residual	40	(28)	0.57459	0.01436		
Total	59	(30)	3.63168			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
s.e.d.	0.0253	0.0309	0.0309	0.0438

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
s.e.d.	0.0438	0.0536	0.0758

(Not adjusted for missing values)

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
l.s.d.	0.0511	0.0625	0.0625	0.0885

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
l.s.d.	0.0885	0.1083	0.1532

(Not adjusted for missing values)

Stratum standard errors and coefficients of variation

Variate: MGT

Stratum	d.f.	s.e.	cv%
Rep	4	0.0371	2.2
Rep.*Units*	40	0.1199	7.1

3.1 (d) Analysis of variance for seedling dry weight (mg seedling^{-1})

2311 “General Analysis of Variance."
2312 BLOCK Rep
2313 TREATMENTS Site*Cultivar*Stress
2314 COVARIATE "No Covariate"
2315 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
2316 PSE=diff,lsd; LSDLEVEL=5] Seedling_dry_weight

Variate: Seedling_dry_weight

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Rep stratum	4		349.83	87.46	2.23	
Rep.*Units* stratum						
Site	1		2528.07	2528.07	64.50	<.001
Cultivar	2		1791.08	895.54	22.85	<.001
Stress	2		420.02	210.01	5.36	0.009
Site. Cultivar	2		1173.82	586.91	14.97	<.001
Site. Stress	2		3490.04	1745.02	44.52	<.001
Cultivar. Stress	3	(1)	1230.74	410.25	10.47	<.001
Site.Cultivar.Stress	3	(1)	366.74	122.25	3.12	0.037
Residual	40	(28)	1567.81	39.20		
Total	59	(30)	7370.92			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
s.e.d.	1.320	1.616	1.616	2.286

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
s.e.d.	2.286	2.800	3.960

(Not adjusted for missing values)

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	40	40	40	40
l.s.d.	2.668	3.267	3.267	4.620

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	40	40	40
l.s.d.	4.620	5.659	8.003

(Not adjusted for missing values)

Stratum standard errors and coefficients of variation

Variate: Seedling_dry_weight

Stratum	d.f.	s.e.	cv%
Rep	4	2.204	3.3
Rep.*Units*	40	6.261	9.5

3.1 (e) Analysis of variance for mean number of seeds (plant^{-1})

```
2443 “General Analysis of Variance."
 2444  BLOCK Rep
 2445  TREATMENTS Site*Cultivar*Stress
 2446  COVARIATE "No Covariate"
 2447  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 2448   PSE=diff,lsd; LSDLEVEL=5] No_of_seeds
```

Variate: No_of_seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	763.4	190.8	1.14	
Rep.*Units* stratum					
Site	1	51.4	51.4	0.31	0.582
Cultivar	2	32372.1	16186.0	96.31	<.001
Stress	2	75103.2	37551.6	223.44	<.001
Site.Cultivar	2	981.6	490.8	2.92	0.061
Site.Stress	2	28.1	14.0	0.08	0.920
Cultivar. Stress	4	15977.1	3994.3	23.77	<.001
Site.Cultivar.Stress	4	1978.5	494.6	2.94	0.026
Residual	68	11428.2	168.1		
Total	89	138683.6			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	68	68	68	68
s.e.d.	2.73	3.35	3.35	4.73

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	68	68	68
s.e.d.	4.73	5.80	8.20

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site

				Cultivar
rep.	45	30	30	15
d.f.	68	68	68	68
l.s.d.	5.45	6.68	6.68	9.45
Table	Site Stress	Cultivar Stress	Site Cultivar Stress	
rep.	15	10	5	
d.f.	68	68	68	
l.s.d.	9.45	11.57	16.36	

