PHYSIOLOGICAL BASIS OF SEED GERMINATION IN *CLEOME GYNANDRA* (L.)

MSc (Plant Pathology), University of Nairobi, Kenya

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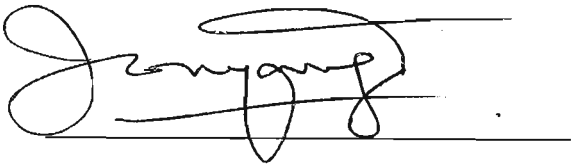
DECLARATION

The experimental work presented in this thesis was carried out at the University of KwaZulu Natal, Pietermaritzburg, from August 2001 to May 2005 under the supervision of Dr Albert T. Modi.

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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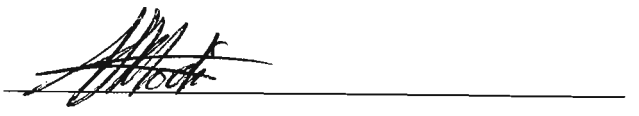
I declare that the above statement is correct.



Dr. Albert T. Modi, Supervisor

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Dedication

This thesis is dedicated to my father

Mr. Thardeus Onyango Omoro Obala,

who at the age of 80 still believed that I should obtain a PhD to be competitive in the world.

Pink stemmed *Cleome gynandra* plant, flowering and podding

ABSTRACT

Dormancy characteristics and optimum conditions for germination of *Cleome gynandra* seeds have not been explained. Seed storage proteins were extracted, analysed with SDS-PAGE and sequenced. Seed proteins of *Cleome* were characterised by comparison with those of wild mustard (*Brassica kaber*). Wild mustard showed seed proteins composed of two α-chains of molecular weight (24-32 kDa) and another two β-chains of 18-22 kDa. The seed proteins of *Cleome* comprised two α-chain polypeptides of molecular weight (25-30 kDa), two β-chain polypeptides of molecular weight (18-20 kDa) and a smaller β-chain of 13-15 kDa. The storage proteins occurred in the seeds as dimeric complexes of molecular weight 40-65 kDa, which were broken into polypeptide chains of approximately 20 and 30 kDa by the reducing action of DTT. Comparison with proteins in the proteome library and similarity index further confirmed that the seed proteins of *Cleome* had similarities with those of wild mustard. Two-dimensional SDS-PAGE showed that the two species have nine similar polypeptides and four different ones.

Events associated with dormancy release during seed germination still require explanation. Seeds of *Cleome* are characterised by low germination and there has been no explanation for this. Changes in protein expression during germination of *Cleome* in the presence or absence of light and at constant or alternating temperatures were examined. The germination of *Cleome* seeds at 20 °C was inhibited by light, but it was improved at 20 °C in darkness. There was no photoinhibition when seeds were germinated at constant 30 °C or alternating 20/30 °C (16 h night and 8 h day) for 10 days. Four proteins were observed to decrease in expression as germination progressed, but remained unchanged during photoinhibition. Photoinhibition was expressed more in seeds that were harvested late, after the pods had turned brown. These seeds showed a fifth, low molecular weight protein (13 kDa) that was absent from the immature seeds and embryos. Photinhibition is a pseudo-

dormancy condition during which seed storage proteins are not utilised and the seed coat could partially play a role in it.

The temperatures for the germination of *Cleome* in darkness have been determined. However, prior to this study the effects of temperature, light and pre-germination treatments (chilling, scarification, hydration and germination in the presence of KNO_3 or GA_3) on the germination of the seeds of this species have not been investigated. Seeds were germinated for 10 days and the final count of germination was used to determine seed performance. The highest germination percentage (60% and 80%, for a 2-year old and a 1-year old seed lot, respectively) of untreated seeds was achieved when alternating temperatures of 20/30 °C (16 h/8 h) in the dark or constant 30 °C in the dark were used. Among the pre-germination treatments, only scarification (puncturing of seeds at the radicle end) improved germination. Seeds were found to be negatively photoblastic, and the phenomenon was more pronounced when they were germinated at 20 °C and 12 h photoperiod or longer. Germination of photoinhibited seeds was, however, improved by treatment with GA_3. It is recommended that the germination of *Cleome* be undertaken under conditions of darkness and at either alternating 20/30 °C or continuous 30 °C.

Seed lot vigour and seedling vigour are two important seed quality aspects that are used in defining the seed germination process. Seed germination is appropriately characterised by radicle protrusion and the attainment of normal seedling structures. However, the international rules for testing seeds combine radicle protrusion and normal seedling attainment in separating seed germination into the first and final counts. The challenge to a seed analyst testing the germination of a species whose first and final counts are unknown is that there is no statistical guideline to determine these important stages of seed germination. Cauliflower and broccoli, for which the first and final counts are published in the international rules for testing seeds and *Cleome*, for which there is no data on the first and

final counts, were examined to determine the statistical significances of the first and final counts. Analysis of variance, logistic regression, 'broken-stick' regression models and survival analysis procedures were used. Analysis of variance showed that there were no differences between the germination percentages on the fourth, fifth and seventh days of germination. Low and stable standard deviations were recorded when evaluating germination after the fourth day. The germination curves of broccoli and cauliflower did not fit the Gompertz curve but fitted the exponential curve. The broken-stick model 'broke' the cumulative germination curve for the *Cleome* seed lots into two linear curves that were significantly different, but failed to break those for broccoli and cauliflower. However, this study confirmed the first and final counts for broccoli and cauliflower as determined by the international rules for testing seeds. Broken-stick modelling and life table analyses confirmed the fourth day as being appropriate to determine the first count for *Cleome* germination. There was no evidence of further seed germination after the seventh day as shown by probability density and hazard rate. It is suggested that for *Cleome,* the 'first count' and 'final count' be performed on the fourth and seventh day of the germination, respectively.

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FIELDS OF STUDY

Major Field: Plant Pathology and Crop/Seed Science

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ABBREVIATIONS

ABA	Abscisic acid
APS	Ammonium persulphate
ARC	Agricultural Research Council, South Africa
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
cv	Coefficient of variation
DNase	Deoxyribonuclease
Fluoridone	1-methyl-3-phenyl-5-[3-trifluorylmethyl-(phenyl)]-4-(1H)-pyridinone
g	Gravitational acceleration (9.806 m sec^{-1})
GA	Gibberellic acid
HIR	High irradiance reaction
IEF	Isoelectric focussing
ISTA	International Seed Testing Association
KSC	Kenya Seed Company
LEA	Late Embryogenic Abundant
mA	Milliamperes
M_r	Relative molecular weight
mRNA	Messenger ribonucleic acid
pI	Isoelectric point
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	2-amino-2(hydroxymethyl)-propane-1,3-diol(Tris[hydroxymethyl] aminomethane
Tris-Cl	Tris hydrochloride
Temed	NNN'N' tetramethylethylenediamine
U	Unit
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1. GENERAL INTRODUCTION

Cleome gynandra (L.) belongs to the family *Capparaceae* of the order *Capparales* (Porter, 1967; Cronquist, 1988). *Capparaceae* is made up mostly of two subfamilies: *Capparoideae*, which are mainly woody and *Cleomoidaea*, which are herbaceous (Porter, 1967). *Capparaceae* resembles *Crucifereae* in being herbaceous and having scattered ex-stipulate leaves (Rendle, 1925) and is generally regarded as the ancestral group to the *Brassicaceae* (Porter, 1967). The similarity between *Cleomoideae* and *Brassicaceae* was explained as parallelism or convergence by Cronquist (1981).

Capparaceae are widespread in tropical and subtropical regions, a few are temperate-zone species occurring mainly in arid climates. Some of the species are cultivated as ornamentals (e.g. *Cleome spinosa*) and *Capparis spinosa* is used for seasoning (Porter, 1967). *Cleome gynandra* is wide spread in the tropics as a weed and it originated in the African continent (Chweya and Mnzava, 1997; Fletcher, 1999). It is not cultivated as a commercial crop anywhere in the world, but for years it has been a semi-domesticated volunteer crop in home gardens in many parts of sub-Saharan Africa where its leaves are eaten as spinach. It is used as both food and medicine (Venter *et al.*, 2000; Nesamvuni, Steyn and Potgieter, 2001). Plant extracts of *C. gynandra* were found to be heat stable and fungitoxic (Pandey *et al.*, 1993) and its essential oils exhibited good repellence against the livestock tick (Lwande *et al.*, 1999). It was recently noted by Jansen van Rensburg *et al.* (2004) that indigenous leafy vegetables, which are rich in micronutrients and vitamins, could play an important role in alleviating hunger and malnutrition in sub-Saharan Africa.

The fast-increasing global population has put enormous pressure on available resources and created not only total food deficits, but also widespread deficiency of micronutrients in developing countries (Graham, Welch and Bouis, 2000). Graham *et al.* (2000) noted that plant breeding should concentrate on developing nutrient-dense staple foods that give resource-poor populations a better-balanced nutritional base to their diet. However, such a scenario is precarious because the adoption and impact of such varieties is not predictable and will take some time. Questions also arise as to whether additional nutrients in these varieties will be in sufficient magnitude and be sufficiently bioavailable, and whether such changes could alter seed quality. Therefore, diversification through domestication of locally adaptable wild species of known nutrient quality could be more appropriate. Domestication of indigenous vegetables may progress through a cycle of changing human interests and dependence: from home garden to minor crop, then to major crop and finally to even a broader ecogeographical scope (Bretting and Duvic, 1997). Jacobsen and Bach (1998) noted that *Chenopodium quinoa*, which was originally from the Andean region, is a very palatable and nutritious vegetable, and recommended its domestication and production in Europe. The indigenous African vegetables have the potential to be cultivated, can provide food security and improve the health and living standards of the resource-poor people.

There is an increase in acceptance and utilization of indigenous African vegetables in the east and southern Africa (Fletcher, 1999; Jansen van Rensburg *et al.*, 2004). These indigenous vegetables can be conserved as seed and preserved by timely planting (Seme *et al.*, 1992). Therefore, information on seed biology and technology of these vegetables is important. Plant breeding and seed production by farmers and private seed companies

should be stimulated (Grubben and Almekinders, 1997) in order to reduce the risk of genetic erosion of indigenous vegetables. It has been suggested that studies conducted on *Cleome* should concentrate on production technology packages and post-harvest handling of the crop (Jansen van Rensburg *et al.*, 2004). National seed companies in both Kenya and Zambia are producing and marketing indigenous plant species in small quantities and could make use of information on seed technology. The domestication and improved production of indigenous vegetables could be a step towards self-sufficiency in food production and hence reduce the amount of foreign exchange used in seed importation. However, information on seed technology for indigenous vegetables in the region is scarce (Nono-Womdim and Opena, 1997). This plant species has been shown to exhibit poor germination (Börhinger *et al.*, 1999; Chweya and Mnzava, 1997). Seed dormancy and methods to break it have not been studied in *C. gynandra*. A study investigating the effects of temperature and light on seed germination of *C. gynandra* (Börhinger *et al.*, 1999) did not produce conclusive results.

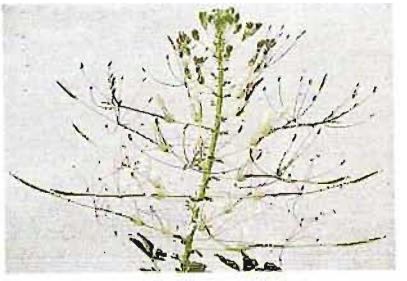
Cleome has small round or circular black seeds that resemble the shell of a snail and have a rough surface. It has a tough brittle seed coat, which is shiny black on the inside and has a curved worm-like embryo enveloped in a semi-permeable cell membrane (Figure 1.1). The transverse cross-section of a mature *Cleome* seed is similar to that of beet (*Beta vulgaris*) (Taylor *et al.*, 2003) and chicory (*Cichorium intybus*) (Pimpini *et al.*, 2002) and *Arabidopsis* mutants (Debeaujon and Koornneef, 2000; Debeaujon, Leon-Kloosterziel and Koornneef, 2000) (Figure 1.2). Since *C. gynandra* belongs to the *Capparaceae* family, which together with *Brassicaceae* form the order *Capparales*

(Cronquist, 1988), it is of interest to compare the seed physiology of *C. gynandra* with that of the *Brassica* species.

Cleome gynandra has the potential to be an important local vegetable in Kenya and sub-Saharan Africa. Seed production is a key element in successful commercialisation of any crop. It was surmised that explanation of the physiological basis for *C. gynandra* seed germination would increase knowledge about its propagation and seed storage aspects. The specific objectives of the study were:

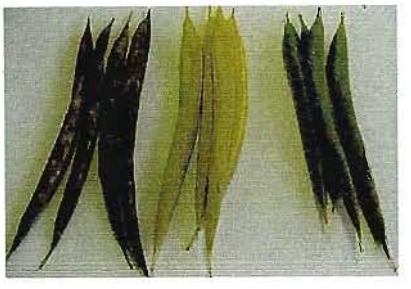
1. To investigate the environmental conditions required to germinate *C. gynandra* seeds, with particular focus on light and temperature
2. To examine various dormancy breaking treatments required to germinate *C. gynandra* seeds
3. To investigate the accumulation and/or expression of storage proteins in the seeds and how these proteins relate to dormancy, germination and seed quality aspects
4. To use statistical analysis to determine the first and final counts during germination of *C. gynandra.*

Welbaum (1998) stated that even the best experimental systems failed to identify the key events controlling seed germination. This study attempted to provide knowledge of seed germination in a hitherto wild species with a great potential to alleviate food insecurity in sub-Saharan Africa.



The inflorescence of green-stemmed *Cleome* plant showing flowers, flower buds and pods

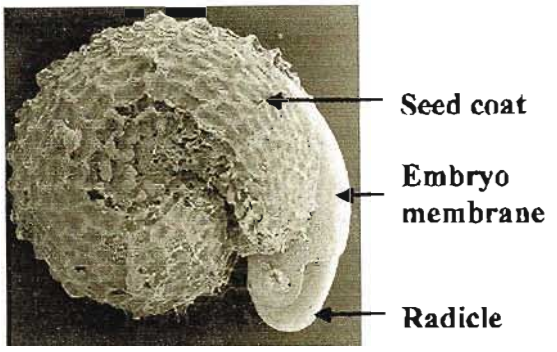
Open flower showing the anthers, filaments and pistil; and flower buds (from IPGRI bulletin)



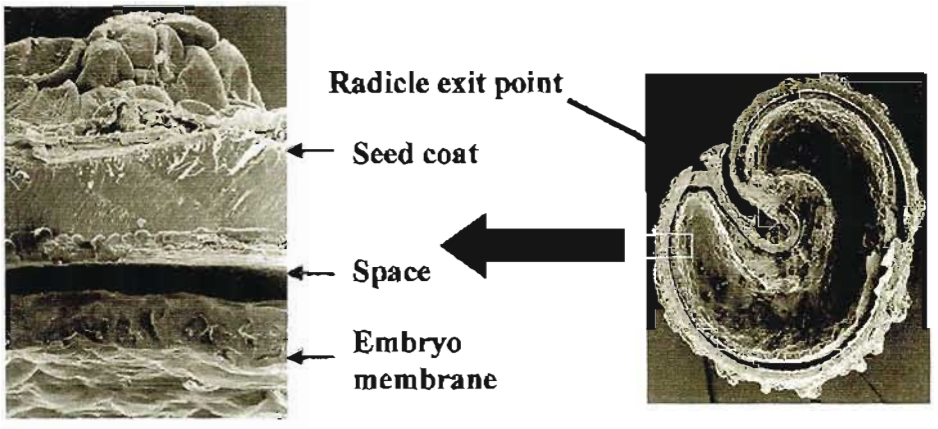
Brown, yellow and green pods

The worm-like, rough surfaced seed of *Cleome*

Figure 1.1. The reproductive structure of *Cleome gynandra*. Note the different stages of pod maturity (green, yellow and brown) from which harvest-mature seeds are found.



Germinating seed after 24 h of incubation



(c) Transverse cross-section of seed coat. (Magnification X 500)

(b) Longitudinal cross-section of seed coat. (Magnification X 40)

Figure 1.2. The morphology of *Cleome* seed 24 hours after imbibition (a), longitudinal (b) and transverse (c) cross-sections of a mature dry seed obtained by Environmental Scanning Electron Microscopy.

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CHAPTER 2. LITERATURE REVIEW

2.1. Seed development and maturation

Seed quality is determined by the sum total of several endogenous and external factors that influence seed development and maturation. The last two stages of seed development encompass the embryo maturation stage, when the embryo increases in weight, and the desiccation stage, when there is a marked decrease in water content (Harada, 1997). During seed maturation there are significant changes in embryo size and weight due to accumulation of storage reserves until physiological maturity is attained. Potential seed quality is established at physiological maturity when 100% of the seeds germinate and produce normal seedlings with maximal vigour (Hilhorst and Toorop, 1997).

Maximum seed quality is attained at different times during seed maturation in different plant species. Harrington (1972) stated that seeds attain maximum quality at the end of the seed filling phase but this does not appear to hold for all crops. It was observed by Siddique and Goodwin (1980) that the optimum harvest date was when the inflorescences start to turn yellow in beans. Nkang and Umoh (1996) showed that harvesting at agronomic maturity after physiological maturity (PM) gave the best quality (potential longevity) seed lots of soybean in the humid tropics of South East Nigeria. 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 of the seed on the mother plant was allowed. Still and Bradford (1998), using hydrotime and ABA-time models to provide a biological basis on which to determine harvest time in *Brassica,* showed that physiological maturity was attained

several days after maximum seed dry mass in rapeseed while in red cabbage it occurred at or after maximum seed dry mass. Lima *et al.* (2000) made similar observations in Sumauma (*Ceiba pentandra*) and Norway maple (*Acer plantanoides*), both of which are not true orthodox seeds. While Coste, Ney and Crozat (2001) observed that seed quality and seed weight can simultaneously be at a maximum only for a short period – at the beginning of desiccation phase in determinate beans.

The condition under which the mother plant (seed crop) is grown has great influence on the quality of seeds produced. Jacobsen *et al.* (1994) found that although *Chenopodium quinoa* is well adapted to poor soils, seed yield was increased when nitrogen was added. High temperatures during seed development cause early pod ripening and rapid seed maturation, resulting in small, poor quality seeds in beans (Siddique and Goodwin, 1980) and in annual ryegrass (Steadman *et al.*, 2004). Bean seeds matured at 33/28 °C were also more susceptible to deterioration and mechanical damage when harvesting was delayed (Siddique and Goodwin, 1980). Climatic changes like elevated temperatures of 40/30 °C may alter seed composition. Thomas *et al.* (2003) noted 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 40/30 °C, then decreased sharply. It was stated by Hilhorst and Toorop (1997) that high temperature during seed development alleviates dormancy and low temperature increases it. This was supported by the observation by Steadman *et al.* (2004) that annual ryegrass plants grown in warm temperatures produced less dormant seeds than those from cool temperatures. This temperature effect on dormancy has often been associated with membrane properties, which are modified by temperature and drought stress during the seed filling phase (Hilhorst and Toorop, 1997). However, maximum

potential longevity increased in cooler temperature regimes during seed maturation in *Phaseolus vulgaris* (Siddique and Goodwin, 1980; Sanhewe and Ellis, 1996) and strong positive correlation was obtained with 58 kDa heat shock protein (Sinniah, Ellis and John, 1998).

Therefore, two important seed quality aspects, germination capacity and vigour are determined during seed maturation (Harada, 1997; Hilhorst and Toorop, 1997). By mid embryo maturation, abscisic acid (ABA) levels reach a maximum and as desiccation stage is approached it decreases sharply while the late embryogenesis abundant (*Lea*) proteins, which impart desiccation tolerance to mature seeds, accumulate (Dure, 1997; Harada, 1997). The embryo then becomes fully germinable and accumulation of food reserves occurs. Such mature seeds may be dormant or non-dormant (quiescent) depending on their response to environmental and endogenous factors.

2.2. Seed dormancy and germination

Primary dormancy may be due to hard/thick seed coat, immature embryo or suppression of precocious germination, regulated mainly by the plant growth hormone, ABA (Bewley and Black, 1994). Several studies have been carried out to understand the involvement of the growth hormones abscisic acid and gibberellins in dormancy and germination (Demir and Ellis, 1992; Hilhorst and Karssen, 1992; Groot and Karssen, 1992; Ni and Bradford, 1993; Hilhorst and Toorop, 1997). Plant physiological studies show that gibberellins do not break primary dormancy (Hilhorst and Karssen, 1992) but enhance germination and seedling vigour (Karssen, *et al.*, 1989b; Hilhorst and Toorop, 1997). This GA effect is similar to that of seed priming (McDonald, 1999). Dormancy is often relieved by imbibition at low temperatures, also referred to as cold

stratification. Jäger and van Staden (2002), studying dormancy release mechanisms in lettuce showed that the escape time, the period that dormancy is broken, occurred around 5 h after the start of imbibition The release of dormancy is accompanied by the initiation of growth that results in radicle emergence (Finkelstein and Lynch, 2000). Progression through phase II of germination, which experiences increased metabolic activity and initiation of growth, is tightly controlled by environmental and hormonal signals, because seedlings become committed to growth upon entry into the final phase of germination. Low concentrations of exogenous sugars suppressed the inhibitory effects of ABA on radicle protrusion (Finkelstein and Lynch, 2000) and the levels of sucrose increased until radicle emergence, and decreased thereafter (Jäger and van Staden, 2002). Hilhorst and Toorop (1997) hypothesized that conditions that relieve seed dormancy might have long-term effects beyond germination. Robinson and Hill (1999) supported this hypothesis by showing that *abi3* mutants, characterized by their ability to germinate in the presence of ABA (reduced dormancy), partitioned more resources into seed development than the wild type and this enhanced the production of high quality seeds.

After physiological maturity, the physiological status of the seed frequently changes depending on the prevailing environmental conditions (Hilhorst and Karssen, 1992; Baskin and Baskin, 1998). Non-dormant seeds germinate when all factors required for germination are present (Figure 2.1, step 6). If germination factors are not all present, germination is passively inhibited and seeds are in a pseudo (or forced) state of dormancy (Figure 2.1, step 5) (Karssen, 1995). After prolonged inhibition of germination, seeds may gradually enter a state of secondary dormancy (Figure 2.1, step 4) that often resembles primary dormancy. Secondary dormancy is often induced by

elevated temperatures and was described as the narrowing of the germination temperature 'window' by Hilhorst and Karssen (1992) and Baskin and Baskin (1998).

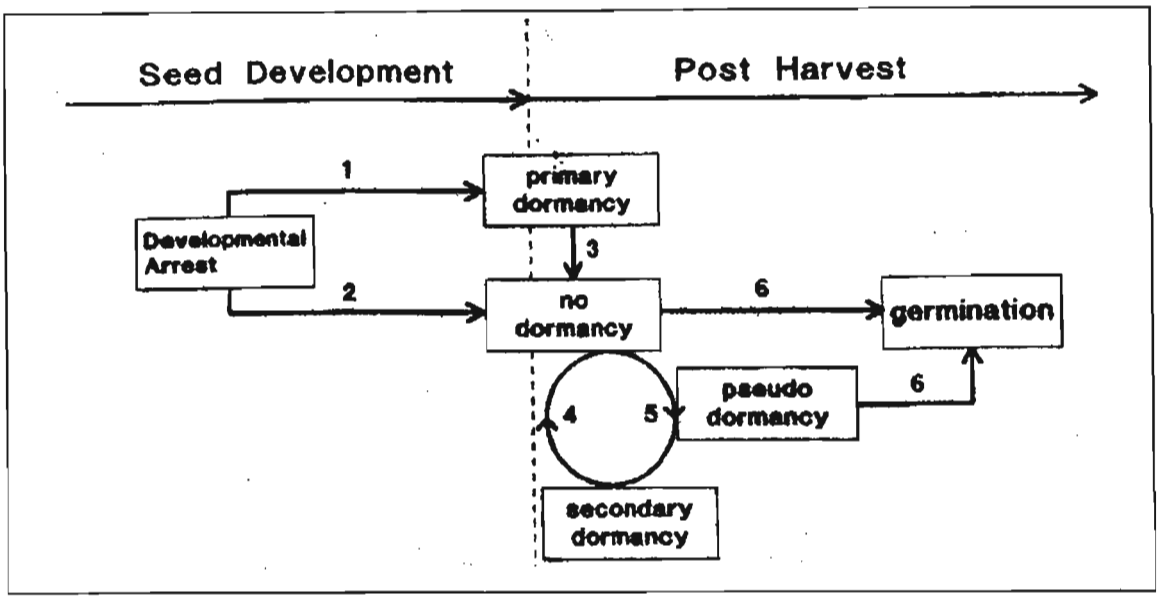

Figure 2.1. Schematic presentation of transitions between different states of dormancy and germination. The dotted line presents the phase during development in which seeds are hydrated (Hilhorst and Karssen, 1992).

2.3. Physiology of seed germination

Seed germination commences with the uptake of water by imbibition of the dry seed, followed by expansive growth of the embryo (Leubner-Metzger, 2003). This culminates in the rupture of the seed coat layers and emergence of the radicle is generally considered as the completion of germination. Catabolic processes such as degradation of reserve foods by hydrolytic enzymes characterize the germination phase. Degradation products such as sugars provide energy while the proteins are used to produce enzymes and building blocks for new cells, and nutrients for embryo development and seedling growth. When isolated from their protective structures, embryos will germinate soon after the histodifferentiation phase, but they will not form normal seedlings due to lack of food reserves (Kermode, 1995; Hilhorst and Toorop, 1997). Soltani *et al.* (2002) suggested that the advantage of large seeds with respect to

seedling growth rate is due to their higher content of storage reserves and in their ability to provide energy to the growing seedlings at a faster rate.

During seed germination the content and expression of proteins and enzymes keep changing, new ones appear while others disappear, and yet others remain constant throughout the process (Gallardo *et al.*, 2001). Most of the components for further protein synthesis for germination are also present in the dry seed, including mRNA. Di Nola, Mischke and Taylorson, (1990) observed that the transition of seeds from dormant to non-dormant state was associated with the synthesis of specific proteins and a decrease in content of others in the plasma membrane. An increase in the synthesis of proteins in the 20 kDa range was observed in the intracellular membranes of germinating seeds. Polysomes, which are absent in dry seeds, appear soon after the lag phase and continue increasing thereafter (Bewley and Black, 1994). During the period of germination there are two sets of proteins produced: "House keeping" proteins – enzymes and other proteins needed for basic needs of living plants and the proteins involved specifically with germination. Two "house keeping" enzymes observed in *Arabidopsis* seeds were Methionine synthase and S-adenosylmethionine synthase, which accumulated during seed germination (Gallardo *et al.*, 2002). Delseny *et al.* (1977) indicated that polyadenylic acid and mRNAs disappear early in the germination of radish embryo axis. De Jimenez and Aquilar (1984) showed in maize that a protein (~37 kDa) disappeared within 18-24 h of incubation. Gallardo *et al.* (2001) observed that late embryogenesis abundant proteins and mRNAs first become evident as seed maturation begins but disappear within the first 24-36 h of germination. Gallardo *et al.* (2001) identified eight out of 14 proteins whose accumulation declined during the radicle emergence step of germination in *Arabidopsis* seeds as 12 S cruciferin variants. Globulins of molecular weight 46-49, 38.2-40, 25-27, 27-29 and

22.5-23.5 kDa were reported by Klimaszewska *et al.* (2004) as the most prominent protein sets in the zygotic embryos of *Pinus strobus* that decreased during germination.

2.3.1. Influence of temperature on seed germination

Plants have evolved highly complex sensory mechanisms to monitor their surroundings and adapt their growth and development appropriately to the prevailing conditions. The plasma membrane is the first site in seed cells which detects the stimulus from external factors affecting seed dormancy (Di Nola *et al.*, 1990). The plants are able to coordinate their development with favourable seasonal conditions by integrating information from multiple environmental cues, through cross-talk regulation (Franklin and Whitelam, 2004). Under natural conditions, seed germination occurs when the environment ensures the survival of the plant (species) and this surety is obtained through cross-talk. The existence of some type of cross-talk regulation of the FsACO1 gene by GAs and ethylene during the breaking of dormancy/onset of germination in *Fagus sylvitica* was suggested by Calvo *et al.* (2004).

There is a minimum temperature below which seeds of a given species will not germinate and a maximum above which there is no germination. These temperatures were reported to be 13 °C and 37 °C, respectively for *Cleome gynandra* seeds, and there is an optimum range of 19-31°C (Böhringer *et al.*, 1999). Within the optimum temperature range, studies with *Chenopodium quinoa* showed that there was a log-linear relationship between germination rate and temperature (Jacobsen and Bach, 1998). High and low temperatures reduce both speed of germination and final germination percentage and long exposure often induces seed dormancy.

High temperatures of 45 °C induced thermoinhibition in T*agetes minuta* (Hills, van Staden and Viljoen, 2001) and secondary dormancy in *A. caudatus* seeds, which was

corrected through scarification (Kępczyński and Bihun, 2002). In both cases, the seeds recovered their germination ability when transferred to optimum germination conditions. Thermoinhibition in lettuce seed germinated at 33 °C was caused by both high ABA content and high ABA sensitivity (Gonai *et al.*, 2004). In order to restore germination, the seeds were treated with fluridone and exogenous GA.

2.3.2. Effects of light on seed germination

Some plant species require light for seed germination while darkness enhances germination in others. Milberg, Anderson and Thompson (2000) compared the germination of seeds of 54 species, which accumulate persistently in seed banks, in light and darkness after cold stratification and observed that germination became less dependent on light with increasing seed mass. These findings suggest that light response and seed size may have coevolved. However, most seeds have some degree of dormancy and require alternating temperatures coupled with light or darkness for maximum germination. Zhou, Deckard and Ahrens (2005) reported that hairy nightshade (*Solanum sarrachoides*) germinated equally well under both a 14 h photoperiod and continuous darkness, indicating that its seeds are not photoblastic.

In light requiring species, *Sisymbrium officinale* and *Arabidopsis*, light (Pfr) plays an important role in the biosynthesis of GAs and also increases the sensitivity of seeds to GAs (Hilhorst, Smitt and Karssen, 1986; Hilhorst and Karssen, 1988; Baskin and Baskin, 1998; Yamaguchi and Kamiya, 2002). These species germinated in darkness when exogenous GA was applied. Hilhorst (1990) suggested that the induction and breaking of secondary dormancy was phytochrome controlled in light-requiring seeds. This was also shown in guava seeds (*Psidium guajava*), where germination was induced by high R/FR ratio and alternating temperatures (Sugahara and Takaki, 2004).

Seed germination in many plant species is inhibited by continuous white light and such seeds germinate well in darkness (Bewley and Black, 1994). Earlier, Salisbury and Ross (1992) concluded that dark-germinating seeds become dormant after long exposure to light. Such exposure to light could prevent the germination of negatively photoblastic seeds until factors like high or low temperatures induced the seeds to secondary dormancy (Baskin and Baskin, 1998). This negative photosensitivity shown by such seeds became known as photoinhibition.

2.3.3. Photoinhibition of germination

There are many reports on the effects of the interaction between temperature and light on seed germination (Gutterman, Corbineau and Côme, 1992; Bewley and Black 1994; Benvenuti, Simonelli and Macchia, 2001a). Photoinhibition is caused by the length of white light exposure, is fluence-rate dependent and there are indications that the embryo must be under stress (Bewley and Black, 1994). The germination of *Amaranthus* seeds was inhibited by continuous white light at low temperatures (Gutterman *et al.*, 1992). Negative photosensitivity or photoinhibition has been reported in Mediterranean maritime plants (Thanos *et al.*, 1991), watermelon (Thanos and Mitrakos, 1992), *Matthiola tricuspidata* (Thanos, Georghiou and Delipetrou, 1994) and *Passiflora incarnata* (Benvenuti *et al.*, 2001a). Photoinhibition of seeds is also influenced by the seed coat. Takaki and Gama (1998) demonstrated the effect of seed coat on photoinhibition through chemical scarification of seeds of *Lactuca sativa* cv. Grand Rapids. Bewley and Black (1994) reported that seeds are susceptible to photoinhibition during cell elongation and this was supported by the observation that shoot elongation was inhibited by exposure to red light (Kato-Noguchi, 2002).

The condition under which excessive light caused photosynthesis to cease is also referred to as photoinhibition (Gray *et al.*, 2003; Govindachary *et al.*, 2004). It is dependent on environmental conditions and is accelerated at low or high temperatures and during drought, when the D1 protein is degraded followed by the inhibition of the electron transport reactions (Mamedov and Styring 2003; Bergo *et al.*, 2003). Chlorophyll fluorescence imaging showed that *A. thaliana* plants, which were cold acclimated at 4 °C or cold-shifted to 4 °C, fully recovered from the effects of photoinhibition (Gray *et al.*, 2003). However, chilling-induced photoinhibition in *Valonia utricularis* (Chlorophyta) from different climatic regions did not show the same level of recovery after transfer to optimal temperatures of 25 °C (Eggert, van Hasselt and Breeman, 2003). These observations seem to suggest that the phenomenon of photoinhibition in photosynthesis is not the same as in seed germination. The main difference between photosynthesis photoinhibition and germination photoinhibition is that photoinhibition in photosynthesis has always been reported when plants are stressed.

2.3.3.1. *Role of phytochrome in photoinhibition*

Photoinhibition of seed germination by prolonged illumination is a manifestation of high irradiance reaction (HIR) of photomorphogenesis. It involves the operation of phytochromes A and B, and its effectiveness depends on the duration of exposure and fluence rate (Bewley and Black, 1994; Franklin and Whitelam, 2004). Seeds that germinate in darkness initially have no dormancy because they mature on the parent plant with sufficient Pfr or because they contain phytochrome intermediates that generate Pfr on hydration (Bewley and Black, 1994). Phytochrome activation occurred during the second stationary phase of water uptake, just before the activation of radicle

elongation in watermelon (Thanos and Mitrakos, 1992). The far-red wavelengths of sunlight were most inhibitory because they decrease the amount of Pfr in the seeds to a level below that needed for germination (Salisbury and Ross, 1992). The red and far-red light photoreceptor phytochrome regulated GA biosynthesis in germinating lettuce and *Arabidopsis* seeds (Yamaguchi and Kamiya, 2002). There are indications that light signals induced germination in sorghum through the enhancement of GA synthesis (Pérez-Flores *et al.*, 2003). Also, the biosynthesis of ABA is through the terpenoid pathway, which is influenced by light. The regulatory effects of light were manifested at the transcript level of carotenogenic genes, especially the first two enzymes phytoene synthase (psy) and phytoene desaturase (pds). These genes were strongly down-regulated in the dark, leading to completely stalled carotenoid biosynthesis in pepper (*Capsicum annum*) leaves (Simkin *et al.*, 2003), which can affect the maternal contribution of ABA in the seed coat (Karssen, 1995).

2.3.3.2. *Role of ABA/GA balance in photoinhibition*

One suggestion made to explain secondary dormancy was the existence of an unfavourable balance between growth-promoting and growth-inhibiting substances (Copeland and McDonald, 1995). Among these substances, ABA and GAs act antagonistically and the prevailing ABA/GA ratio either suppressed or enhanced seed germination in tomato (Ni and Bradford, 1993), maize (White *et al.*, 2002), oats (Poljakoff-Mayber *et al.*, 2002) and lettuce (Gonai *et al.*, 2004). Jacobsen *et al.* (2002) suggested that GAs did not appear to play a role in seed dormancy but may be required for germination and seedling growth in barley, and Hays *et al.* (2002) showed that it played a role in cell elongation in *Brassica napus*. However, Gonai *et al.* (2004) indicated that increased GA content in the embryo enhanced the catabolism of ABA,

hence playing some role in dormancy release. The ABA/GA balance was shown to influence the activity of two enzymes, end-β-manannase and β-mannosidase, which are responsible for the weakening of the micropylar endosperm to allow radicle protrusion (Mo and Bewley, 2003). There are indications that ABA could interfere directly with GA biosynthesis as it suppressed GA 20-oxidase gene expression in sorghum embryos (Pérez-Flores *et al.*, 2003).

Light plays an important role in the biosynthesis of both ABA and GA as indicated in Section 2.3.2. Finkelstein and Lynch (2000) observed that light affected the suppressive action of glucose on the inhibition of germination (radicle emergence) in *Arabidopsis* by ABA. Tomato plants grown under enhanced UV-B radiation produced seeds with more ABA and higher ABA/GAs ratio, which resulted in delayed seed germination (Yang *et al.*, 2004). The effects of light on the ABA/GA balance should be interpreted in the light of whether the seeds are light or dark germinating.

2.4. Statistical determination of seed germination testing method of new species

Seed technologists take the germination process beyond radicle emergence in order to determine the planting value of a seed lot. To adequately assess this seed quality aspect, both seed and seedling vigour are examined. Hence, the use of intermediate and final seed germination counts (ISTA) in tests to separate seed lots on quality basis. The seed germination process has been best described by non-linear growth curves (Scotts and Jones, 1982; Scotts *et al.*, 1984; Bewley and Black, 1994; Drapers and Smith, 1998). Non-linear regression with the Weibull function provided very good fit and description of the germination process in *C. gynandra* (Böhringer *et al.*, 1999). The cumulative germination of *Amaranthus retroflexus* also fitted the Weibull distribution better than the exponential one (Mas and Verdú, 2002). Zhou *et al.* (2005) used non-linear analysis

to determine the effect of pH, temperature and osmotic stress on seed germination in *Solanum sarrachoides*.

In recent years, image analysis systems have increasingly been used in studying the seed germination process. While studying the process of imbibition in white cabbage seeds, Dell' Aquila, van Eck and van der Heijden (2000) found computer image analysis technique to be versatile and sensitive. Geneve and Kester, 2001, Sako *et al.* (2001) and McDonald, Evans and Bennett (2001), using computer-aided analysis of digital images and scanners coupled with computer software, respectively, noted that seed and seedling evaluation was greatly improved. Later, computer-aided analysis of digital images enabled Hoffmaster *et al.* (2003) and Oakley, Kester and Geneve (2004) to achieve accurate and standardized assessment of seedling vigour in soybean and *Impatiens*, respectively. These digital image techniques use computers to analyse seed and seedling parameters such as width, length and area, and use them to compute vigour indices like speed of germination and seedling growth.

Dell'Aquila (2004) used an image analysis system to capture the sequence of images of seed germination in cabbage, lentil, pepper and tomato, and applied linear regression to analyse the data. Image analysis was reported to improve data recording of the germination onset and the rate of radicle elongation through the increase in roundness of the seed. The measurements of seed size and area, and seedling size and area, using computer image techniques, are assessments of vigour through evaluation of germination speed. Vigour index was designed to reflect the speed and uniformity of growth measures that indicated seedling potential for rapid and uniform emergence upon planting in the field (Hoffmaster *et al.*, 2003). The use of statistical analysis in the measurements of germination and seedling growth rate as mentioned focus on comparing seed lots and the various techniques. However, an appropriate statistical

analysis that properly describes the germination process and give indications on when to assess the seedling would be an added advantage to these techniques.

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CHAPTER 3. ACCUMULATION OF SEED STORAGE PROTEINS IN *CLEOME GYNANDRA* L. AND *BRASSICA KABER* L.

3.1 Introduction

Evolutionary changes have occurred in the two ancestral genes that encode vicilin and legumin resulting in the many different globulins recognised today (Bewley and Black 1994). Usually, one type of storage protein dominates the protein bodies of a species (Bewley and Black, 1994) and the dominating storage proteins in several dicotyledonous species are the 11-12 S globulins (Rödin and Rask, 1990; Shutov *et al.*, 2003). It was reported by Bewley and Black (1994) that vicilin had been shown to be absent in the *Brassicas* and *Arabidopsis*. Cruciferin, a legumin-like 12 S storage protein, is the main seed protein in *Raphanus sativus* (Laroche *et al.*, 1984), *Brassica napus* (Rödin and Rask, 1990; Breen and Crouch 1992; Rödin *et al.*, 1992) and *Arabidopsis thaliana* (Gallardo *et al.*, 2001). These species belong to the family *Brassicaceae* that is in the order *Capparales* together with the family *Capparaceae* to which *Cleome gynandra* belongs (Cronquist, 1988). Cronquist (1988) reported that the *Brassicaceae* are related to the *Capparaceae* but are more advanced in some aspects, and hence are more complex..

The 12 S globulin in radish has a molecular weight of about 300-350 kDa and are composed of six dimeric polypeptides of molecular weight ~55 kDa that are hydrogen bonded (Laroche *et al.*, 1984). Two-dimension SDS-PAGE showed that the ~ 55 kDa dimer consisted of 9 polypeptides with molecular weights ranging from 33-30 kDa. The storage protein in radish was found to be similar to that in rapeseed but the latter was more complex (Laroche *et al.*, 1984).

Characterisation of the 12 S storage protein of *Brassica napus* showed that it consisted of pairs of α-chain (30 kDa) and β-chain (20 kDa) polypeptides, linked to each other by disulphide bonds, to form a subunit pair of ~55 kDa (Rödin and Rask, 1990). Further electrophoretic analysis under reducing conditions showed that the α-chain and four β-chain polypeptides split further into four different polypeptides (Rödin *et al.*, 1990, Rödin and Rask 1990). Sjödahl, Rödin and Rask (1991) identified at least three different cruciferin precursors and referred to these as P1, P2 and P3. The precursor P1 gave rise to mature α_1-β_1 chain (subunit Cru1); P2 gave rise to $\alpha_{2/3}$-$\beta_{2/3}$ (subunits Cru2 and Cru3) and P3 to α_4-β_4 (subunit Cru4), resulting in four groups of polypeptides previously observed by Pang, Pruitt and Meyerowitz (1988). In a recent review, Shutov *et al.* (2003) emphasised that during formation and deposition, storage proteins are protected against premature breakdown, whereas they become completely degradable during initiation of germination. However, it was observed that there is limited proteolysis during seed maturation (Shutov *et al.* 2003) and, Wong *et al.* (2004a) argued that the presence of thioredoxins, which are known to reduce disulphide bonds of wheat endosperm proteins including storage proteins, was an indication that limited proteolysis occurs. As stated earlier, analyses of reduced proteins show that the holoprotein is split into smaller polypeptides.

Krochko and Bewley (1988; 2000) successfully used electrophoretic techniques for cultivar identification in alfalfa. Protein profiles obtained by SDS-PAGE of seed extracts of *B. kaber* and *C. gynandra* were compared through the use of similarity index (SI) (Ladizinsky 1983) and pairing affinity (PA) (Das and Mukherjee 1995). These parameters have been used to establish the degree of similarity between and within

species. However, the formula for PA indicates that total protein bands are obtained by adding the bands of species A and B, thereby double counting the bands and obtaining a false low PA value.

The 12 S globulins are synthesised and accumulate during seed development and maturation stages (Laroche *et al.*, 1984; Bewley and Black, 1994). These proteins do not show enzymatic activity and have high solubility in dilute salt solutions, and are hence considered as globulins (Nielsen *et al.*, 1997). Most of the 12 S polypeptides in rapeseed are neutral (pI) but major proteins in radish were found between pI 6.2-8.5 and at 10-12 (Laroche *et al.*, 1984). In this study, seed storage proteins from developing and mature seeds were sequenced after analysis by SDS-PAGE and comparisons were made between the prominent protein bands and spots of *Cleome* to those of wild mustard (*Brassica kaber*).

3.2 Materials and methods

3.2.1 Plant materials

Cleome gynandra: Seeds donated by the Kenya Seed Company (KSC seed lot) were used to produce fresh seeds under rainfed field conditions in Pietermaritzburg (29°35′S30°25′E), South Africa. Seeds were harvested at three pod maturity stages: green, yellow and brown pods. Green, brown and black seeds were present in green pods and only black seeds were present in the yellow and brown pods. Pod and seed colour are indications of the relative physiological maturity of the seeds. Seeds from brown pods were germinated at alternating temperatures 20/30 °C in darkness (Labcon, LTGC 20-40; Johannesburg, South Africa) and were sampled at 24 h intervals for protein extraction.

Wild mustard: The seeds of wild mustard were produced under rainfed field conditions at Umbumbulu, 65 km from Pietermaritzburg and mature dry seeds were harvested when the pods were brown.