Stratum standard errors and coefficients of variation

Variate: No_of_seeds

Stratum	d.f.	s.e.	cv%
Rep	4	3.26	10.2
Rep.*Units*	68	12.96	40.4

3.1 (f) Analysis of variance for seed mass (plant^{-1})

```
2449 “General Analysis of Variance."
 2450  BLOCK Rep
 2451  TREATMENTS Site*Cultivar*Stress
 2452  COVARIATE "No Covariate"
 2453  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 2454   PSE=diff,lsd; LSDLEVEL=5] Yield
```

Variate: Yield

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	1.623E+07	4.058E+06	2.08	
Rep.*Units* stratum					
Site	1	8.717E+06	8.717E+06	4.46	0.038
Cultivar	2	3.001E+08	1.500E+08	76.78	<.001
Stress	2	9.801E+08	4.901E+08	250.78	<.001
Site.Cultivar	2	6.796E+06	3.398E+06	1.74	0.183
Site.Stress	2	1.123E+07	5.613E+06	2.87	0.063
Cultivar. Stress	4	1.063E+08	2.658E+07	13.60	<.001
Site.Cultivar.Stress	4	1.727E+07	4.317E+06	2.21	0.077
Residual	68	1.329E+08	1.954E+06		
Total	89	1.580E+09			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	68	68	68	68
s.e.d.	294.7	360.9	360.9	510.4

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	68	68	68
s.e.d.	510.4	625.2	884.1

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	45	30	30	15
d.f.	68	68	68	68
l.s.d.	588.1	720.2	720.2	1018.6

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	15	10	5
d.f.	68	68	68
l.s.d.	1018.6	1247.5	1764.2

Stratum standard errors and coefficients of variation

Variate: Yield

Stratum	d.f.	s.e.	cv%
Rep	4	474.8	12.7
Rep.*Units*	68	1397.9	37.4

3.1 (g) Data for mean number of seeds re-analysed after for two cultivars (Nzovu and Kenkunde) after the deletion of M66

```
2558 “General Analysis of Variance."
 2559  BLOCK Rep
 2560  TREATMENTS Site*Cultivar*Stress
 2561  COVARIATE "No Covariate"
 2562  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 2563   PSE=diff,lsd; LSDLEVEL=5] No_of_seeds
```

Variate: No_of_seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	1158.3	289.6	1.33	
Rep.*Units* stratum					
Site	1	64.1	64.1	0.29	0.590
Cultivar	1	16467.3	16467.3	75.75	<.001
Stress	2	73510.2	36755.1	169.07	<.001
Site.Cultivar	1	405.6	405.6	1.87	0.179
Site.Stress	2	307.0	153.5	0.71	0.499
Cultivar. Stress	2	8750.0	4375.0	20.12	<.001
Site.Cultivar.Stress	2	747.1	373.6	1.72	0.191
Residual	44	9565.3	217.4		
Total	59	110974.9			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	30	30	20	15
d.f.	44	44	44	44
s.e.d.	3.81	3.81	4.66	5.38

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	10	10	5
d.f.	44	44	44
s.e.d.	6.59	6.59	9.33

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	30	30	20	15
d.f.	44	44	44	44
l.s.d.	7.67	7.67	9.40	10.85

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	10	10	5
d.f.	44	44	44
l.s.d.	13.29	13.29	18.79

Stratum standard errors and coefficients of variation

Variate: No_of_seeds

Stratum	d.f.	s.e.	cv%
Rep	4	4.91	11.8
Rep.*Units*	44	14.74	35.6

3.1 (h) Data for seed yield re-analysed after for two cultivars (Nzovu and Kenkunde) after the deletion of M66

```
2564 “General Analysis of Variance."
 2565  BLOCK Rep
 2566  TREATMENTS Site*Cultivar*Stress
 2567  COVARIATE "No Covariate"
 2568  ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32;
CONTRASTS=7; FPROB=yes;\
 2569   PSE=diff,lsd; LSDLEVEL=5] Yield
```