3.2.2 Protein extraction and polyacrylamide gel electrophoresis

Three sub-samples each of 50 dry seeds or germinated seeds were ground in liquid nitrogen and total proteins extracted in thiourea/urea lysis buffer as described by Harder *et al.* (1999). To the lysis buffer was added 18 mM Tris-HCl, 1% (v/v) Ampholytes (pH 3-10) (Amersham Pharmacia Biotech, UK), 0.2% (v/v) Chaps, protease inhibitor "complete mini" cocktail from Roche Diagnostics GmbH, 4.9 U/ml of Rnase A, 53 U/ml of Dnase I and 0.2% (v/v) Triton X-100. The lysis solution was added to the ground seed sample and then shaken. After 10 min, 1% (w/v) dithiothreitol (DTT) was added and extraction proceeded at room temperature with intermittent shaking (Gallardo *et al.*, 2001, with modifications from Görg *et al.*, 2003). The non-reduced form of the proteins was obtained without incorporating DTT into the extraction buffer. Extraction was carried out at room temperature for 1 h with intermittent shaking. The extract was centrifuged (Beckman J2-21M) two times at a speed of 32,000 g for 10 min at 4 °C to obtain a clear supernatant, which constituted the soluble fraction of the proteins. Protein content of the extracts was determined using the Pierce Micro BCA™ Protein Assay Reagent Kit with bovine serum albumin (BSA) as a standard, according to the manufacturer's instructions. Protein samples of 20 µg per well were analysed with 10% SDS-PAGE run in Multiphor II vertical electrophoresis apparatus (Mighty Small) according to Laemmli (1970) system at 20mA, 300V for 3 h. The gels were stained

overnight with standard Coomassie (R250) blue and then de-stained with Destaining solution II until the gel background became clear.

Bands on the SDS-PAGE gels were determined with Quantity One computer package (2004), which also quantified average band intensity. This package is able to match protein bands with those of the molecular weight marker used and indicate the relative weights of the unknown bands on the gel. Quantity One was used to determine the total number of protein bands on three replicate gels of the two species *Cleome* and *B. kaber*. Analysis of variance and similarity index (SI) were used to compare band variations between the two species. The SI was calculated from electrophoretic band spectra of the gels on Figure 3.2 by the formula (Ladizinsky, 1983):

$$SI = \frac{a*100}{a+b}$$

where, a is the number of common bands and

b is the number of uncommon bands

The SI value was used to determine the relatedness of the species to each other and the higher the index, the closer are the species.

3.2.3 Two-dimensional SDS-polyacrylamide gel electrophoresis

Two-dimensional SDS-PAGE was performed according to the manufacture's instructions (Pharmacia Biotech SE 260 System). The first dimension isoelectrofocusing (IEF) was performed on IPG strips (immobilized pH gradient) loaded with protein extracts; each strip with one sample extract. The Immobiline^{TM} DryStrip (pH 3-10, 11 cm long) was rehydrated with 40 µl of sample dissolved in rehydration solution (8 M Urea, 2% CHAPS, 0.2% Ampholyte, 0.5% IPG buffer and 0.5% DTT) in the reswelling tray

overnight at about 22 °C. The strip was washed three times in distilled water and placed in the IEF apparatus. The conditions for IEF on Multiphor II System (Pharmacia) were 500V for 4.5 h in Phase I & II and 2000V for 11.5 h in Phase III & IV, giving a total run time of 16 hours, 20100 Vh. Two-step equilibration procedures followed the isoelectrofocusing of the IPG strip before the second dimension electrophoresis was performed. In the first step the IPG strip was placed into a tube with 20 ml equilibration solution I containing equilibration buffer (6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8) plus 50 mM DTT and shaken for 10 min. The strip was removed and placed into another test tube with 20 ml equilibration solution II containing equilibration buffer plus 4% (w/v) iodoacetamide and bromophenol blue for another 10 min on the shaker. The strip was removed and rinsed in distilled water and placed on moist filter paper to dry. The strip was placed horizontally on top of a 10% SDS-PAGE gel of 1 mm diameter, cast without the stacking gel, at a height of 10 cm. The second dimension was run vertically at 300V and 20mA constant current for 2 h. The gels were stained with standard Coommasie (R250) blue overnight, followed by de-staining with Destain II until the gel background became clear.

3.2.4 N-terminal amino acid sequencing

In order to improve protein resolution, 15% SDS-PAGE gels were prepared and stored in a fridge at 4 °C overnight to polymerise completely. Proteins were extracted as before and 20 µg of protein per well was analysed by SDS-PAGE. Thioglycolate (0.1 mM) was added to the upper running buffer prior to electrophoresis. Proteins were electroblotted onto the Sequi-Blot PVDF Membrane (Bio-Rad) for sequencing. The hydrophobic PVDF

(polyvinylidene difluoride) membrane binds proteins tightly, thereby providing excellent support for amino-terminal protein sequence. The membrane was dipped in 100% methanol until it became translucent. It was floated on the transfer buffer (3-[cyclohexylamino]-1-propanesulphonic acid buffer - CAPS) until it was completely equilibrated. Once the membrane was wet, it was placed on the gel which already had the proteins and, they were placed between layers of wet blotting papers and transferred to a gel holder cassette. The cassette was then placed into the protein transfer unit (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). The transfer was run according to the Boi-Rad Instructions Manual for 2 h at maximum current and 90V. The membrane was removed and allowed to dry on the bench overnight. The distinctly separated protein bands were cut from the membrane and protein sequencing was performed using Procise 491 Protein Sequencer (Applied Biosystems, USA). The sequence analysis results provided 10 amino acids from the N-terminal, which were used to search for sequence identity (homology). The search was carried out at the site http://www.expasy.org/cgi-bin/blast.pl.

3.2.5 Statistical analysis

Three replications in the form of three gels were obtained and variations in the occurrence of the prominent bands was analysed. Analysis of variance was performed on the presence or absence of nine bands at molecular weight of about 50, 30, 27, 25, 24, 22, 20, 18, and 15 kDa and the same bands were used in the similarity index (SI) calculations. Analysis of variance was done on the original and transformed data using GenStat statistical package (2000) and mean values were used in SI analyses.

3.3 Results

In the electrophoretic analysis of the non-reduced proteins, bands of M_r 40-65 kDa were present in both species (Figure 3.1). *Cleome* had prominent storage proteins at 48.44, 40.61, 28.13, 25.42, 17.90 kDa and 15.80 kDa, while wild mustard showed prominent bands at 64.60, 50.60, 30.14, 27.88, 24.89, 17.00 kDa and 15.13 kDa.

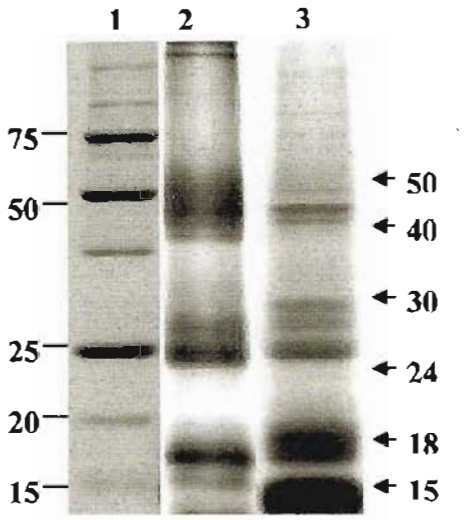

Figure 3.1. One-dimensional gel electrophoresis of proteins extracted from *Cleome gynandra* and *Brassica kaber* mature seeds. Proteins were non-reduced and loaded at 20 µg per lane and proteins were detected with standard Coomassie blue stain after electrophoresis. Bands were determined by Quantity One computer package (2004). Storage proteins are identified by arrows on the right. Lane 1, molecular weight marker; Lane 2, *Cleome* seeds and Lane 3, *B. kaber* seeds.

When the protein samples were reduced, the protein bands of M_r 41-48 kDa disappeared and a band of 62.56 kDa appeared. There was increased accumulation of polypeptides of molecular weight 27.50 kDa, 18.85 kDa and 13.73 kDa in *Cleome* seeds (Figures 3.2; Appendix 3.2c). Wild mustard seeds showed increased accumulation of proteins at molecular weight 30.14 kDa, 26.16 kDa and 18.04 kDa, while a band of 72.64 kDa appeared. Five protein bands of M_r 14, 18, 25, 27 and 30 kDa, which were consistently present in *Cleome*, were also observed in wild mustard, but at higher

accumulation levels in the latter. This suggests that there are similar peptide components in the storage proteins of these two species.

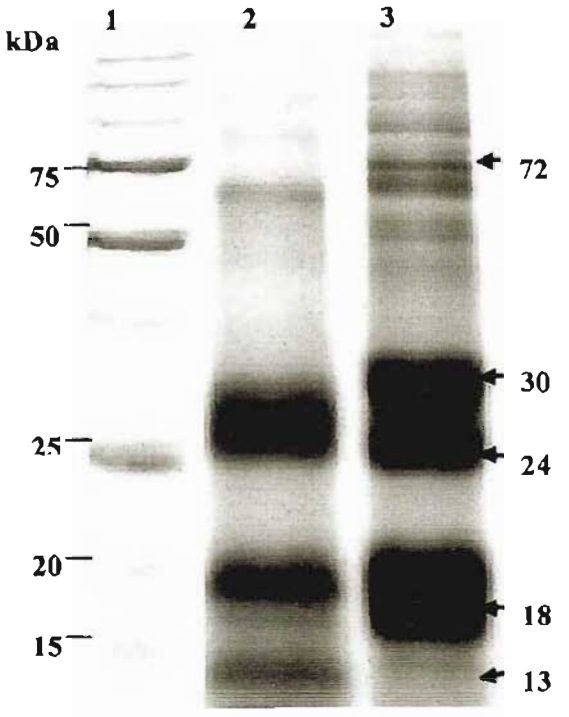

Figure 3.2. One-dimensional gel electrophoresis of proteins extracted from *Cleome gynandra* and *Brassica kaber* mature seeds. Proteins were reduced and loaded at 20 µg per lane and proteins were detected with standard Coomassie blue stain after electrophoresis. Storage proteins are identified by arrows on the right. Lane 1, molecular weight marker; Lane 2, *Cleome*; Lane 3, *B. kaber*.

Analysis of variance of the presence or absence of nine relatively prominent bands showed that the two species were dissimilar at 10% probability level (Appendix 3.2a). There was no interaction between species and bands, meaning that there was consistency in the appearance of the bands in the two species. To further support this view, an average similarity index value was obtained from the electrophoretic band spectra of *Cleome* and wild mustard from Figure 3.2. A similarity index of 64.3% was calculated

and this value is relatively low because wild mustard seeds had more polypeptides than *Cleome* seeds.

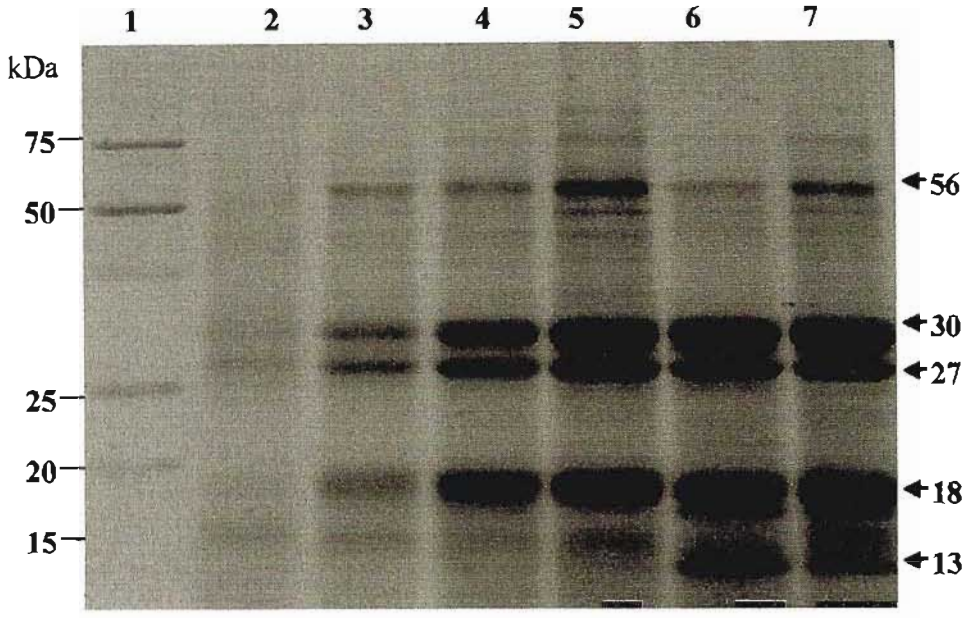

Figure 3.3. One-dimensional gel electrophoresis of proteins isolated from *Cleome gynandra* seeds during development. Proteins were loaded at 10 µg per lane and proteins were detected with standard Coomassie blue stain after electrophoresis. Storage proteins are identified by arrows on the right. Lane 1, molecular weight marker in kilodaltons; 2, 3 and 4, green, brown and black seeds selected from green pods; 5, seeds from green pods; 6, black seeds from yellow pods; 7, black seeds from brown pods. Arrows indicate the prominent proteins.

The content of storage proteins increased as the seeds of *Cleome* matured as shown by sequential increase in the number of bands (Figure 3.3). Green seeds from green pods showed the lowest number of protein bands. Brown seeds selected from the green pods accumulated more seed proteins compared to the green seeds, but less than the black seeds. Black seeds selected from green pods and those obtained from yellow and brown pods had four protein bands with molecular weights of 55.19 kDa, 30.06 kDa, 26.63 kDa and 17.76 kDa. The seeds from yellow and brown pods further accumulated a low molecular weight protein band of about 13.31 kDa. The intensity of band expression

increased significantly as the seeds matured (Figure 3.4). The green seeds from green pods showed the lowest expression whereas the black seeds from yellow and brown pods showed the highest protein accumulation.

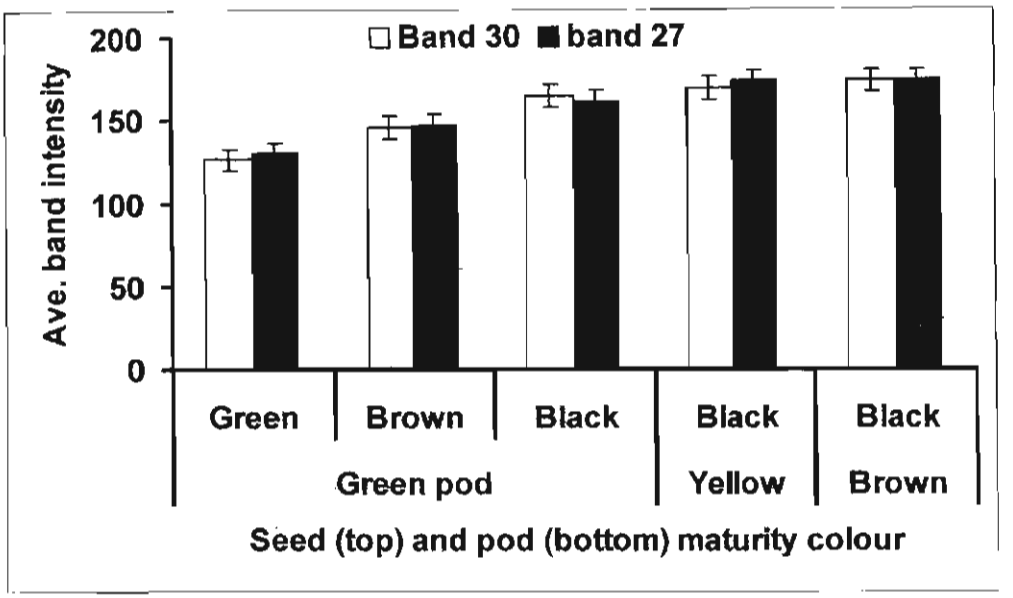

Figure 3.4. Average accumulation intensity of bands of molecular weight of about 30 and 27 kDa from Figure 3.3. The intensity was determined with Quantity One and the error bars represent SE, n = 3.

Analysis of the seeds of wild mustard with two-dimensional SDS-PAGE showed that it had more polypeptides than *Cleome* (Figure 3.5). There were three polypeptides of M_r 30 kDa, p*I* 6.8-7.5 (circled) and one at p*I* 8.5 (arrow). Two polypeptides were shown at M_r 27 kDa, p*I* 7.0-7.5 (circled) and the other at p*I* 9.6 (arrow). Two polypeptides of M_r 17 kDa showed low expression at p*I* 9.0-9.6 (circled). Two polypeptides at M_r 18 kDa appeared separately at p*I* 6.8 and p*I* 8. Three other polypeptides of M_r 18 kDa were shown at p*I* 8.8-9.8 (circled). The four consistent protein bands from *Cleome* seeds split into nine prominent polypeptides according to their isoelectric points (Figure 3.6). Three polypeptides occurred at M_r 30 kDa and two at M_r 27 kDa, at p*I* 7.0-7.5 (circled), while

two polypeptides each appeared at M_r 18 kDa and 22 kDa, at p*I* 9.0-9.6 (circled). The polypeptides that are circled were common to both *Cleome* and wild mustard seeds (Figures 3.5 and 3.6). However, four additional polypeptides absent in *Cleome* seeds were observed in wild mustard (Figure 3.5, arrows).

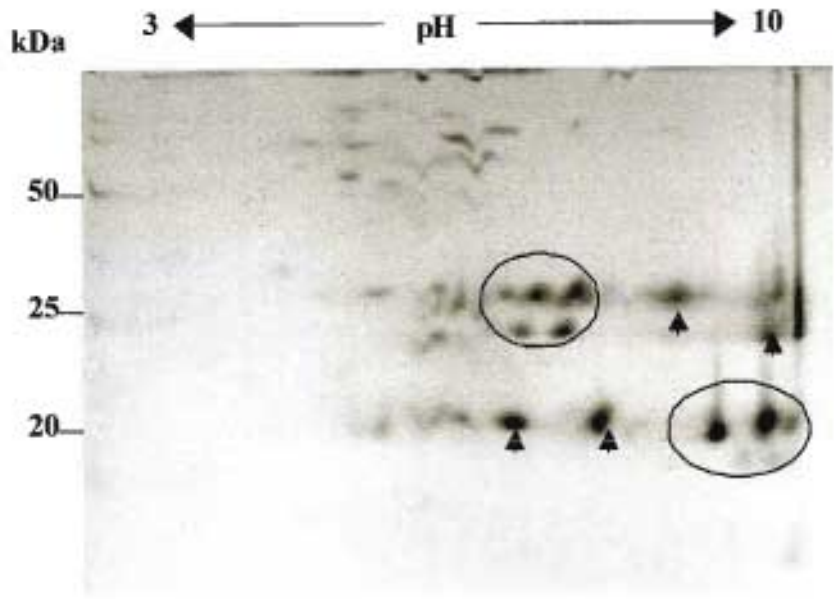

Figure 3.5. Two-dimensional gel electrophoresis of proteins extracted from mature *B. kaber* seeds. Proteins were reduced and 40 µg of protein was loaded per IPG strip, and proteins were detected by standard Coomassie blue staining after electrophoresis. The storage proteins that are common to both *C. gynandra* and *B. kaber* are identified by circles on the gel and the arrows identify the proteins that are absent in *C. gynandra* seeds.

The seeds of *Cleome* started germinating on the second day and by the third day had reached a germination percentage above 70%. The mobilisation of seed proteins became evident at this point of the germination process as the proteins were utilised. The expression of the proteins of M_r 30 and 27 kDa was diminished by 72 h when seeds were germinated at alternating temperatures 20/30 °C in light or darkness, as shown in the circle in Figure 3.7.

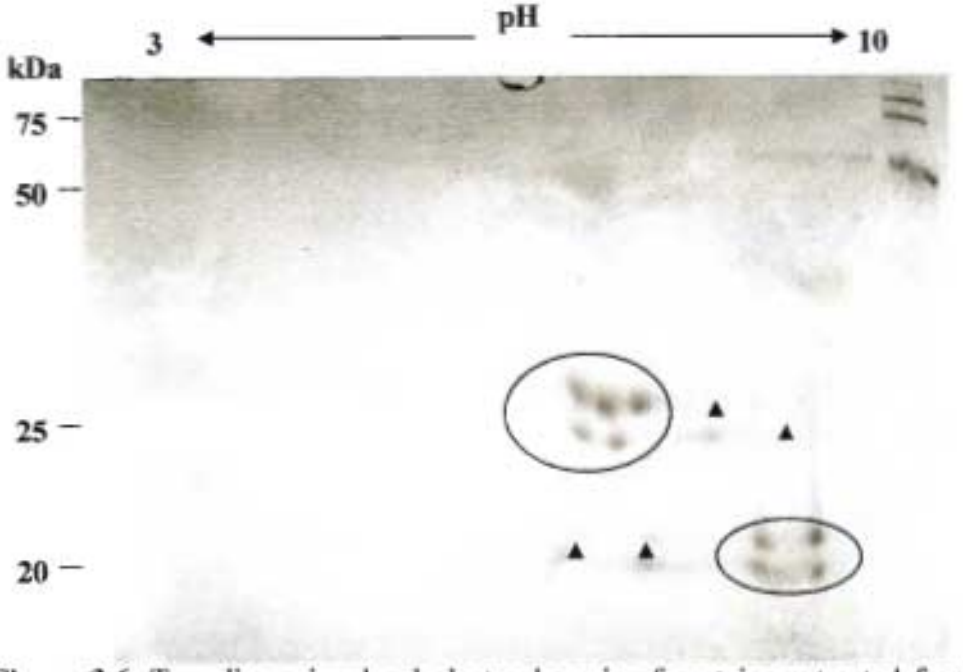

Figure 3.6. Two-dimensional gel electrophoresis of proteins extracted from mature *C. gynandra* seeds. Proteins were reduced with DTT and 40 µg of protein was loaded per IPG strip, and proteins were detected by standard Coomassie blue staining after electrophoresis. The storage proteins are identified by circles on the gel. The arrows indicate positions of protein that are absent or showing low protein expression in *Cleome* but present in *B. kaber*.

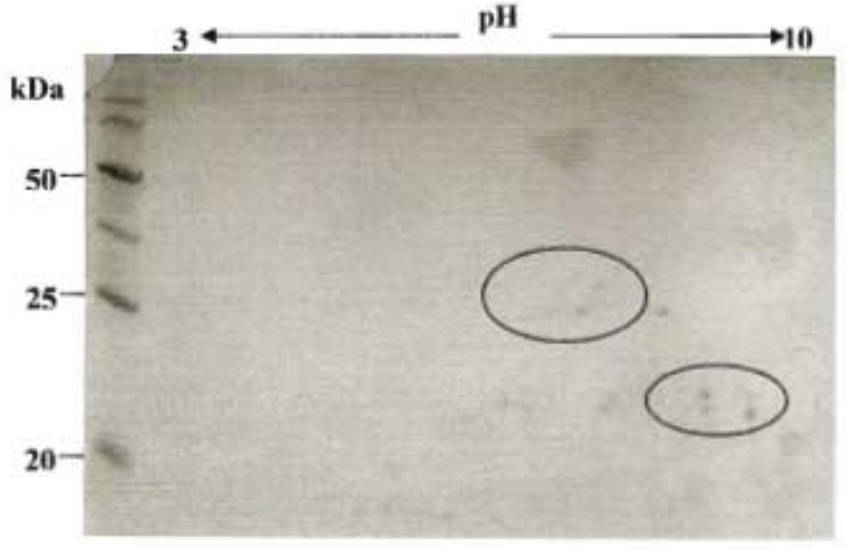

Figure 3.7. Two-dimensional gel electrophoresis of proteins extracted from seeds of *C. gynandra* germinated at 20/30 °C in darkness for 72 h. Proteins were reduced with DTT and 40 µg of protein was loaded per IPG strip, and proteins were detected by standard Coomassie blue staining after electrophoresis. Storage proteins identified by the circles on the gel are diminished.

Comparisons of the N-terminal amino acid sequences obtained from the four prominent proteins in *Cleome* seeds with those in the proteome library are shown in Table 3.1. Apart from the expansin-like protein on Band II of 28.64 kDa and lectin-like protein of 22.48 kDa on Band III, the table showed other homologues as either hypothetical proteins or proteins with unknown functions. Unedited full sequences of these proteins are shown in Appendix 3.1 and it was observed that proteins of molecular weight 21.254, 21.640 and 22.094 kDa shared a section of the sequence. Others that shared portions of their sequence are 25.943 kDa & 11.433 kDa and 25.741 kDa & 31.505 kDa, respectively.

Table 3.1. Four protein bands of M_r 18, 21, 27 and 30 kDa in *Cleome* seeds were separated by 15% SDS-PAGE and sequenced. The N-terminal amino acid sequences obtained were compared with the proteins in the proteome library. The proteins tabulated below showed homology with those from *Arabidopsis*. The unedited sequences are shown in Appendix 3.1.

Mol wt (kDa)	Identities (%)	Amino acids	Protein type
***I =30.14 (α)**			
31.601	85	286	Hypothetical protein [AT2G42710]
31.505	100	279	Protein [AT2G22890]
31.316	100	278	Hypothetical protein [T8E3.21]
30.717	100	268	Hypothetical protein [T8E3.4]
30.515	83	269	Protein [AT2G01790]
30.214**	100	265	Protein [AT2G05410]
II=26.60 (α)			
28.642	85	265	Expansin-like 2 protein [Q9SVE5]
26.035**	88	226	Hypothetical protein [AT4G05220]
25.943	83	236	Hypothetical protein [AT2G45740]
25.712	47	224	Hypothetical protein [F9D24.230]
24.620	85	219	Hypothetical protein [F9D24.280]
24.518	100	217	Protein [AT2G05400]
III=21.43 (β)			
22.480	100	194	Lectin-like protein [T16H5.210]
22.094	83	197	Protein [F14D16.29]
21.782**	100	194	Hypothetical protein [AT2G05400]
21.640	83	192	Hypothetical protein [AT1G19130]
21.254	83	187	Hypothetical protein [Q8LDX4]
21.974	21	188	Hypothetical protein [Q84RE3/ AT2G05430]
20.779	100	203	Hypothetical protein [AAC80588.1]
IV=18.04 (β)			
19.861	100	175	Hypothetical protein [T8E3.3]
19.724	34	173	Hypothetical protein [Q9M2I3; F9D24.240
17.831**	29	153	Hypothetical protein [Q9M2J3; F9D24.140
16.693	41	146	Hypothetical protein [AT3G58220; F9D24.130

* I-IV represents the four proteins consistently observed in SDS-PAGE gels.

** Polypeptides of molecular weights marked in red were experimentally determined.

3.4 Discussion

Prominent protein bands that were observed at M_r ~50 kDa in *Cleome* (Figure 3.1) were reduced and resulted in the increased expression of M_r 27-30 kDa, 18 kDa and 13-15 kDa polypeptides in Figure 3.2. Polypeptides of M_r 25-30 kDa and 18-20 kDa were shown in the unreduced sample (Figure 3.1) and their accumulation levels increased when extraction was done with a reduction agent (Figure 3.2). This suggests that the storage protein of *Cleome* consists of dimeric polypeptides at ~50 kDa, which are broken by the reducing agent DTT into bands of M_r 30, 28, 18 kDa and 13-15 kDa. This is similar to the observations made in radish (Laroche *et al.*, 1984), rapeseed (Rodin *et al.*, 1990) and many dicotyledonous plants (Bewley and Black, 1994). Rödin and Rask (1990) observed that the unreduced sample of *B. napus* seeds showed polypeptides of molecular weight 48-54 kDa, 29-33 kDa and 21-23 kDa. Klimaszewska *et al.* (2004) also observed that 11 S globulins of molecular weight 59.6 kDa dissociated under reducing conditions to 38.2-40.0 and 22.5-23.5 kDa range polypeptides in *Pinus strobus*. Rödin *et al.* (1990) showed that the 30 kDa α- and 20 kDa β-polypeptides in rapeseed further split into polypeptide chains of slightly different molecular weights after reduction and alkylation.

It is evident from Figures 3.1 and 3.2 that the protein bands of M_r 25-30 and 18 kDa were present before the reduction process. Wong *et al.* (2004b) observed that proteins are synthesized in the reduced state during seed development and oxidised during maturation and drying. Wong *et al.* (2004a) observed that thioredoxin reduces disulphide bond of target proteins during germination and argued that its presence in mature seeds implied that there was some protein reduction going on. Adachi *et al.* (2003) observed that this

could happen when the pH in the vacuoles become acidic. Shutov *et al.* (2003) explained that there is limited proteolysis of the storage proteins in dry seeds.

The number of protein bands in *Cleome* increased as the seed matured and the accumulation of low molecular weight bands increasing later during maturation (Figure 3.3). Four prominent protein bands of estimated molecular weights of 30 kDa, 28 kDa, 18kDa and 17 kDa were present in the mature seeds of *C. gynandra*. The seed lots of *Cleome* produced in different regions contained similar protein bands. Ladizinsky (1983) observed that seed protein profiles are highly stable within species and Krochko and Bewley (2000) showed that there were consistent similarities among cultivars and between subfamilies in *Medicago sativa*. Thomas *et al.* (2003) showed that the concentration of crude protein in soybean seeds did not change significantly when the glasshouse was kept at average day/night temperatures of 23 to 31 °C, but decreased with further temperature increases. Johansson *et al.* (2003) showed that environmental influences that gave rise to variations in protein content also changed total amounts of glutenins and gliadins in wheat. Furthermore, Triboï, Martre and Triboï-Blondel (2003) observed that the variations in protein fraction composition in mature wheat grains were mainly due to differences in the total quantity of N per grain. It is noteworthy that these observations were not made specifically on storage proteins, but on crude protein content (Thomas *et al.*, 2003; Triboï *et al.*, 2003) and on gluten and gliadin (Johansson *et al.*, 2003).

Two-dimensional SDS-PAGE showed polypeptides of M_r 30, 27, 21 and 18 kDa, and wild mustard seeds accumulated more polypeptides than *Cleome* (Figures 3.5 and 3.6). Laroche *et al.* (1984) observed that the storage proteins in radish and rapeseed were

similar, and that rapeseed accumulated more polypeptides. However, Sjodahl *et al.* (1991) reported that two different, but highly similar proteins might be able to co-migrate to the same position on SDS-PAGE gel.

The amino acid sequences obtained from the four prominent bands separated discretely by 15% SDS-PAGE showed homology with many proteins in the proteome library, including expansins and lectins (Table 3.1). Expansins are enzymes that are structurally similar to glycosidases, and promote cell wall loosening and extension, normally expressed during seed germination (Chen, Dahal and Bradford, 2001; Ludidi *et al.*, 2002). These functions are pH dependent, with an acidic optimum. High transcript levels of expansin are found in the growing regions of internodes, leaves and roots (Lee and Kende, 2002). The proteins under study were extracted from non-germinated dry seeds and appeared at the neutral pH, spreading to the basic region of the SDS-PAGE gel. Lectins, on the other hand, are a group of non-enzymatic glycoproteins that bind to specific carbohydrates in plants, animals and microorganisms. In plants, their function is not well defined (Bewley and Black, 1994; Herve and Lescure, 1995). Adeli and Altosaar (1984) showed that vicilin-like polypeptides in *Avena sativa* were glycosylated like those in *Pisum sativum* and *Glycine max* unlike 12 S legumin. This could imply that lectins are mostly glycosylated vicilins.

The four amino acid sequences generated had homology with some proteins of unknown functions in the proteome library. For example, Laroche *et al.* (1984), assuming a molecular weight of 30 kDa, obtained a total amino acid of 251 in radish, which is comparable to the value of 265 at molecular weight 30.2 kDa obtained during this study. Rödin *et al.* (1990) using cDNA cloning of 12 S cruciferin of *B. napus* obtained a β-chain

of 190 amino acids and molecular weight of 21.2 kDa. This is comparable with the β-chain of 187, 192 and 194 amino acid of molecular weight of 21.25 kDa, 21.64 kDa and 21.78 kDa respectively, reported in this study. Sjödahl *et al.* (1991) used cDNA cloning to show the evolutionary close relationship between 11-12 S globulins in *Arabidopsis thaliana* and *Brassica napus* and, deduced that the Cru4 precursor was composed of 465 amino acid residues. Upon proteolytic cleavage of a single peptide bond, this residue gave rise to an α-chain of 254 amino acid residues of 28.1 kDa and β-chain of 189 residues of molecular weight 20.9 kDa. This compares with the α-chain of amino acid 265 and 263 (29.64, 29.50 kDa) and β-chain of 187, 192 and 194 amino acid (21.25, 21.64, 21.78 kDa). Most of the homology between the Blast search results and the N-terminal amino acid sequences obtained in this study showed high percent identities of 83-100% (Table 3.1).

Laroche *et al.* (1984) showed that the 12 S globulins in radish were similar to those in rapeseed. Similarity index obtained in this study and the analysis of variance confirmed that wild mustard is closely related to *Cleome,* and also supports their different classification into different families by Cronquist (1988). It also supports the observation by Johansson *et al.* (2003) that cultivar influences can give rise to variations in both protein components and concentration.

3.5 Conclusions

In this study, it has been shown that the seed storage proteins in *Cleome gynandra* have some similarities with those of wild mustard (*Brassica kaber*). The sequence results consistently showed that the seed proteins of *Cleome* also have similarities to those of

radish, rapeseed and *Arabidopsis* (Laroche *et al.*, 1984; Rödin *et al.*, 1990; Rödin and Rask, 1990; Sjödahl *et al.*, 1991; Rödin *et al.*, 1992). The storage proteins in *C. gynandra* consist of polypeptides of M_r 41-48 kDa (α/β), 27-30 kDa (α), 17-18 kDa (β), and one smaller chain of 13-15 kDa. Seeds of *Brassica kaber* consist of polypeptides of M_r 50-65 kDa (α/β), 30 kDa, 24-28 kDa (β), 17-18 kDa (α), and a smaller chain of 13 kDa. The polypeptides of M_r 27-30 kDa occurred at p*I* 6.5-7.5 and those of M_r 17-22 kDa occurred at p*I* 8.5-9.6.

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CHAPTER 4. LIGHT-INDUCED TRANSIENT DORMANCY INFLUENCES PROTEIN UTILISATION DURING SEED GERMINATION OF *CLEOME GYNANDRA* L.

4.1 Introduction

Physiologically mature seeds are either dormant or non-dormant and will germinate once the dormancy is relieved or optimum conditions are provided. However, embryos germinate soon after the histodifferentiation phase when isolated from their protective structures, but they do not form normal seedlings due to lack of nutrient reserves (Kermode, 1995; Hilhorst and Toorop, 1997). Successful completion of the developmental phase ensures the synthesis of nutrient reserves, a prerequisite for normal germination. During germination, catabolic processes result in degradation of reserve nutrients by hydrolytic enzymes that provide nutrients for embryo and seedling growth. Early in germination, polyadenylic acid and mRNAs disappeared in radish embryo axis (Delseny, Aspart and Guitton, 1977). In maize, the axes initiated radicle protrusion by 18-24 h of incubation at which time some proteins (~37 kDa) disappeared, while a group of house keeping proteins were synthesized (De Jiménez and Aguilar, 1984). These findings demonstrated that during germination, the expression of seed proteins might change or remain constant. The change in expression may be related to functions during germination or early seedling growth (Gallardo *et al.*, 2002; Wu *et al.*, 2003).

Eight out of 15 proteins, whose accumulation varied during radicle protrusion in *Arabidopsis* seeds including the 12 S storage protein (cruciferin) precursors, were identified by Gallardo *et al.* (2001). According to Gallardo *et al.* (2001) the abundance of

14 polypeptides changed during germination *sensu stricto*, six of which belonged to α- and β-cruciferins. Guy and Black (1998) showed that some proteins varied in abundance during imbibition and germination in wheat seeds, but they did not identify the proteins. Although many reports of increased protein levels during seed priming have been reported (McDonald, 1999; Wu *et al.*, 2003), 2-dimension SDS-PAGE indicated that there were no specific priming-induced proteins. Gallardo *et al.* (2001, 2003) identified polypeptides whose abundance increased during priming as degradation products of 12 S cruciferin β-subunits, which suggested that there was already mobilisation of these proteins.

Seeds of *Nemophila insignis, Amaranthus caudatus* and many cultivars of lettuce germinate normally in the dark but their germination is inhibited by light. Such seeds are referred to as negatively photoblastic (Bewley and Black, 1994; Baskin and Baskin, 1998). It was observed in *Nemophila* that the inhibitory effect of light is probably on cell elongation (Bewley and Black, 1994), which would mean that the expression of some proteins that facilitate or enhance this process is affected. White light had a marked inhibitory effect on the germination of *A. caudatus* at 20 °C and this was attributed largely to its effect on seed coat and endosperm (Gutterman, Corbineau and Côme, 1992). Dormancy in annual ryegrass was released through wet-stratification in darkness (Steadman, 2004). A larger proportion of annual ryegrass seeds failed to germinate when wet-stratification was performed in the presence of light at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 37 °C (Steadman, 2004). *Cleome* exhibited negative photosensitivity on exposure to continuous white light beyond 12 h at 20 °C during germination, but

photoinhibition was greatly reduced at alternating 20/30 °C or at constant 30 °C (Ochuodho and Modi, 2005).

Photoinhibition can be compared to thermoinhibition, because in both cases the seeds do not germinate. However, Hills, van Staden and Viljoen (2001) showed that 10 polypeptides, which were expressed during thermoinhibition in *Tagetes minuta*, disappeared when these seeds were transferred to optimal germination temperature. Earlier, Cantliffe, Fischer and Nell (1984) showed that cell elongation and division in lettuce were inhibited during thermoinhibition and that primed seeds germinated at the inhibitive temperature of 35 °C. De Klerk and Smulders (1984) also observed that protein synthesis during the lag phase of germination was inhibited at the high temperature of 30 °C and in dormant seeds of *Agrostemma githago*. Kępczyński and Bihun (2002) stated that the seed coat was partially responsible for the secondary dormancy that was induced in *A. caudatus* when the seeds were incubated at 45 °C. Gonai *et al.* (2004) showed that the biosynthesis of ABA was enhanced and the sensitivity of the seeds to it increased during thermoinhibition at 33 °C in lettuce. However, Xia, Stewart and Kermode (2002) reported that there was efficient post-germinative mobilisation of storage proteins in yellow cedar after dormancy-relieving treatments.

Previously, Ochuodho and Modi (2005) showed that a large proportion of mature seeds of *Cleome* failed to germinate at 20 °C in continuous light. The objective of the present study was to examine how the utilization of native protein reserves, which are metabolised during germination, is influenced by photoinhibition. The response of seeds harvested at different pod maturity stages to light and temperature interactions during germination was also investigated.

4.2 Materials and methods

4.2.1 Plant material

Two seed lots of *Cleome* were each donated by Kenya Seed Company (KSC), Kenya and Agricultural Research Council (ARC), South Africa in 2002. Fresh seeds were produced from the KSC seed lot at the University of KwaZulu Natal farm in Pietermaritzburg. Pods were harvested separately at different maturity levels depicted by pod colour – mature green, yellow and brown. Another lot of seeds was grown in glasshouses at day/night temperatures of 21/16 °C, 27/22 °C and 33/28 °C, and harvested when the pods had turned brown. The glasshouses experienced the natural day length during the months of July to October 2002 (Pietermaritzburg: 29°35′S30°25′E) and a controlled relative humidity of 60%.

4.2.2 Seed germination

Four replicates of 50 seeds were germinated at 20 °C or alternating 20/30 °C (16 h night/8 h day, respectively) in continuous white light or in darkness (Labcon LTGC 20-40; Johannesburg, South Africa). Seeds were removed from incubation after 18 h, 24 h, 36 h, 40 h, 48 h, 72 h or 96 h and non-imbibed seeds were used for control. The seed lots from the glasshouses were germinated at alternating 20/30 °C in the presence of light or in darkness for 10 days. Subsamples from these seed lots were incubated at 20 °C in continuous light for 3 or 7 days and then transferred to 20/30 °C in darkness for 10 days. The seed was considered germinated when radicle protrusion was evident and germination percentage was determined 10 days after incubation. The seeds for protein

analysis were ground in liquid nitrogen and stored at -20 °C until extraction was performed.

4.2.3 Extraction of proteins

Extraction buffer was prepared by dissolving 21.02 g urea and 7.61 g thiourea in distilled water. To this buffer was added 0.2% v/v CHAPS, 1% v/v carrier ampholytes (pH 3-10), 18 mM Tris, one tablet proteinase inhibitor (complete mini), 53 U/ml DNase and 4.9 U/ml RNase and lastly 1% w/v dithiothreitol (DTT) (Gallardo *et al.*, 2002 modified according to Görge *et al.*, 2003). The ground seed was added to 1.2 ml of the extraction buffer in a 1.5 ml centrifuge tube. Extraction was carried out at room temperature for 1 h with intermittent shaking. The extract was centrifuged (Beckman J2-21M) at a speed of 32,000 g for 10 min at 4 °C to obtain a clear supernatant. Protein content of the extracts was determined using the Pierce Micro BCA™ Protein Assay Reagent Kit with bovine serum albumin (BSA) as a standard, according to the manufacturer's instructions.

4.2.4 Polyacrylamide gel electrophoresis

To separate the proteins on weight basis, the SDS-PAGE gel (Laemmli, 1970) system was employed, according to the manufacturer's instructions (Amersham Pharmacia Biotech SE 260). Extracts containing equal amounts of protein were resolved with 10% SDS-PAGE gels cast with a 7 cm resolving gel and 1 cm stacking gel. A sample of 20 µl containing approximately 20 µg protein was loaded and the gels were run at 300V, 20mA constant current for 3 h. The gels were stained with Coomasie blue (R250) and viewed through a UVP GDS 5000 Image Store apparatus and saved on a diskette. Band density

analysis was performed using the computer program AnalySIS 3.2 (SOFT IMAGING SYSTEM) on a picxel scale of 0-255 (where black is given value 0 and white, 255). Thirty readings were made on each band and the inverse of each value recorded. Using inverse values made it possible to have the light (poorly expressed) protein bands to correspond with low values. The average and standard deviation were used to present the intensity of each protein band.

4.2.5 Statistical analysis

Analysis of variance was used to quantify the differences between seed lots from ARC & KSC and those obtained from green, yellow and brown pods. Band intensity of gels obtained from seeds whose germination was stopped at various intervals and measured by AnalySIS was also analysed. GenStat statistical package (2000) was used to analyse germination percentages obtained and Excel was used to compute standard deviations, used to form the error bars.

4.3 Results

4.3.1 Germination of *Cleome* seeds

Seeds from the ARC showed a germination of 57% and 41% at alternating 20/30 °C in darkness and in continuous light, respectively, after four days of incubation. Germination at 20 °C was 43% in the dark but it decreased to 2% in continuous light (Figure 4.1A).

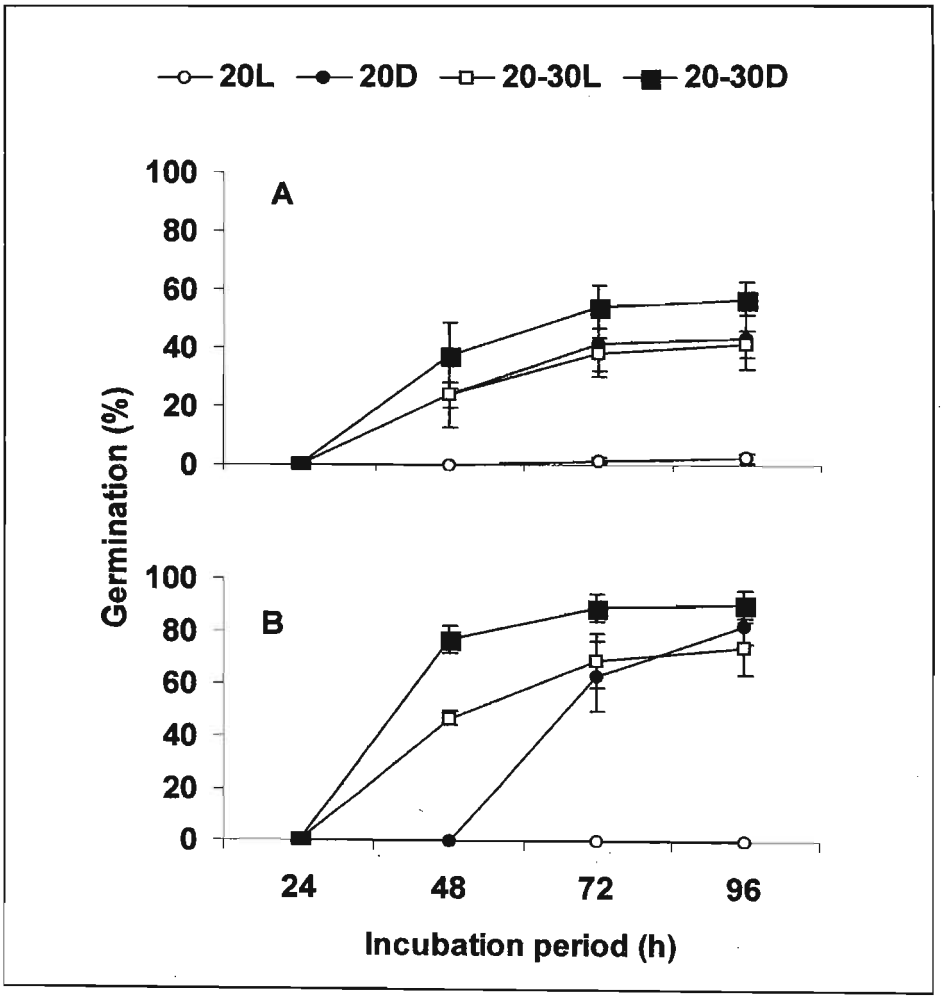

Figure 4.1. Germination of the original *Cleome* seeds from ARC (A) and KSC (B) at 20 °C or alternating 20/30 °C (16/8 h) in continuous white light or darkness. (○) 20 °C with light, (●) 20 °C in darkness, (□) 20/30 °C with light and (■) 20/30 °C in darkness. Error bars represent SD, n = 6.