Variate: Yield

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	2.558E+07	6.396E+06	2.66	
Rep.*Units* stratum					
Site	1	1.488E+06	1.488E+06	0.62	0.436
Cultivar	1	1.320E+08	1.320E+08	54.83	<.001
Stress	2	8.712E+08	4.356E+08	180.97	<.001
Site.Cultivar	1	2.542E+06	2.542E+06	1.06	0.310
Site.Stress	2	6.704E+05	3.352E+05	0.14	0.870
Cultivar. Stress	2	4.767E+07	2.383E+07	9.90	<.001
Site.Cultivar.Stress	2	5.779E+06	2.889E+06	1.20	0.311
Residual	44	1.059E+08	2.407E+06		
Total	59	1.193E+09			

Standard errors of differences of means

Table	Site	Cultivar	Stress	Site Cultivar
rep.	30	30	20	15
d.f.	44	44	44	44
s.e.d.	400.6	400.6	490.6	566.5

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	10	10	5
d.f.	44	44	44
s.e.d.	693.8	693.8	981.2

Least significant differences of means (5% level)

Table	Site	Cultivar	Stress	Site Cultivar
rep.	30	30	20	15
d.f.	44	44	44	44
l.s.d.	807.3	807.3	988.8	1141.7

Table	Site Stress	Cultivar Stress	Site Cultivar Stress
rep.	10	10	5
d.f.	44	44	44
l.s.d.	1398.3	1398.3	1977.6

Stratum standard errors and coefficients of variation

Variate: Yield

Stratum	d.f.	s.e.	cv%
Rep	4	730.1	15.5
Rep.*Units*	44	1551.5	33.0

Appendix 4.1 ANOVA TABLES (CHAPTER 4)
4.1 (a) Analysis of variance for EC before and after accelerated aging test

4039 “General Analysis of Variance."
4040 BLOCK Rep
4041 TREATMENTS Site*Cultivar*Days*Interval
4042 COVARIATE "No Covariate"
4043 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
4044 PSE=diff,lsd; LSDLEVEL=5] EC

Variate: EC

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	9	5.059E+07	5.621E+06	26.87	
Rep.*Units* stratum					
Site	1	1.100E+08	1.100E+08	525.82	<.001
Cultivar	4	1.609E+08	4.022E+07	192.26	<.001
Days	1	2.694E+08	2.694E+08	1287.61	<.001
Interval	24	1.883E+09	7.846E+07	375.06	<.001
Site.Cultivar	4	2.328E+07	5.820E+06	27.82	<.001
Site. Days	1	4.812E+07	4.812E+07	230.01	<.001
Cultivar. Days	4	4.020E+07	1.005E+07	48.04	<.001
Site. Interval	24	2.461E+07	1.025E+06	4.90	<.001
Cultivar. Interval	96	4.106E+07	4.277E+05	2.04	<.001
Days. Interval	24	3.316E+07	1.382E+06	6.61	<.001
Site.Cultivar.Days	4	6.319E+07	1.580E+07	75.52	<.001
Site.Cultivar.Interval	96	9.542E+06	9.939E+04	0.48	1.000
Site.Days.Interval	24	7.999E+06	3.333E+05	1.59	0.033
Cultivar.Days.Interval	96	1.462E+07	1.523E+05	0.73	0.978
Site.Cultivar.Days.Interval	96	2.072E+07	2.158E+05	1.03	0.397
Residual	4491	9.395E+08	2.092E+05		
Total	4999	3.740E+09			

Standard errors of differences of means

Table	Site	Cultivar	Days	Interval
rep.	2500	1000	2500	200
d.f.	4491	4491	4491	4491
s.e.d.	12.94	20.45	12.94	45.74

Table	Site Cultivar	Site Days	Cultivar Days	Site Interval
rep.	500	1250	500	100
d.f.	4491	4491	4491	4491
s.e.d.	28.93	18.29	28.93	64.68

Table	Cultivar	Days	Site	Site

	Interval	Interval	Cultivar Days	Cultivar Interval
rep.	40	100	250	20
d.f.	4491	4491	4491	4491
s.e.d.	102.27	64.68	40.91	144.63
Table	Site Days Interval	Cultivar Days Interval	Site Cultivar Days Interval	
rep.	50	20	10	
d.f.	4491	4491	4491	
s.e.d.	91.47	144.63	204.54	