The seeds from KSC showed a similar germination pattern to the ARC seeds under the same conditions (Figure 4.1B). At alternating 20/30°C, the KSC seeds had a germination of 90% in darkness and 74% in light, after four days. Seed germination at 20 °C was 82% in the dark, but the seeds failed to germinate in continuous white light. In both seed lots (ARC and KSC), the interaction between temperature and light was highly significant ($P < 0.001$) and the germination response over days was quadratic (Appendix 4a and b).

The negative effect of light was also shown in mature fresh seeds harvested from green, yellow and brown pods grown in the field (Figure 4.2). Mature seeds from green pods from the field attained more than 90% germination within 48 h of incubation at alternating 20/30 °C in the presence of light or in darkness. The germination of seeds from yellow pods was intermediate, reaching about 70% in both light and darkness, after four days.

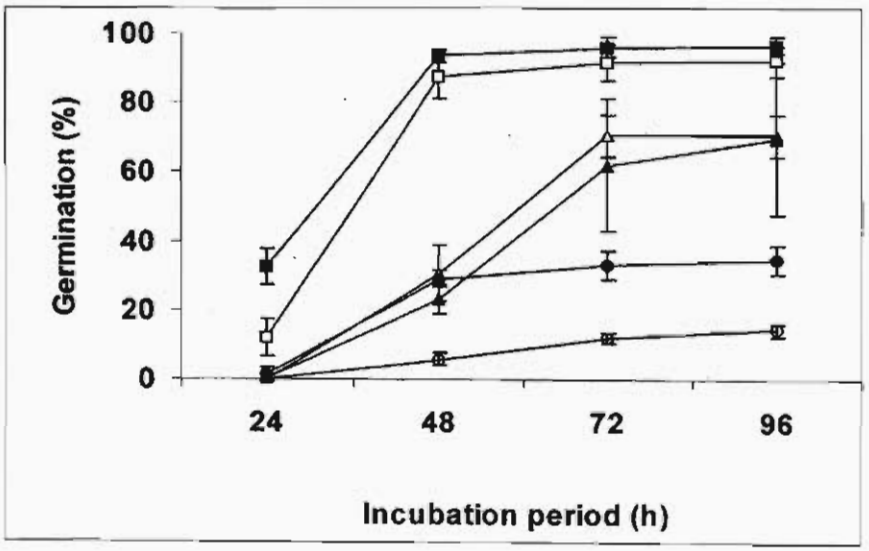

Figure 4.2. Germination of black seeds of *Cleome* grown in the field and harvested from green pods incubated at alternating 20/30 °C (16 h night/8 h day) in continuous white light (□) or darkness (■), yellow pods incubated at 20/30 °C in continuous white light (△) or in darkness (▲) and brown pods incubated at 20/30 °C in continuous white light (○) or in darkness (●). Error bars represent SD, n = 4.

However, the seed from brown pods showed a poor germination of 35% in darkness and the germination was reduced to 15% in the presence of light. Analysis of variance showed that the germination of seeds from the three pod maturity stages followed a quadratic response. There was a significant three-way interaction between exposure x lots x days ($P < 0.001$). This can be interpreted to mean that there was no consistency in the germination responses of the seed lots over days regardless of whether the incubation was in darkness or continuous light (Appendix 4c)

It appears that physiological maturity was attained between the green and the yellow pod stages, when seed germination and the TSW (thousand seed weight) reached the maximum (Figure 4.3). The highest germination was showed by black seeds from green pods and germination dropped significantly when the pods were yellow.

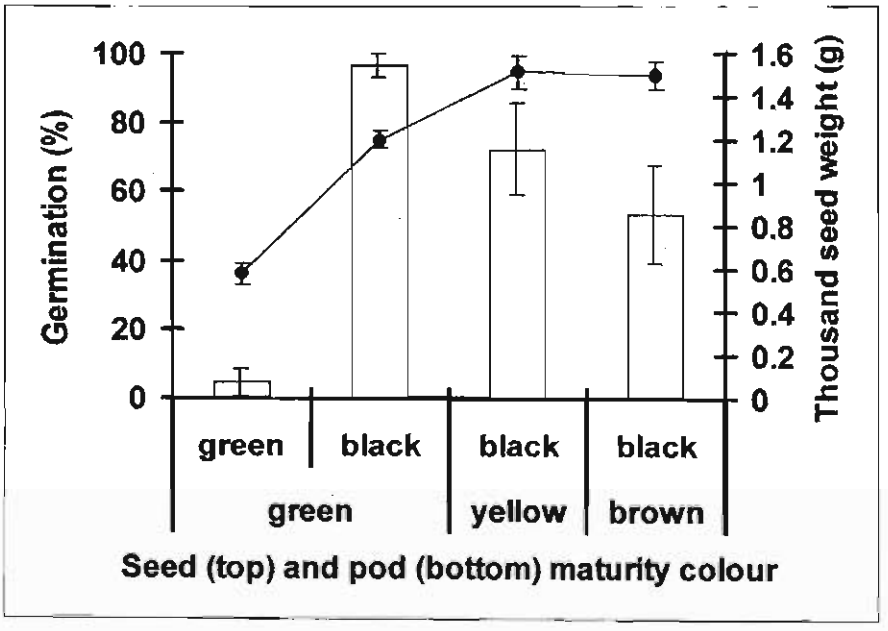

Figure 4.3. Germination (bar graph) of *Cleome* seeds at alternating 20/30 °C in darkness after 10 days and thousand seed weight (line graph) at different seed and pod maturity stages, as categorised by colour. Error bars represent SD, n = 4.

Seeds grown in the glasshouses at day/night temperatures of 21/16 °C, 27/22 °C and 33/28 °C and harvested when the pods were brown had lower germination in the presence of light than in darkness (Table 4.1). The seeds produced at 21/16 °C showed 76% germination; those produced at 27/22 °C had a germination of 93% while those produced at 33/28 °C obtained a germination of 84% at the germination temperature of 20/30 °C after 4 days of incubation in darkness. In continuous light and 20/30 °C germination temperature, the seeds produced at 21/16 °C showed a germination of 40%, those produced at 27/22 °C had a germination of 55% and the germination of those produced at 33/28 °C decreased to 37%. The seeds of *Cleome* showed better germination at 20 °C in darkness compared to that at alternating 20/30 °C in continuous light. The germination of seeds from 21/16 °C, 27/22 °C and 33/28 °C was 54%, 76% and 69%, respectively, after four days. However, the seeds failed to germinate at 20 °C in continuous light but on transfer to alternating 20/30 °C in darkness, the seeds had a germination of 23%, 39% and 40% respectively.

Table 4.1 Germination of seeds produced in glasshouses at different temperatures, germinated in different conditions. Values represent means ± SD, n = 4.

Incubation condition	**Temperature of production (day/night °C)**		
	21/16	**27/22**	**33/28**
20/30 °C dark	**76.0 ± 9.033**	**93.0 ± 3.464**	**83.5 ± 7.550**
20/30 °C light	**40.0 ± 5.888**	**53.5 ± 3.416**	**35.5 ± 1.915**
20 °C dark	**53.5 ± 8.226**	**76.0 ± 1.633**	**69.0 ± 7.605**
20 °C light	**0.00**	**0.00**	**0.00**
7 days 20 °C light transfer to 20/30 °C dark	**23.0 ± 6.831**	**39.0 ± 5.773**	**40.5 ± 5.291**

4.3.2 Expression of storage proteins during seed germination

Protein bands of KSC seeds incubated at 20/30 °C in darkness and in the presence of light had diminished by 72 h of germination (Figure 4.4). However, the analysis of band intensity using the computer package AnalySIS showed that the proteins of seeds germinated in darkness diminished faster (24 h) than the bands of seeds incubated in the presence of light (48 h) (Figure 4.5). Analysis of variance of the bands showed that band intensities at 24 h in darkness were significantly lower than those in light (Appendix 4d). By 72 h of germination, the intensity of the bands in both light and dark-germinated seeds was less than half the original values (Figure 4.5).

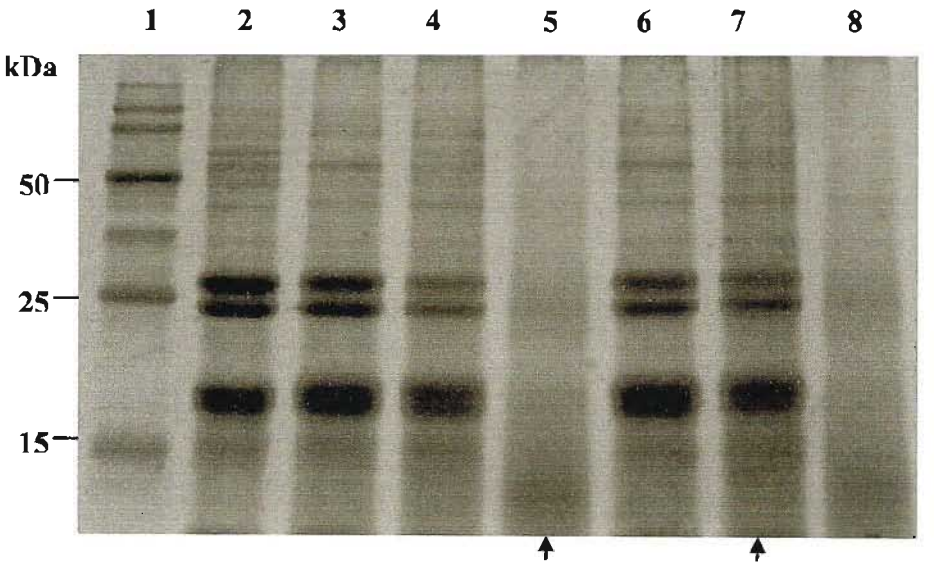

Figure 4.4. Analysis of the proteins of KSC seeds with10% SDS-PAGE gel. Lane 1, molecular weight marker, dry seeds (Lane 2). Seeds germinated at alternating 20/30 °C in continuous white light for 24 h (Lane 3), 48 h (Lane 4) and 72 h (Lane 5). Seeds germinated at alternating 20/30 °C in darkness for 24 h (Lane 6), 48 h (Lane 7) and 72 h (Lane 8). Arrows indicate the lanes where the protein expression diminished.

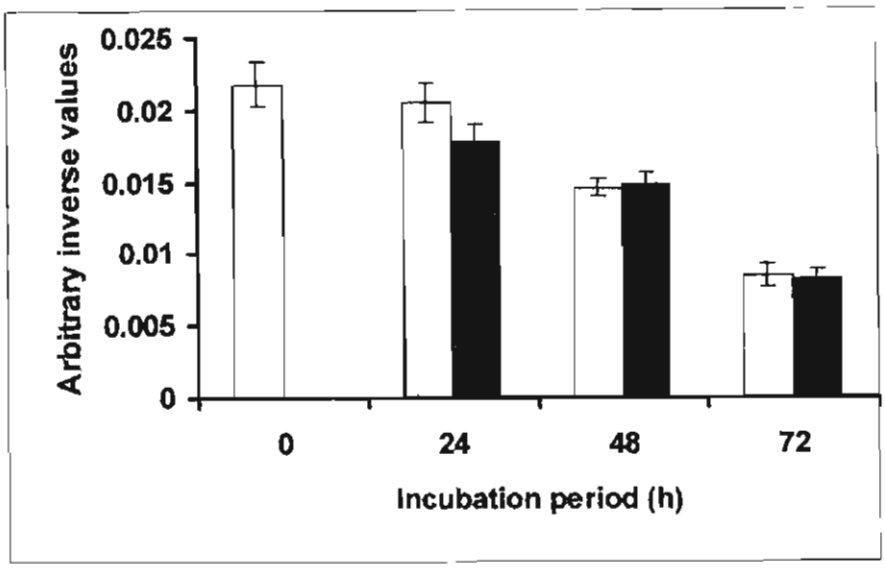

Figure 4.5. Analysis of band intensity of the gel in Figure 4.4 using computer soft image system (AnalySIS). Seeds were germinated at alternating 20/30 °C with light (□) or in darkness (■). Seed proteins were extracted at intervals and analysed by SDS-PAGE. Error bars represent SD, n =30.

There was an apparent difference between the seed lots, as the proteins of the KSC seeds germinated at 20/30 °C in darkness diminished faster than those of the ARC seeds (Figure 4.6). The intensity of the protein bands of the KSC seeds was significantly lower than those of ARC seeds after 48 h of germination ($P < 0.05$) (Appendix 4e), but the difference could only be discerned visually after 72 h (Figure 4.7). The bands of the ARC seeds were still visible after 72 h.

The proteins of the mature black seeds from green pods started diminishing between 24-48 h irrespective of whether germination occurred in darkness or light. Seeds harvested from the yellow and brown pods did not show any change in protein expression in 48 h (Figure 4.8). Whether germinated in light or darkness, the expression of the higher molecular weight protein of M_r 28 kDa diminished before the lighter protein of M_r 24 kDa (Figure 4.8, Lanes 4 and 6).

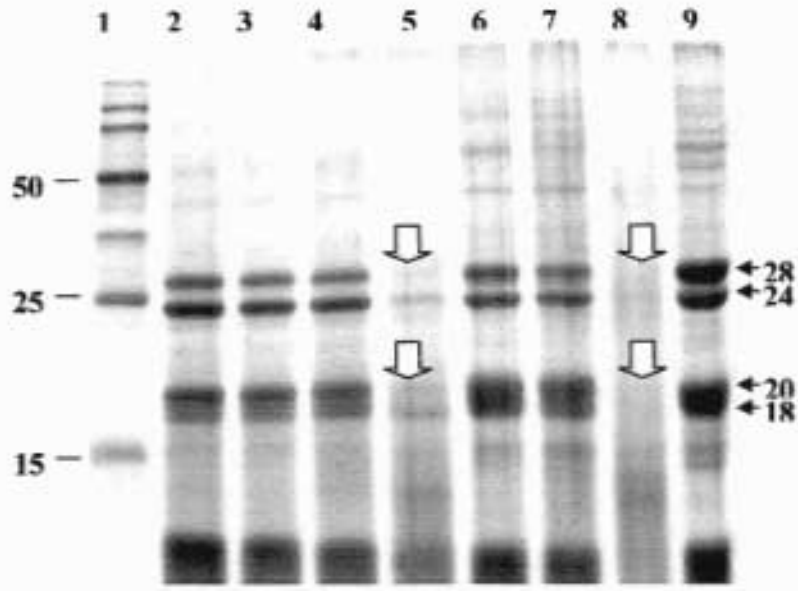

Figure 4.6 Analysis of the proteins of ARC and KSC seeds germinated at alternating 20/30 °C in darkness with10% SDS-PAGE gel. Lane 1, molecular weight marker. ARC seeds: Lane 2, dry seed; Lanes 3, 4, and 5, seeds incubated for 24 h, 48 h and 72 h, respectively. KSC seeds: Lanes 6, 7, and 8, seeds incubated for 24 h, 48 h and 72 h, respectively; Lane 9, dry seed. Open arrows indicate the protein bands that diminished and closed arrows indicate prominent proteins.

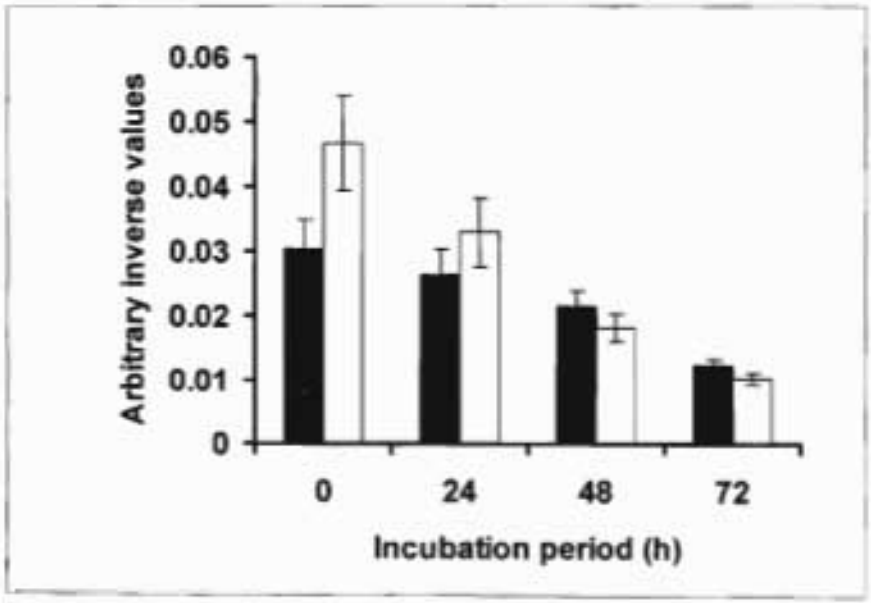

Figure 4.7. Analysis of band intensity of the gel in Figure 4.6 using computer soft image system (AnalySIS). Seeds from ARC (■) and KSC (□) were germinated at alternating 20/30 °C in darkness. Seed proteins were extracted at 24 h intervals and analysed by SDS-PAGE. Error bars represent SD, n = 30.

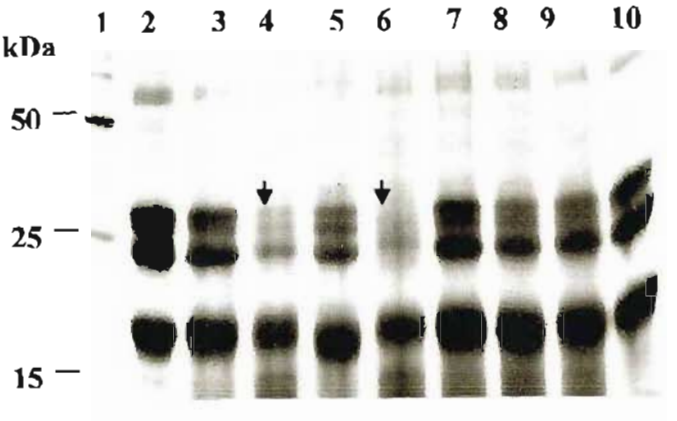

Figure 4.8. Proteins extracted from fresh seeds germinated at 20/30 °C for different periods and analysed with 10% SDS-PAGE gel. Lane 1, molecular weight marker; Lane 2, dry seeds. Lanes 3 and 4, seeds from green pods germinated in light for 24 h and 48 h; Lanes 5 and 6, seeds germinated in darkness for 24 h and 48 h, respectively; Lanes 7 and 8, seeds from yellow pods germinated in darkness for 24 h and 48 h, and Lanes 9 and 10, seeds from brown pods germinated in darkness for 24 h and 48 h, respectively. Arrows indicate the bands that diminished.

Cleome seeds incubated at 20 °C in continuous light failed to germinate or had low germination as shown in Figure 4.1 and Table 4.1. The seeds did not show any protein accumulation change on SDS-PAGE analysis and the seeds had protein bands similar to those of the dry ungerminated seeds (Figure 4.9). However, the high molecular weight proteins of 28 kDa began diminishing by the fourth day (96 h) in the seeds that were germinated in darkness.

A comparison between the protein expressed by dry ungerminated seeds and those from seeds that had failed to germinate at 20 °C in continuous white light showed no difference. Photoinhibition of seed germination for up to 7 days neither changed the number nor band intensity of the storage proteins expressed (Figure 4.10).

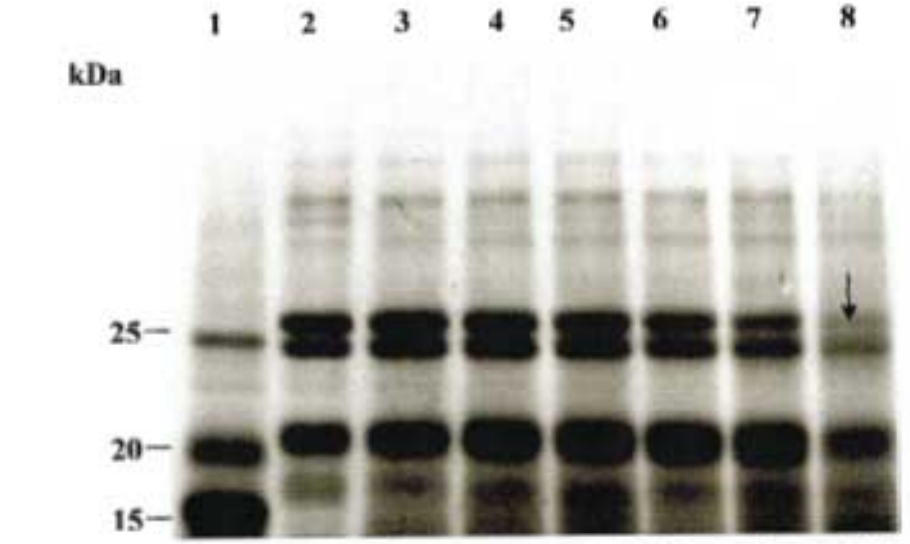

Figure 4.9. Seeds from KSC germinated at 20 °C in continuous light and in darkness. Lane 1, molecular weight marker; Lane 2, dry seed; Lanes 3, 4 and 5, seeds incubated with light for 48, 72 and 96 h, respectively; Lanes 6, 7 and 8, seeds incubated in darkness for 48, 72 and 96 h, respectively. Arrow indicates the band that diminished.

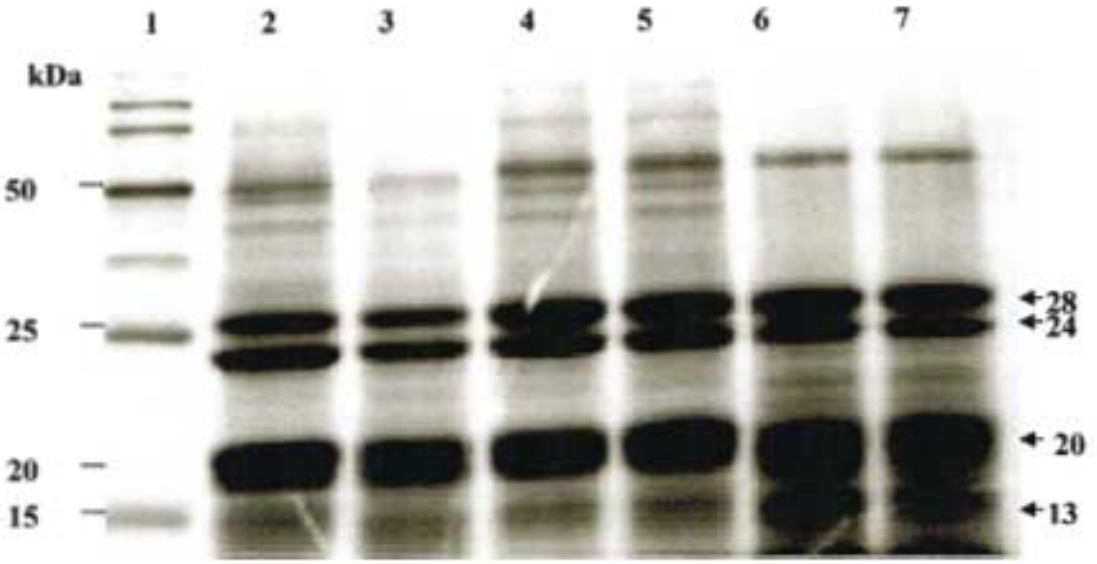

Figure 4.10. Proteins extracted from mature dry seeds grown in glasshouses at day/night temperatures of 21/16 °C, 27/22 °C and 33/28 °C. The seeds were harvested when the pods were brown and analysed with 10% SDS-PAGE gel. Lane 1, molecular weight marker. Proteins from nongerminated seeds that were produced at 21/16 °C, 27/22 °C and 33/28 °C are shown in Lanes 2, 3 and 4, respectively. Proteins from seeds produced at 21/16 °C, 27/22 °C and 33/28 °C, and germinated at 20 °C in continuous white light for 7 d are shown in Lanes 5, 6 and 7, respectively. Arrows indicate the sizes of proteins that were differentially expressed due to germination treatment.

4.4 Discussion

The results indicate that the germination of *Cleome* seeds was negatively photosensitive and that photoinhibition was more pronounced at 20 °C (Figure 4.1). It has been shown that photoinhibition of germination in *Cleome* was pronounced at 20 °C, but minimally expressed at optimum germination temperatures (Ochuodho and Modi, 2005). Gutterman *et al.* (1992) and Steadman (2004) obtained similar results in *A. caudatus* and annual ryegrass, respectively. There was also an indication that delayed harvesting influenced photoinhibition (Figure 4.2). There are many reports that physiological maturity is reached after maximum seed dry mass in several plant species (Hilhorst and Toorop, 1997; Still and Bradford, 1998) and in this study physiological maturity may have been attained before yellow pod stage in *Cleome* (Figure 4.3). These observations suggest that black seeds selected from the green pods may have attained physiological maturity and hence their germination percentage was higher. This observation corroborates the earlier suggestion that physiological maturity fell between mature green and yellowing pods, when the seeds turned black. Seeds harvested at the brown pod stage showed strong negative response to continuous light during germination. Germination can be delayed, reduced or may fail to occur depending on the physiological status of the seeds and, the light and temperature at which the test is carried out also has an effect (Figures 4.1 and 4.2).

The temperature at which seeds are produced influenced the quality of seed, especially, germination (Table 4.4). The seeds with the lowest germination were produced at the lowest temperature of 21/16 °C and were also the most photosensitive. These results support observations made by Steadman *et al.* (2004) in annual ryegrass

that seed maturation environment, particularly temperature, had a significant effect on seed numbers and seed dormancy characteristics.

It is not clear whether the difference in protein expression between KSC and ARC seeds is related to seed quality. However, from Figure 4.1 the KSC seeds showed better germination than ARC seeds. Also, the black seeds from green pods showed better germination compared to those from yellow and brown pods (Figure 4.2). The seed proteins from both KSC and black seeds from green pods diminished earlier during germination compared to those from ARC, yellow and brown pods (Figures 4.4-4.8). Guy and Black (1998) demonstrated that the content of stored and new mRNA in wheat embryos changed depending on seed vigour, and Xia *et al.* (2002) showed that after breaking dormancy in yellow-cedar, protein bands of the seed lot with higher percent germination diminished faster. Similarly, Klimaszewska *et al.* (2004) observed that the bands of the prominent protein complex 46-49, 38.2-40, 25-27, 27-29 and 22.5-23.5 kDa disappeared rapidly during germination in the zygotic embryos of *Pinus strobus*.

Seed germination proceeds slowly at low temperatures and the accumulation of proteins in seeds germinated in darkness did not diminish immediately (Figure 4.9). The expression of the 28 kDa protein gradually decreased as the expression of the 20 kDa protein gradually increased, and the seed progressed towards germination by 96 h (Figure 4.9). This observation apparently contradicts the findings by Guy and Black (1998) that the number of polypeptides increased during imbibition of wheat in darkness. Guy and Black (1998) did not identify the proteins but seemed to be dealing with enzymes and mRNAs involved in the germination process. The observation agrees with Gallardo *et al.* (2003) on the increased accumulation of the subunits of seed storage proteins in

Arabidopsis. Bewley and Black (1994) and Gallardo *et al.* (2003) observed that the mobilisation of reserve proteins began with the hydrolysis of the larger molecules into smaller subunits.

The changes in the embryo axis during imbibition reported by Delseny *et al.* (1977) in radish and De Jiménez and Aguilar (1984) in maize focused on the accumulation of old and new mRNAs during the radicle protrusion phase. The changes observed in this study involved storage proteins and may or may not have occurred if seeds were investigated in the reports by the two groups above. Hilhorst and Toorop (1997) reported that immature embryos may germinate but will not develop normal seedlings due to insufficient nutrient reserves. Adequate storage reserves are, therefore, essential for the development and sustenance of a viable normal seedling and seed storage proteins form an important component of these reserves. The mobilisation of the seed storage reserves is associated with germination and embryo growth and does not occur in dry or imbibed seeds or ungerminated dormant seeds (Bewley and Black, 1994; Shutov, *et al.*, 2003). Genuine storage proteins act as amino acid reserves which are mobilised to nourish the embryo and the young seedling (Shutov, *et al.* 2003). Seed storage proteins remained unchanged when germination was carried out at 20 °C in continuous light because there was no radicle protrusion.

Photoinhibition is also referred to as pseudo-dormancy, where seeds fail to germinate because conditions of incubation are not optimum. Gutterman *et al.* (1992) showed that the inhibitory effect of light on germination of *Amaranthus caudatus* seeds was enhanced in low oxygen or at low temperatures. The seeds of *Cleome* showed low germination percentage when transferred to optimum conditions but this did not elicit discernible

changes of protein levels. Figure 4.10 indicated that the photoinhibited seeds showed the same proteins as those of the dry seeds. Thermoinhibition as shown by Hills *et al.* (2001) in *Tagetes minuta* seeds is different from photoinhibition because no new specific polypeptides were expressed. The proteins that accumulated during thermoinhibition were absent from dry seeds and disappeared during subsequent germination.

Seed dormancy differs from photoinhibition because dormancy has to be relieved through external treatment before seed germination can occur. Most of the seeds in the photoinhibited seed lot did not regain their germination capability when transferred to optimum germination conditions (Table 4.1). Photoinhibition may be a 'conditional dormancy' and because conditional dormancy is progressive, some of the seeds could have attained secondary dormancy (Baskin and Baskin, 1998). Analyses of seed proteins by SDS-PAGE showed consistent five bands of M_r 24-28 kDa, 18-20 kDa and 13-15 kDa. The 13-15 kDa protein was prominent in KSC seeds and the seeds from brown pods produced in both the field and the glasshouse conditions. This observation could imply that the protein was more abundant in the seed lots that were more sensitive to photoinhibition and its accumulation increased after physiological maturity. The seed coat has been reported to play a partial role in photoinhibition (Gutterman *et al.*, 1992) and, in the induction of secondary dormancy in *A. caudatus* (Kępczyński and Bihun, 2002) and to influence seed dormancy, germination and longevity in *Arabidopsis* (Debeaujon *et al.*, 2000). It is speculated that the accumulation of the 13-15 kDa protein could be high in the seed coat.

Seed priming is a popular technology by which seeds are subjected to controlled hydration and germination proceeds up to phase II. During phase II, new physiological

activities associated with germination are initiated and minimal changes in protein levels were observed (Cantliffe *et al.*, 1984, McDonald, 1999; Wu *et al.*, 2003). Gallardo *et al.* (2003) observed that during hydropriming, there was an accumulation of subunits of cruciferins and tubulins, indicating that germination had began. No such change in the content of seed proteins was observed in photoinhibited seeds (Figure 4.10). The quality of primed seeds is affected by the conditions, especially the temperature, of priming. The best results for seed priming were obtained at priming temperatures considered optimum for germination of four grass species (Hardegree, 1996) and tomatoes (Ozbingol *et al.*, 1998). As stated earlier, photoinhibition was more pronounced at suboptimal temperature of 20 °C in continuous light in *Cleome*. Chilling on the other hand is carried out at temperatures below the minimum required for germination. However, when chilled seeds are transferred to optimum germination temperatures, their germination can be improved (Steadman, 2004). Seeds of *Cleome* that were chilled in darkness showed the same germination as non-chilled (Ochuodho and Modi, 2005). It can, therefore, be stated that while priming or pre-chilling generally improved seed germination, photoinhibition affected germination negatively in *Cleome*.

4.5 Conclusions

Cleome seeds responded negatively to continuous white light during germination at 20 °C. This photoinhibition was more evident in seeds harvested when the pods had turned brown than the mature black seeds selected from green pods. During photoinhibition four proteins of M_r 28, 24, 20 and 18 kDa were consistently observed in mature *Cleome* seeds but these proteins diminished during germination. These proteins are within the same

range of molecular weight as storage proteins found in radish, *Brassica napus* and *Sinapis arvensis,* which suggest that they may be storage proteins. The fifth protein of M_r 13-15 kDa was more pronounced in seeds from brown pods than those from immature seeds and may be composed of proteins of the seed coat.

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CHAPTER 5. TEMPERATURE AND LIGHT REQUIREMENTS FOR THE GERMINATION OF *CLEOME GYNANDRA* SEEDS

5.1 Introduction

In the majority of plant species, seed development and germination are separated by a period of low metabolic activity referred to as dormancy or quiescence. Dormancy is the inability of the embryo to germinate due to some inherent inadequacy even when given optimum conditions (Bewley and Black, 1994). It allows for the distribution of germination in time and space. In weedy species, seed dormancy secures long-term survival by allowing the seeds to germinate over a long period. Seeds show a continuum of responses as they go from nondormancy to dormancy and from dormancy to nondormancy, coupled with changing sensitivity to environmental factors (Baskin and Baskin, 1998). However, in commercial seed production seed dormancy lowers the quality of seed by decreasing crop stand.

The seed coat protects the embryo against adverse environmental conditions, but it also imposes dormancy through impermeability to water and/or oxygen and resistance to radicle protrusion. Debeaujon, Leon-Kloosterziel and Koornneef (2000) demonstrated seed-coat dormancy using *Arabidopsis thaliana* mutants with different testa characteristics. Using the same mutants, Debeaujon and Koornneef (2000) showed that gibberellic acid overcame the germination constraints imposed by both seed coat and ABA-related embryo dormancy.

External stimuli such as light and alternating temperatures have been shown to reduce dormancy (Hilhorst and Karssen, 1992). Seed treatments such as after-ripening and light may lead to degradation of abscisic acid (ABA), and pre-chilling may induce leakage of

ABA, thus breaking dormancy (Groot and Karssen, 1992). While light interacts with high temperatures to break dormancy, it induces secondary dormancy at low temperatures in some species. The germination of *Amaranthus caudatus* seeds was found to be fastest and highest at 35 °C in darkness and in the presence of light (Gutterman, Corbineau and Côme, 1992). Germination of *Amaranthus caudatus* was also found to be inhibited by continuous white light at 20 °C. Similar results were obtained in maritime plants, including *Brassica tournefortii* (Thanos *et al.*, 1991), *Citrullus vulgaris* (Thanos and Mitrakos, 1992), *Matthiola tricuspidata* (Thanos, Georghiou and Delopetrou, 1994) and *Passiflora incarnata* (Benvenuti, Simonelli and Macchia, 2001).

Dormancy that is controlled by an inhibitor-promoter balance could be corrected by exposing dry seeds to higher temperatures or imbibing at low temperatures (stratification) (Copeland and McDonald, 1995). Poljakoff-Mayber *et al.* (2002) showed that the initial amount of endogenous ABA in dormant and non-dormant oat seeds was similar, but ABA almost disappeared within the first 16 h of imbibition in the non-dormant seeds as GA_1 was produced to allow germination. Similar results were obtained in *Onopordum nervosum* (Fernandez *et al.*, 2002) and in tall and dwarf peas (Kato-Noguchi, 2002), where shoot elongation was not realized as the biosynthesis of GA_1 was inhibited by exposure to red light. Hays, Yeung and Pharis (2002) demonstrated that GA_1 plays a role in cell elongation at the embryo axis in *Brassica napus*.

However, Caboche *et al.* (1998) observed that dormancy is a complex quantitative character controlled by several genes, some of which are in turn controlled by environmental factors. These authors proposed that the best model established for explaining maintenance and breaking of dormancy should involve the equilibrium

between ABA and GA. A combination of the dormancy breaking treatments not only shifts the seed from dormancy to a quiescence state, but it also induces germination. Work has been done to break dormancy and improve germination through the use of various temperatures, light, growth regulators, and inorganic salts in species other than *Cleome* (Thanos *et al.*, 1991, 1994; Benvenuti, Macchia and Miele, 2001; Mackay, Davis and Sankhla, 2001; De Villiers, van Rooyen and Theron, 2002; Corbineau *et al.*, 2002; Veassey and Texeira de Freitas, 2002).

Cleome is wide spread in the tropics as a weed, but for years it has been a semi-domesticated volunteer crop in home gardens in many parts of sub-Saharan Africa. Its origin is unclear as it is reported to be native to North, West, Central and East Africa, African Islands, Middle East and Asia (Chweya and Mnzava, 1997; Fletcher, 1999). This plant flowers indeterminately for about three months and the seeds may take up to one year, post-harvest, to reach maximum germination (Chweya and Mnzava, 1997). Böhringer, Lourens and Jansen van Vuuren (1999) obtained maximum germination of only 25% at 31 °C in darkness six months after harvest. The poor seed germination in *Cleome* could be due to the hard seed coat, immature embryos or induced secondary dormancy. The objective of this study was to determine the effects of temperature and light on the germination of *Cleome gynandra* seeds. The effects of temperature and light were investigated concurrently with pre-germination treatments: chilling, scarification, hydration and germination in the presence of KNO_3 or GA_3.

5.2 Material and methods

5.2.1 Plant material

Seeds of *Cleome gynandra* (L.) were obtained from the Agricultural Research Council (ARC), South Africa and Kenya Seed Company (KSC), Kenya. The seeds from the ARC were harvested in 2001 and those from Kenya in 1999. Seeds were stored in a refrigerator (5 °C) until the experiments commenced.

5.2.2 General germination conditions

Four replications of 50 seeds were placed in petri dishes on three Whatman No.1 filter papers moistened with 7 ml of distilled water. The seeds were incubated in growth chambers (Labcon, LTGC 20-40; Johannesburg, South Africa) at 20 °C or 30 °C, either in darkness or continuous light, and at 20/30 °C alternating temperature regimes, 16 h (20 °C) and 8 h (30 °C) either in darkness or continuous light. Germination was allowed to continue for 10 days. To investigate the effects of light exposure period on germination, seeds were incubated in light for 24 h, 16 h, 12 h, 8 h and 0 h (24 h dark), respectively, at 20 °C or 20/30 °C during 10 days of germination. Germination testing after seed pre-treatments, however, was conducted at alternating 20/30 °C in darkness only. Germination counts were conducted at 2-day intervals. Seeds were considered germinated when the radicle had protruded.

5.2.3 Pre-germination treatments

Three replications of 50 seeds from both the ARC and KSC seed lots were placed on three filter papers moistened with 7 ml of distilled water, 0.1% GA_3 solution or a solution

of 0.2% KNO_3 and incubated for 14 days at 20 °C in continuous light. These seeds were then transferred to 20/30 °C alternating temperatures in darkness to allow germination for 10 days.

Sub-samples of the ARC seed lot were scarified using a razor blade and sulphuric acid. Three replications of 50 seeds each were scarified by making a transverse cut at the radicle end (point-pricking) with a razor blade. Another three replications of 50 seeds were soaked in concentrated sulphuric acid (98%) for 5, 10, 20 or 30 minutes and then washed in running distilled water for five minutes. The seeds were then germinated at alternating temperatures of 20/30 °C in darkness for 10 days.

Other pre-treatment methods applied to the seeds from ARC to improve seed germination were pre-chilling, pre-hydration, GA and potassium nitrate. Three replications of 50 seeds were placed on moist filter papers in petri-dishes and incubated in a cold room at 5-10 °C for five or seven days. The pre-hydration treatment was performed on four replications of 50 seeds. The seeds were placed between two filter papers moistened with 7 ml of distilled water and incubated at 25 °C for 4 h or 18 h. After these treatments, the seeds were germinated at alternating temperatures of 20/30 °C in the dark. Lastly, sub-samples of the ARC seed lot were treated with potassium nitrate or GA. Four replications of 50 seeds each were placed on three filter papers soaked with 0.2% KNO_3 or 0.1% GA_3 and, the seeds germinated at alternating temperatures of 20/30 °C in darkness.

5.2.4 Statistical analysis

Analysis of variance was performed using GenStat statistical package (2000). Differences between treatments were determined by LSD (P = 0.05) or standard deviations.

5.3 Results

The total germination of the KSC seed lot was significantly higher than that of the ARC seed lot, regardless of the light and temperature conditions during germination (Figure 5.1). However, the germination response to temperature and light showed the same pattern for both seed lots.

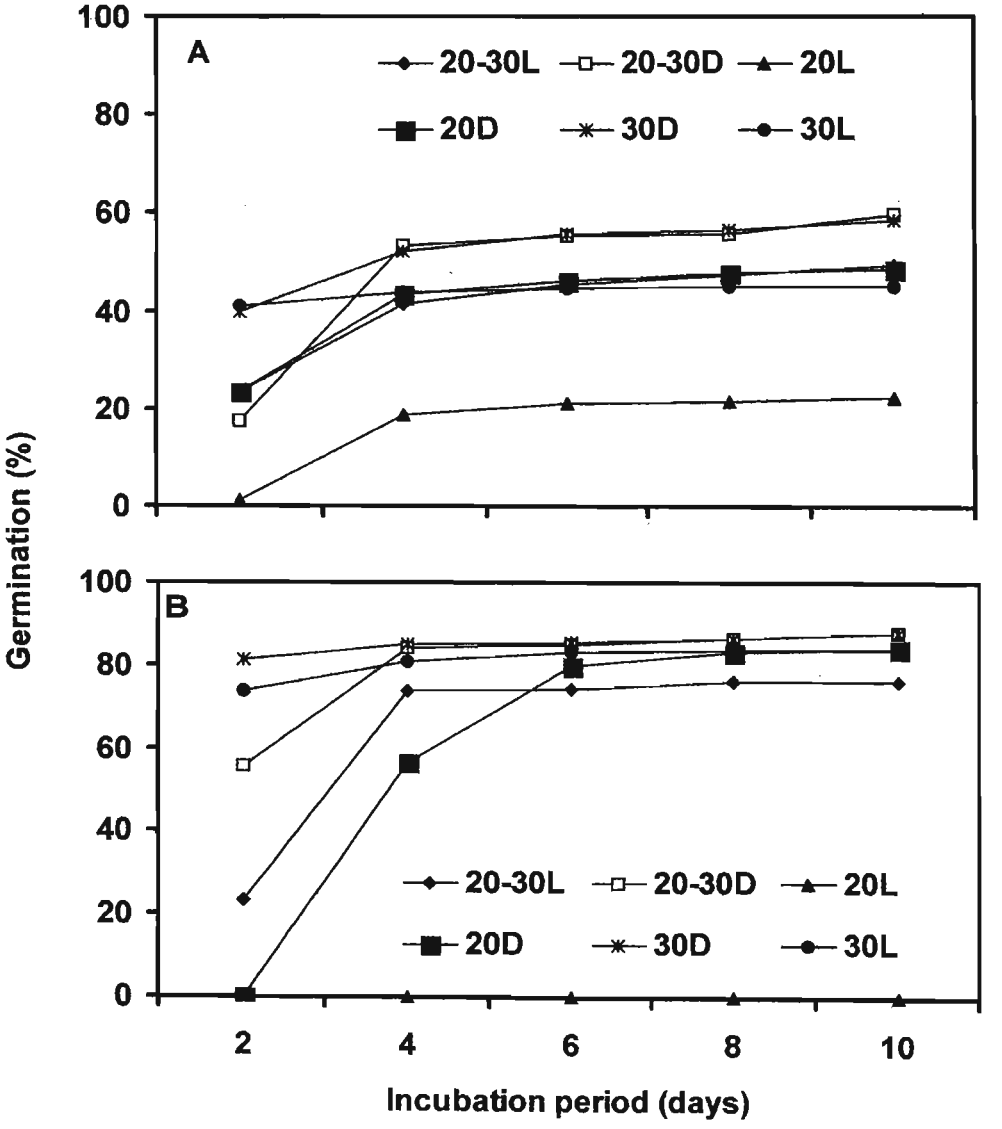

Figure 5.1. Germination of ARC (A) and KSC (B) seeds under various light and temperature combinations: 20 °C and 30 °C in continuous white light (20L, 30L) or darkness (20D, 30D); 20/30 °C alternating temperature in continuous white light (20-30 L) or darkness (20-30 D). Note: $LSD_{0.05} = 4.43$ (ARC) and 7.94 (KSC).

The ARC seed lot attained a maximum germination of 60% in response to alternating temperatures of 20/30 °C or to 30 °C in darkness after 10 days (Figure 5.1A).

For the KSC seed lot, the maximum germination of 80% was attained in response to 20/30 °C or 30 °C in darkness (Figure 5.1B). For both seed lots, the lowest germination was observed in response to 20 °C in continuous light (22.3% and 0% for the ARC and KSC seed lots, respectively). This was significantly ($P < 0.001$) lower compared to the germination at 20/30 °C in continuous light (47.2% and 76%, for the ARC and KSC seed lots, respectively), 20 °C in darkness (46.7% and 89%, for the ARC and KSC seed lots, respectively) or 30 °C in light (45% and 83.5%, for the ARC and KSC seed lots, respectively) (Appendix 5.1).