Least significant differences of means (5% level)

Table	Site	Cultivar	Days	Interval
rep.	2500	1000	2500	200
d.f.	4491	4491	4491	4491
l.s.d.	25.36	40.10	25.36	89.67
Table	Site Cultivar	Site Days	Cultivar Days	Site Interval
rep.	500	1250	500	100
d.f.	4491	4491	4491	4491
l.s.d.	56.71	35.87	56.71	126.81

Table	Cultivar Interval	Days Interval	Site Cultivar Days	Site Cultivar Interval
rep.	40	100	250	20
d.f.	4491	4491	4491	4491
l.s.d.	200.50	126.81	80.20	283.55
Table	Site Days Interval	Cultivar Days Interval	Site Cultivar Days Interval	
rep.	50	20	10	
d.f.	4491	4491	4491	
l.s.d.	179.33	283.55	401.00	

Stratum standard errors and coefficients of variation

Variate: EC

Stratum	d.f.	s.e.	cv%
Rep	9	106.03	9.3
Rep.*Units*	4491	457.37	40.3

4.1 (b) Analysis of variance for germination capacity before and after accelerated aging test (Transformed data)

4536 “General Analysis of Variance."
4537 BLOCK Rep
4538 TREATMENTS Cultivar*Day
4539 COVARIATE "No Covariate"
4540 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
4541 PSE=diff,lsd; LSDLEVEL=5] Germination_%_angular_transformation

Variate: Germination_%_angular_transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	133.17	33.29	0.38	
Rep.*Units* stratum					
Cultivar	4	875.67	218.92	2.51	0.044
Day	3	151988.29	50662.76	580.64	<.001
Cultivar. Day	12	4657.19	388.10	4.45	<.001
Residual	176	15356.65	87.25		
Total	199	173010.98			

Standard errors of differences of means

Table	Cultivar	Day	Cultivar Day
rep.	40	50	10
d.f.	176	176	176
s.e.d.	2.089	1.868	4.177

Least significant differences of means (5% level)

Table	Cultivar	Day	Cultivar Day
rep.	40	50	10
d.f.	176	176	176
l.s.d.	4.122	3.687	8.244

Stratum standard errors and coefficients of variation
Variate: Germination_%_angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	4	0.912	1.7
Rep.*Units*	176	9.341	17.9

4.1 (c) Analysis of variance for germination index (GI) before and after accelerated aging test (Transformed data)

8703 “General Analysis of Variance."
8704 BLOCK Rep
8705 TREATMENTS Cultivar*Day
8706 COVARIATE "No Covariate"
8707 ANOVA [PRINT=aovtable,information,means,%cv,missingvalues; FACT=32; CONTRASTS=7; FPROB=yes;\
8708 PSE=diff,lsd; LSDLEVEL=5] GI_angular_transformation

Variate: GI_angular_transformation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	0.5990	0.1498	0.43	
Rep.*Units* stratum					
Cultivar	4	6.0053	1.5013	4.28	0.003
Day	3	707.0804	235.6935	671.61	<.001
Cultivar. Day	12	14.7537	1.2295	3.50	<.001
Residual	176	61.7649	0.3509		
Total	199	790.2034			

Standard errors of differences of means

Table	Cultivar	Day	Cultivar Day
rep.	40	50	10
d.f.	176	176	176
s.e.d.	0.1325	0.1185	0.2649

Least significant differences of means (5% level)

Table	Cultivar	Day	Cultivar Day
rep.	40	50	10
d.f.	176	176	176
l.s.d.	0.2614	0.2338	0.5228

Stratum standard errors and coefficients of variation

Variate: GI_angular_transformation

Stratum	d.f.	s.e.	cv%
Rep	4	0.0612	1.5
Rep.*Units*	176	0.5924	14.7