Subjecting seeds to light for ≥ 12 h day $^{-1}$ at 20 °C continuously, significantly ($P < 0.001$) reduced germination compared to those exposed to ≤ 8 h day $^{-1}$ at the same temperature (Figure 5.2A; Appendix 5.2). However, there were no significant differences between light exposure periods, with respect to seed germination, in response to 20/30 °C alternating temperatures (Figure 5.2B). When the seeds were incubated at 20 °C in continuous light while imbibed in water, KNO_3 or GA_3, the KSC seeds failed to germinate, whereas the ARC seeds treated with GA_3 showed 13% germination. After they were transferred to 20/30 °C in darkness, the KSC seeds imbibed in water attained a germination of 56%, which was not different from the germination of the seeds that were imbibed in KNO_3 (Figure 5.3). The KSC seeds imbibed in GA_3 showed an improved germination of 73% and the ARC seeds achieved a germination of 52%. It is important to note that the improvement in response to pre-germination treatment did not equal seed germination attained in response to 20/30 °C in darkness (Figure 5.1).

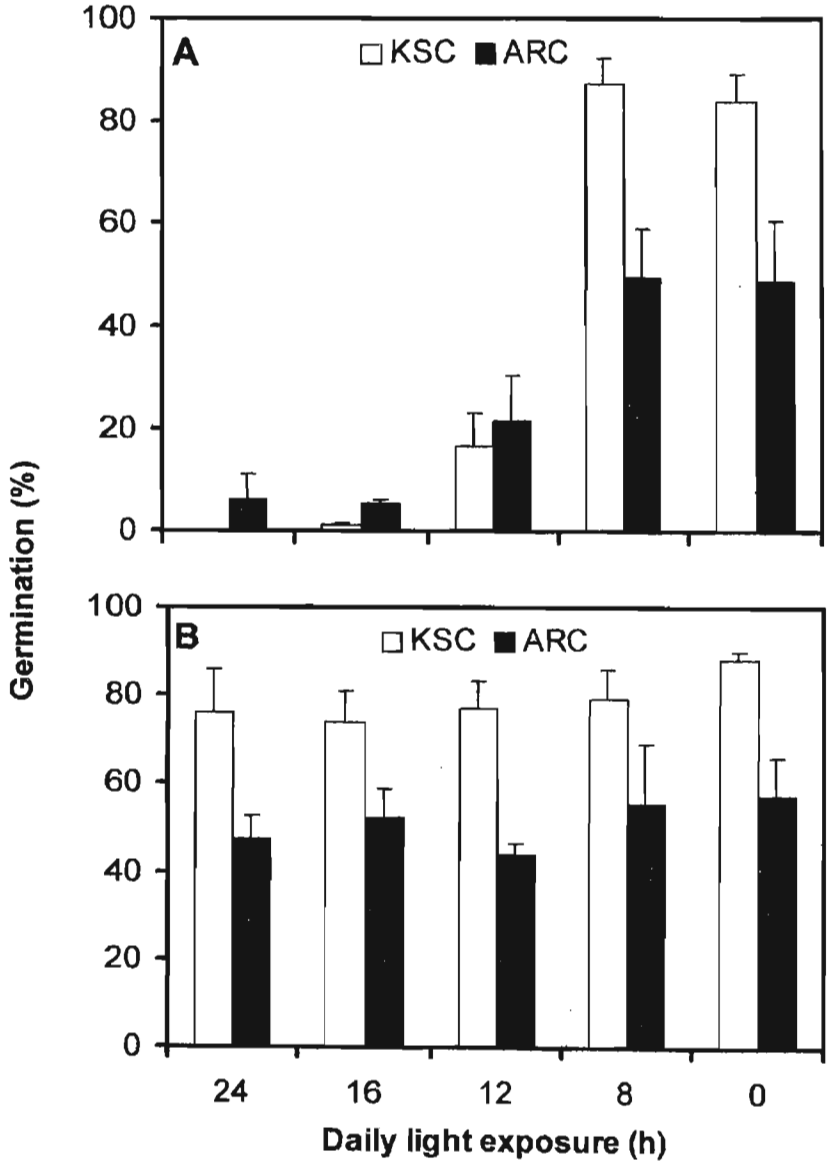

Figure 5.2. Effect of light exposure per day on germination of ARC and KSC seeds incubated at 20 °C (A) or alternating 20/30 °C (B).

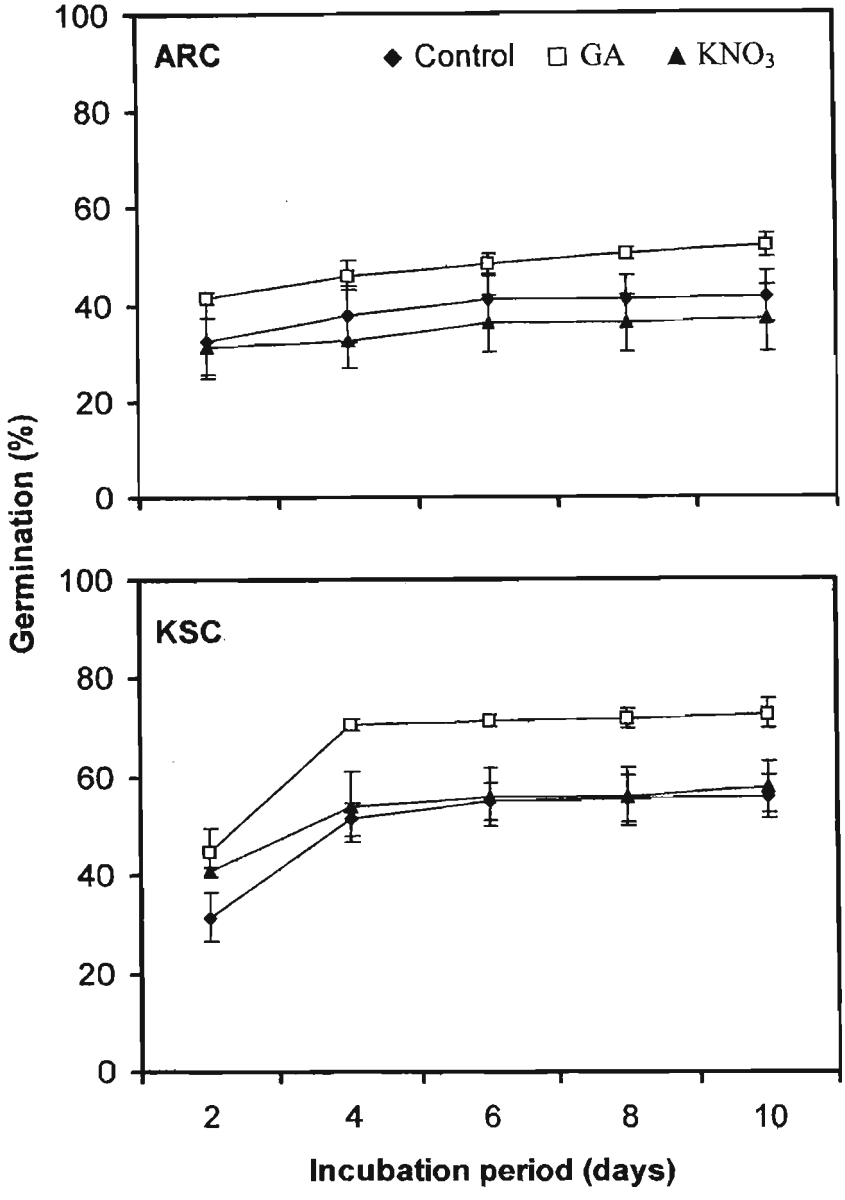

Figure 5.3. Germination of ARC and KSC seeds at alternating temperatures of 20/ 30 °C in darkness, after the seeds had been imbibed in GA_3 or KNO_3 at 20 °C in continuous white light for 14 days.

Acid scarification did not change the trend nor improve total germination of the ARC seed lot. However, the seeds that were treated with acid for 10 min had a similar germination percentage to the control seeds (Figure 5.4). Treatment of the seeds with acid

for 20 min drastically reduced germination, and the seeds given 30 min of acid scarification failed to germinate. Compared with acid scarification, pricking the seed at the radicle end significantly ($P < 0.05$) improved germination to 68% (Appendix 5.3a). Germination was significantly reduced when seeds were chilled for 7 days or hydrated for 4 h (Figure 5.5). However, the germination of seeds chilled at 5°C -10 °C for five days was as good as the control (Appendix 5.3b).

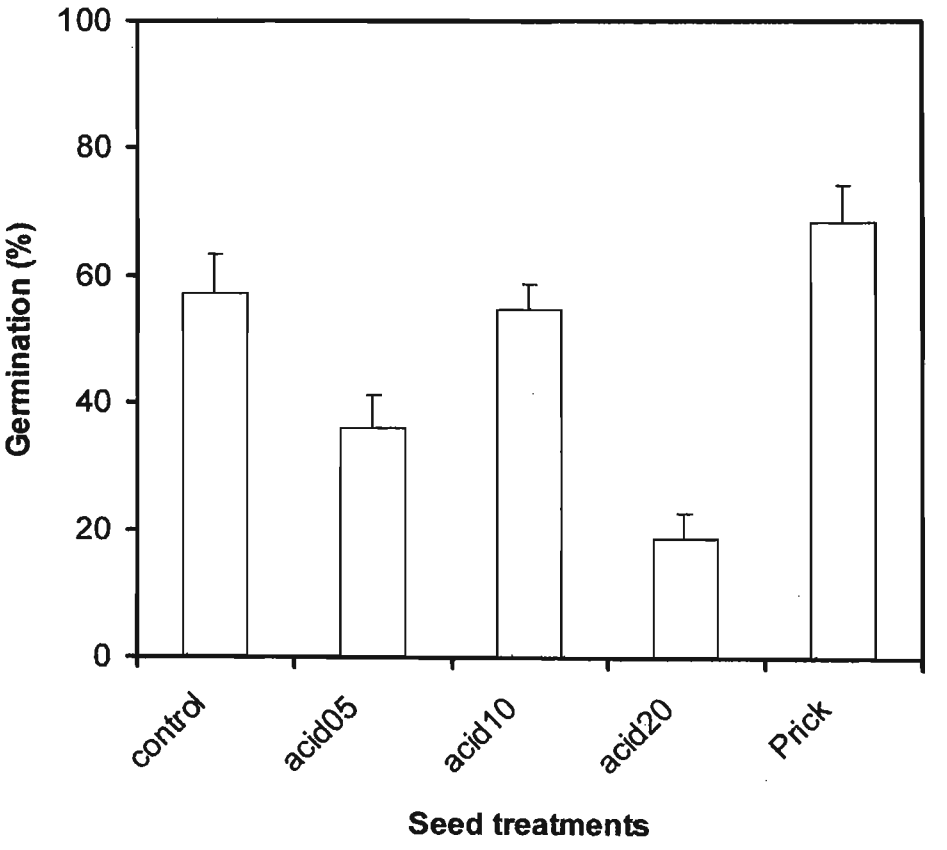

Figure 5.4. Germination of ARC seeds after treatment with Sulphuric acid for 5, 10 or 20 min (acid05, acid10, acid20, respectively) and the seeds cut with a razor blade at the radicle end (Prick). The seeds were germinated at alternating temperatures of 20/30 °C in darkness.

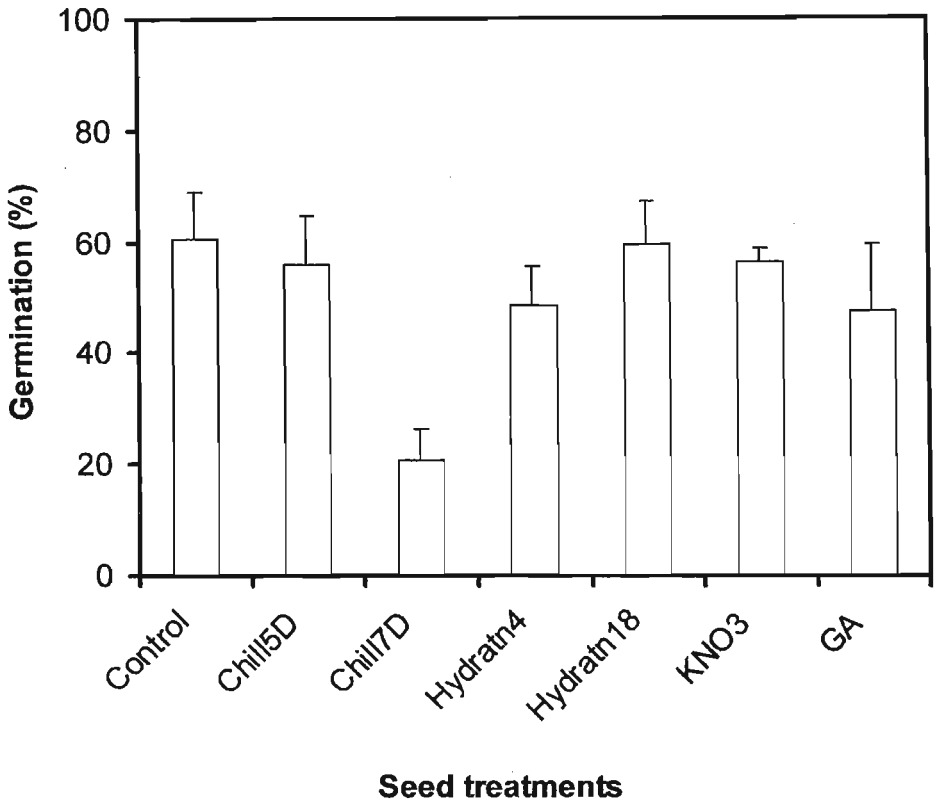

Figure 5.5. Germination of ARC seeds after pre-chilling at 5 °C -10 °C for 5 or 7 days (Chill5D, Chill7D); pre-hydration in distilled water for 4 h or 18 h (Hydratn4, Hydratn18); treated with KNO_3, or GA_3. The seeds were germinated at alternating temperatures of 20/30 °C in darkness.

5.4 Discussion

The germination of *Cleome* found in this study was higher than what was reported by Böhringer *et al.* (1999). This may be related to the fact that the present seed lots had been in storage for a longer period (1 to 2 years) compared to the seeds used by Böhringer *et al.* (1999), which were only six months old. The long period of storage may have allowed most of the immature embryos to reach maturity (Chweya and Mnzava, 1997). However, the ARC seed lot still showed a higher level of dormancy compared to the KSC seed lot, as shown by its slow rate of germination and low final germination percentage. It is not clear whether the difference between the seed lots was due to environmental conditions during seed development or storage, or was of a genetic nature. The two seed lots were obtained from very different locations and it is possible that the environmental conditions during seed development could have imposed dormancy on the ARC seed lot. The highest and the most rapid germination were obtained in darkness in both seed lots.

The tetrazolium test conducted on these seeds gave negative results (data not shown) as it failed to stain these seeds, perhaps due to the impermeability of the seed coat membrane to the tetrazolium salt solution as explained by Debeaujon *et al.* (2000) in *Brassica*. Debeaujon *et al.* (2000) noted that even within *Brassica campestris*, dark coloured seeds did not take up the stain as compared to yellow seeds, and this was also true for *B. napus* and *B. napobrassica*. This indicates that the tetrazolium test may not be useful in giving indications on *Cleome* seed viability.

In both seed lots, germination increased generally with temperature in continuous light (Figure 5.1). The lowest germination percentage was observed in response to 20 °C in continuous light, and this was 45.9% of the germination of the ARC seeds in the dark.

In response to 30 °C, germination in the presence of light was 78% of that in the dark. However, the total germination in response to 20 °C in darkness was higher compared to that in response to 30 °C in light, showing an interaction between light and temperature. The situation was similar with the seeds from KSC, except that at 20 °C in continuous light, no germination was observed. The germination of *Cleome gynandra* seeds was, therefore, shown to be negatively photoblastic (Baskin and Baskin, 1998). Similar results were obtained in *Brassica tournefortii* (Thanos *et al.*, 1991), *Citrullus vulgaris* (Thanos and Mitrakos, 1992), *Amaranthus caudatus* (Gutterman *et al.*, 1992), *Matthiola tricuspidata* (Thanos *et al.*, 1994) and *Passiflora incarnata* (Benvenuti *et al.*, 2001).

Photoinhibition occurred progressively as temperature decreased and was most evident at 20 °C. The seeds became more negatively photosensitive when exposed to light for more than 12 h in a day (Figure 5.2). This response to light exposure could be explained as a survival adaptation of *Cleome gynandra* because most of its seeds are found on the soil surface. Such seeds will not germinate until conditions are optimal, whether in darkness or light conditions as observed by Thanos *et al.* (1991) in maritime plants and Gutterman *et al.* (1992) in *A. caudatus*. When the seeds are buried in the soil, they experience darkness and there is no photoinhibition (Thanos *et al.*, 1991; Bewley and Black, 1994). Burial may ensure enough moisture for germination and seedling establishment (Schütz, Milberg and Lamont, 2002). These observations may explain why the seeds will germinate even at 20 °C in darkness. However, Luna *et al.* (2004) observed that even short exposures of green safelight increased seed germination of some west Mediterranean species and stressed that seeds tested in darkness should not be exposed to any light until the end of the experiment. This trend of argument seems to run counter to

the results reported above, but we think that the species used by Luna *et al.* (2004) could have been the type requiring light to germinate. Similar observations were made when *Solidago* species were tested in green light, white light and darkness (Walck, Baskin and Baskin, 2000). Hence, even momentary exposure to dim light solicited some positive germination response. This line of argument is supported by the statement by Benvenuti, Dinelli and Bonetti (2004) that even low quantities of Pfr are sufficient to promote germination in light requiring grass species, *Leptochloa chinensis*. Benvenuti *et al.* (2004) noted that low phytochrome balance resulting from seed exposure to far-red light caused a much higher germination percentage compared with incubation in darkness.

During photoinhibition the seeds were in a state of suspended germination. This effect of light on seed germination is modulated by temperature and light exposure period (Figures 5.1 and 5.2). These seeds germinated when they were transferred to a higher temperature, and the germination was improved when they were treated with GA (Figure 5.3). According to Bewley and Black (1994) photoinhibition of seed germination by prolonged illumination is a manifestation of the high-irradiance reaction of photomorphogenesis. It is postulated that this negative photosensitivity at 20 °C in continuous white light was due to imbalance in phytohormones, especially ABA and GAs. The biosynthesis of gibberellins, which is mediated through the phytochrome system, may have been inhibited by light exposure (Baskin and Baskin, 1998; Kato-Noguchi, 2002). The results in Figure 5.3 seem to support the involvement of GA in this phenomenon as suggested by Fernandez *et al.* (2002), Hays *et al.* (2002) and Poljakoff-Mayber *et al.* (2002), but due to the hard seed coat of *Cleome*, a longer treatment period was required to significantly improve germination (which was not evident in Figure 5.5).

However, seed germination at optimum temperature was not affected significantly by light, as observed in Figures 5.1 and 5.2. Hilhorst and Toorop (1997) and Baskin and Baskin (1998) suggested that temperature could act on membrane properties to modify the activities of phytochrome receptors.

The various seed treatment methods used in this study did not improve seed germination, except for the point prick scarification, where seeds were punctured at the radicle end with a razor blade (Figure 5.4). This treatment significantly improved the germination of ARC seeds ($P<0.001$). The seeds treated with sulphuric acid for 30min did not germinate, an observation which is contrary to the findings by Mackay *et al.* (2001) and Veasey and Texiera de Freitas (2002) who used legumes with seemingly tougher seed coats. De Villiers *et al.* (2002) showed that pricking and acid scarification improved germination of *Brassica tounefortii,* but this species seems to have a more fortified seed coat as it germinated better with longer treatments (32 min) in sulphuric acid. The International Seed Testing Association (1999) recommends pre-chilling with or without KNO_3 for *Brassica* vegetables. However in the present study, chilling for five days and hydration for 18 h resulted in germination percentages similar to those of the control (Figure 5.5). This finding contradicts the observation by Benvenuti *et al.* (2001) that stratification overcame seed dormancy in wild mustard. That observation however, emanated from field experimental results, which could have been affected by other environmental factors.

5.5 Conclusions

The findings of this study suggest that the germination test for *Cleome gynandra* should be performed at alternating temperatures of 20/30 °C or at 30 °C in darkness. The non-dormant seed lot germinated well between 20 °C and 30 °C in the dark without pre-treatment, while germination of the dormant seed lot was improved by pricking at the radicle end. Longer light exposures, more than 12 h per day, can drastically reduce seed germination or even inhibit it at sub-optimal temperatures due to photo-inhibition. The germination of photo-inhibited seeds was improved when the seeds were pre-treated with GA_3. Chilling, pre-hydration and KNO_3 did not effectively improve germination percentage.

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CHAPTER 6. STATISTICAL EVALUATION OF THE GERMINATION OF *CLEOME GYNANDRA* L. SEEDS

6.1 Introduction

Germination test is the most commonly used method for determining the quality or planting value of a seed lot. Seed germination begins with imbibition and normally ends with radicle emergence or the protrusion of the hypocotyl. International seed testing rules (ISTA, 2004) consider the speed of germination to determine the first count, and the presence or absence of essential seedling structures, coupled with lack of further radicle emergence is used to determine the final count. An intact seedling with well-developed essential structures is referred to as a normal seedling (ISTA, 1979; 2004). Seedlings that have reached the stage when all essential structures can be accurately assessed are removed from the test at 'first count' or any other intermediate counts. This is done largely to avoid entanglement of their roots or collapse of the seedlings or even spread of diseases. When there are no signs of any further germination, all the seeds and seedlings are evaluated as the 'final count' and the test is terminated.

Many parameters have been used to measure and describe the germination process. Goodchild and Walker (1971) measured the changes in the rate of germination and total germination, and concluded that these were the best methods for studying seed germination in conjunction with physiological and biochemical components. In that study (Goodchild and Walker, 1971) the germination process was adequately described by polynomial regression methods of curve fitting. Shortly thereafter, Janssen (1973) suggested that a normal distribution function was most suitable, with the premise that seed germination follows a normal distribution. Richter and Switzer (1982) quantified the heterogeneity of dormancy within seed populations by fitting mathematical functions to cumulative germination curves of *Pinus taeda* L. (loblolly pine). In the same year, Scott and Jones (1982) proposed the use

of survival analysis to evaluate seed germination of tomato species at low temperatures. This method has advantages because it takes account of censored observations, and germination responses need not be normally distributed. Two important parameters are computed by this method: probability function and hazard rate. Scott *et al.* (1984) reiterated the use of survival analysis procedures where censored observations are involved, as is frequently the case in seed germination. Scott *et al.* (1984) suggested that seed germination is a binary response, and when germination is scored once, it can be analysed better by logistic regression rather than analysis of variance. However, Bewley and Black (1994) contended that the behaviour of a seed population with respect to germination could not be adequately explained by one parameter alone.

Other statistical methods have been used to analyse data on seed dormancy and germination of many plant species including *Cleome gynandra* (Böhringer *et al*, 1999; Ochuodho and Modi, 2005). The course of germination of *C. gynandra* was appropriately summarised by a non-linear regression with the Weibull function (Böhringer *et al.*, 1999). Probability density function was used by Pimpini *et al.* (2002) to show that seed colour variations in radicchio affected germination and influenced storability, germination temperature and field performance. Steadman and Pritchard (2003) and Steadman (2004) used logistic curves to predict dormancy release and germination in *Aesculus hoppocastanum* and *Lolium rigidum* seeds, respectively. Larsen and Bibby (2004) estimated the final germination percentage, mean germination time and time from 25 to 75% germination from germination time courses in turfgrass species. The curve fitting procedure used, which was based on an asymmetric multinomial distribution adequately detected cultivar differences in germination characteristics.

The International Seed Testing Association has developed testing methods and regulations for many species, but many new crops and wild plant species are still not included

(Hampton *et al.*, 1996, 1999). For a germination test to be recommended it should allow for the expression of the full potential of a seed sample to produce normal seedlings and there should be minimum variability in the results both within and between laboratories (Hampton *et al.*, 1999). According to ISTA (2004) *Brassica oleracea* (cabbage) and *B. rapa* (Chinese cabbage) are both tested at 30/20 °C (day/night) on top of filter paper (TP), with chilling and/or KNO_3 administered. Under these conditions, the 'first count' is on the fifth day and 'final count' on the tenth and seventh day for common cabbage and Chinese cabbage, respectively. It is recommended (ISTA, 2004) that the final count for oilseed rape (*B. napus)* and *B. juncea* be on the seventh day.

When germinating new or wild species it is logical to use the method of a known species of the same genera or similar species. However, this might lead to false results because germination responses differ within genera and even between species or cultivars. Work has been reported on how to analyse germination data of unknown species but there is no statistical guideline to determine neither the first nor the final germination counts. The objective of this study was to establish the first and final counts during the germination of *C. gynandra*. In order to determine these intervals, the germination of *Cleome* was compared with those of closely related plant species of the *Brassicaceae* family, cauliflower and broccoli, for which ISTA has established seed testing regulations.

6.2 Materials and methods

6.2.1 Plant material

Two seed lots of *Cleome gynandra* were donated by the Kenya Seed Company (KSC) and the Agricultural Research Council (ARC), South Africa. Fresh seeds were produced from the KSC seed lot at the University of KwaZulu Natal farm in Pietermaritzburg. Preliminary studies showed that mature seeds of *Cleome* could be obtained from green, yellow and brown

pods. For the purposes of this study, seeds were harvested from brown pods. Hence three *Cleome* seed lots were used: KSC, ARC and brown. When this study was conducted, the KSC seed lot was about two years old, the ARC was about one year old and the brown seed lot was less than a year old. One seed lot each of cauliflower and broccoli was obtained from Starke Aryes Seed Company (Greytown, South Africa). Both the cauliflower and broccoli seed lots were less than one year old when this study was conducted.

6.2.2 General germination conditions

Four replications of 50 *Cleome* seeds were placed in petri dishes on three Whatman No.1 filter papers moistened with 7 ml of distilled water. The seeds were incubated for 10 days in growth chambers (Labcon, LTGC 20-40; Johannesburg, South Africa) at 20/30 °C (night/day) in alternating darkness and light (Ochuodho and Modi, 2005). Seeds were considered germinated when the radicle protruded about 2 mm and germination count was performed daily. Seedling evaluation was performed from the seventh to tenth day based on guidelines for the *Brassicas* (ISTA, 2004). Broccoli and cauliflower seeds were pre-treated with 0.1% KNO_3 and germinated on top of filter paper under conditions as recommended by ISTA. The germination tests were repeated seven times, herein referred to as runs.

6.2.3 Statistical analysis

Analysis of variance was performed on transformed (Angular) germination percentages but the differences were discussed using the original data. Standard deviation (SD) was used to test the extent of daily germination score variations within each seed lot. Each SD value was calculated from 28 observations, which were obtained from four replications in seven experiments (RUNS). Logistic regression analyses: exponential, Gompertz (Draper and Smith, 1998) and broken stick were performed on cumulative germination percentages.

'Broken-stick' regression is a modelling procedure of the GenStat statistical package that 'breaks' a non-linear curve into two linear components:

The Model ..... $E(Y) = \beta_0 + \beta_1 Z_1 + \beta_2 Z_2 + \beta_3 Z_3$ (Dicks, 2002)

The first line ..... $E(Y) = \beta_0 + \beta_1 Z_1$

The second line..... $E(Y) = (\beta_0 + \beta_3) + \beta_2 Z_2$

and the point of intersection t is ... $\beta_1 t = \beta_3 + \beta_2 t$

$$t = \frac{\beta_3}{\beta_1 - \beta_2}$$

Where, E (Y) = estimated percent germination

β_0 = is the intercept of the first trend line

β_1 = slope of the first trend line (linear curve)

β_2 = slope of the second trend line

β_3 = change in the intercept of the second line

Z_1, Z_2, Z_3 = Dummy variables

t = point of intersection of the two linear curves and represents the point in time and percent germination, when the first line ends and the second line begins.

The model was used to find the point of inflection on the cumulative germination curves and this provided an appropriate point to evaluate the seedlings. The point of inflection divides the germination curve into two distinct functions that explain separate aspects of the seed lot.

Life table was formulated using daily germination counts from which probability density and hazard rate were computed. Peaks in the density function indicated high germination rates, while the horizontal scale was indicative of the spread of germination within a population of seeds. The probability of an outcome is often expressed as the integral between two points on the probability density function (Johnson *et al.*, 1995). A completely randomised design with four replications was used for all the germination experiments. GenStat statistical package (2000) and Excel (Windows 2002) were used for the analyses.

6.3 Results

6.3.1 Cumulative germination curves

Seeds of broccoli and cauliflower showed faster rates and higher germination percentages compared to the seeds of *Cleome* (Figure 6.1). The broccoli and cauliflower seed lots had germinated by the first day and attained maximum percentage of germination on the second day after which no increase in germination was observed. The ARC, brown and KSC seed lots showed low germination on the second day but none on the first day. The ARC seed lot attained the lowest final germination percentage, followed by the brown, while the seed lot from KSC showed intermediate germination percentage.

Cumulative germination of cauliflower and broccoli seed lots significantly fitted the exponential curve ($P < 0.001$; Appendix 6.2) and the Lot x Day interaction was not significantly non-linear (Figure 6.1). However, the *Cleome* seed lots significantly fitted the Gompertz curve and Lot x Day interaction resulted in a significantly non-linear curve ($P < 0.001$) (Appendix 6.1). Due to this non-linear germination, the 'broken stick' model was able to break the germination curves of *Cleome* seed lots into their linear components.

6.3.2 Standard Deviation

The daily seed germination counts during the first four days of incubation fluctuated greatly in all the seed lots. The largest variation in germination as measured by the standard deviation (SD) was observed on the first day in cauliflower and broccoli seed lots (Figure 6.2) but reduced to < 5 by the second day. The seed lots of *Cleome* showed the highest variation in germination on the second day. By the third day, the SD had reduced to below 10 and stabilized at < 8 from the fourth day onwards.

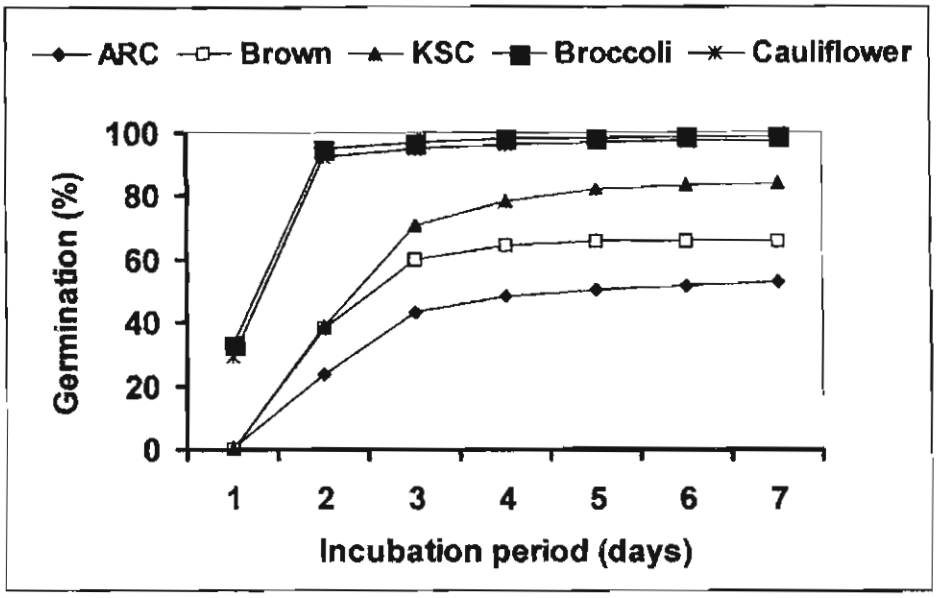

Figure 6.1. Germination of *Cleome* (ARC, Brown and KSC), broccoli and cauliflower seed lots. Seeds were germinated for 7 days at alternating temperature [30/20 °C day/night (8/16 h)]. Data points are means of four replications of seven experiments. Note: Standard deviations are presented in Figure 6.2.

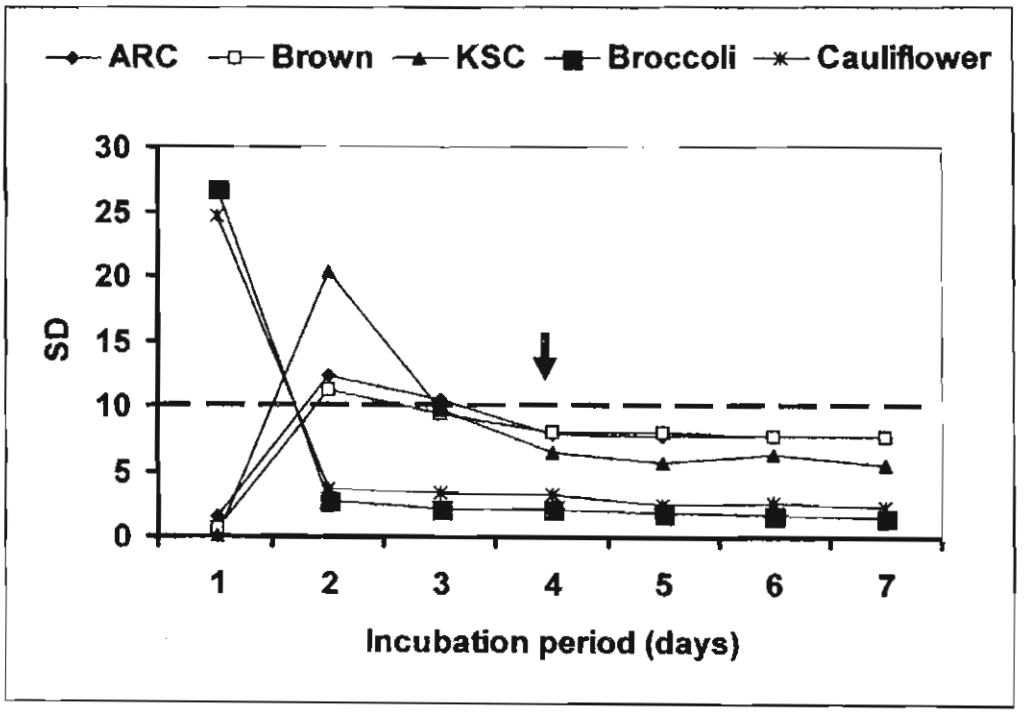

Figure 6.2. The standard deviation (SD) of daily germination counts for *Cleome* (ARC, Brown and KSC), broccoli and cauliflower seed lots. Seeds were germinated for 7 days at alternating temperatures [30/20 °C day/night (8/16 h)] in four replications for seven experiments. The arrow indicates the fourth day when all seed lots showed the lowest fluctuation in daily germination count for the first time. The horizontal dotted line denotes the maximum tolerance value of 8% allowed by ISTA (2004) for germination tests on two different submitted samples at 5% significance level.

6.3.3 Analysis of variance

The results of the analysis of variance (Table 6.1) showed that none of the seed lots experienced a significant increase in germination after the fourth day. The contrast between the third and fourth day was highly significant ($P < 0.05$) yet the contrasts between the fourth and fifth day, and the fifth and seventh day were not significant. The interaction Lot x Contrast (the fourth and fifth day) was also not significant and this meant that the above observation was true for all the seed lots used. There were no significant differences between germination percentages on the fourth and fifth days and, fifth and seventh days.

Table 6.1. Analysis of variance for germination of *Cleome* seed lots (ARC, KSC and brown), cauliflower and broccoli. The seeds lots were germinated at alternating temperatures [30/20 °C day/night (8/16 h)] using four replications in seven experiments (RUNS).

Source of variation	d.f.	s.s.	m.s.	v.r.	Fprob.
RUNS	6	3217.57	536.26	3.79	
REP	3	281.38	93.79	0.66	
RUNS X REP	18	2549.52	141.64	1.65	
LOTS	4	369195.45	92298.86	1073.23	<.001
DAYS	6	506317.60	84386.27	981.22	<.001
Contrast A(3 vs 4)	1	1096.13	1096.13	12.75	<.001
Contrast B(3 vs 5)	1	2150.63	2150.63	25.01	<.001
Contrast C(4 vs 5)	1	176.01	176.01	2.05	0.153
Contrast D(5 vs 7)	1	68.01	68.01	0.79	0.374
LOTS X DAYS	24	37304.78	1554.37	18.07	<.001
LOTS X Contrast A	4	485.23	121.31	1.41	0.228
LOTS X Contrast B	4	920.09	230.02	2.67	0.031
LOTS X Contrast C	4	79.20	19.80	0.23	0.921
Residual	918	78949.20	86.00		
Total	979	997815.51			

6.3.4 Broken-stick model

Broken-stick regression model 'broke' the cumulative germination curve into two linear-linear curves with different interceptions and gradients. There were significant regressions ($P < 0.05$) when modelling germination percentages of *Cleome* seed lots on the third and fourth days. This means that the intercepts and/or gradients of the two linear curves obtained were

significantly different (Table 6.2). The curves were not statistically different when modelled on the fifth day and hence were shown not to be biphasic. High percent variations together with low estimated standard error and significant F-probability were instrumental in the establishment of the point of inflection (Appendix 6.3). In all the *Cleome* seed lots analysed, intersection occurred within the third day, towards the fourth day and by the fifth day there was only one curve. The germination curves for broccoli and cauliflower seed lots were not split into linear components and were therefore shown not to be biphasic.

Table 6.2. Broken-stick analysis of germination percentage of the seed lots of *Cleome* (ARC, KSC and Brown), broccoli and cauliflower.

Cultivars	Model with i^{th} day	Point of Intersection (t)		% Variation accounted	Est. SE observed	F prob. for Regression
		Day	Est Y			
ARC	3	3.26	52.8	98	3.14	0.002*
	4	3.74	53.5	92	6.38	0.014*
	5	4.38	54.6	77.6	10.6	0.062
KSC	3	3.17	78.0	99.7	1.81	<0.001*
	4	3.77	81.0	91.7	9.16	0.014*
	5	4.38	82.0	78.3	14.8	0.058
Brown	3	3.08	64.8	97.4	3.96	0.003*
	4	3.69	66.2	84.6	9.61	0.035*
	5	4.4	67.6	66.5	14.2	0.110
Cauliflo	3	2.71	95.4	67.6	14.4	0.105
wer	4	3.39	96.3	40.1	19.6	0.252
	5	4.08	96.8	18.8	22.8	0.381
Broccoli	3	2.71	97.7	66.9	14.1	0.108
	4	3.39	98.2	38.9	19.1	0.259
	5	4.06	98.4	16.8	22.3	0.394

*** F prob. significant at < 0.05**

6.3.5 Life table analysis

Life table analysis allows for the calculation of probabilities that can help in deciding the fate of seeds under test. It takes into account censored observations like un-germinated seeds, dead or dormant and abnormal seedlings (Table 6.3). The probability density, for example, showed that the probability of having a seed germinating after the fourth day tended towards

zero (Figure 6.3A). This statistic has been reported to be a good estimate of the rate of germination and after the seventh day, the rate of germination was predicted to be zero for all the seed lots used. Cauliflower and broccoli seed lots showed similar patterns of germination, which were skewed to the left. The *Cleome* seed lots showed a wider spread of germination compared with the cauliflower and broccoli, but by the fourth day, no germination was observed. The highest germination was recorded on the second day after the start of seed germination in all seed lots. The hazard rate, on the other hand, is the ratio of seeds that germinate to the non-germinated seeds at that interval (Figure 6.3B). This estimates the probability that seeds, which have not germinated up to now, will germinate on that day. Although this value tended to be higher than the corresponding probability density, it was almost zero by the fifth day and was also predicted to be zero after the seventh day.

Table 6.3. Life table analysis for the germination of the Brown *Cleome* seed lot during 10 days of incubation at alternating temperatures [30/20 °C day/night (8/16 h)]. Life table analyses for the other *Cleome* seed lots (ARC and KSC) and cauliflower are shown in Appendix 6.3.

Time interval (h)	Numbers		Proportion			Probability	
	Entering	Terminal	Terminating (q)	Surviving (p=1-q)	Cumul. survival(s)	Prob. density(f)	Hazard rate (λ)
0	200	0	0	1	1	0	0
1	200	3	0.0150	0.9850	0.9850	0.0150	0.0151
2	197	80	0.4061	0.5939	0.5850	0.4000	0.5096
3	117	22	0.1880	0.8120	0.4822	0.1100	0.2075
4	95	3	0.0316	0.9684	0.7863	0.0152	0.0321
5	92	1	0.0109	0.9891	0.9578	0.0086	0.0110
6	91	3	0.0330	0.9760	0.9654	0.0316	0.0334
7	88	0	0	0	0	0	0
8	88	0	0	0	0	0	0
9	88	0	0	0	0	0	0
10	88	0	0	0	0	0	0

q = proportion of incubated seeds that have germinated (for the first day, 3/200=0.015)
h = interval length = 1 day.
For the first day,

$s_i = (p_{i-1}\ p_i) = 1*0.985 = 0.985$

$f_i = (s_{i-1}\ q_i)h_i = 1*0.015*1 = 0.015$

$\lambda_i = 2q_i/h_i(1+p_i) = 2*0.015/1(1+0.985) = 0.01511$

6.3.6 Seedling evaluation

The seeds of broccoli and cauliflower had germinated by the first day but not the seeds from *Cleome* seed lots of ARC, brown and KSC (Figure 6.4). By the second day, the roots were distinct but the cotyledons were still encapsulated by the seed coat. The cotyledons of broccoli and cauliflower were completely freed from the seed coats by the third day and the seedlings were ready for evaluation. However, the cotyledons of *Cleome* took longer to be released from the seed coat and some were released by the fourth day. By this time, the seedlings of *Cleome* were also ready for evaluation.

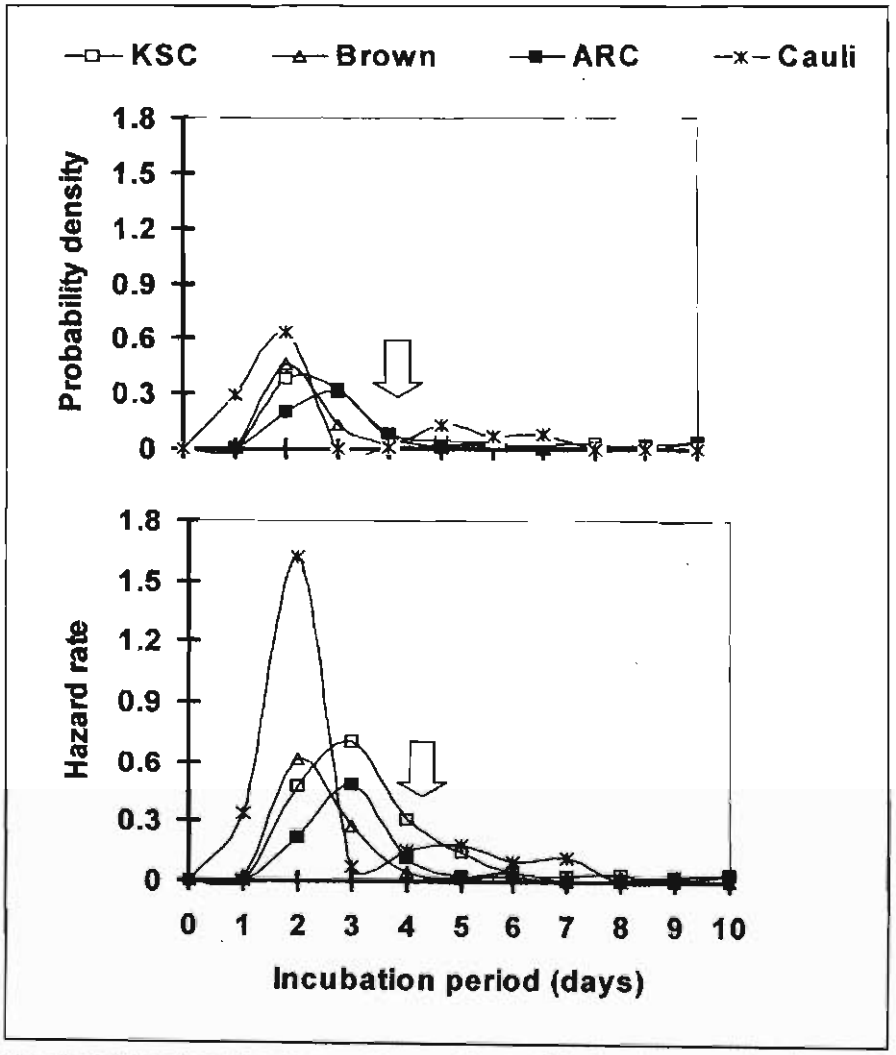

Figure 6.3. Probability density (A) and hazard rate (B) for the germination of *Cleome* seeds lots (ARC, KSC and Brown) and cauliflower. The values were obtained from life table analysis as shown in Table 6.3 and Appendix 6.3. Seeds were germinated for 10 days at alternating temperatures [30/20 °C day/night (8/16 h)] in four replications and repeated seven times. The open arrows indicate the fourth day when the probability density is lowest for the first time for all the seed lots.

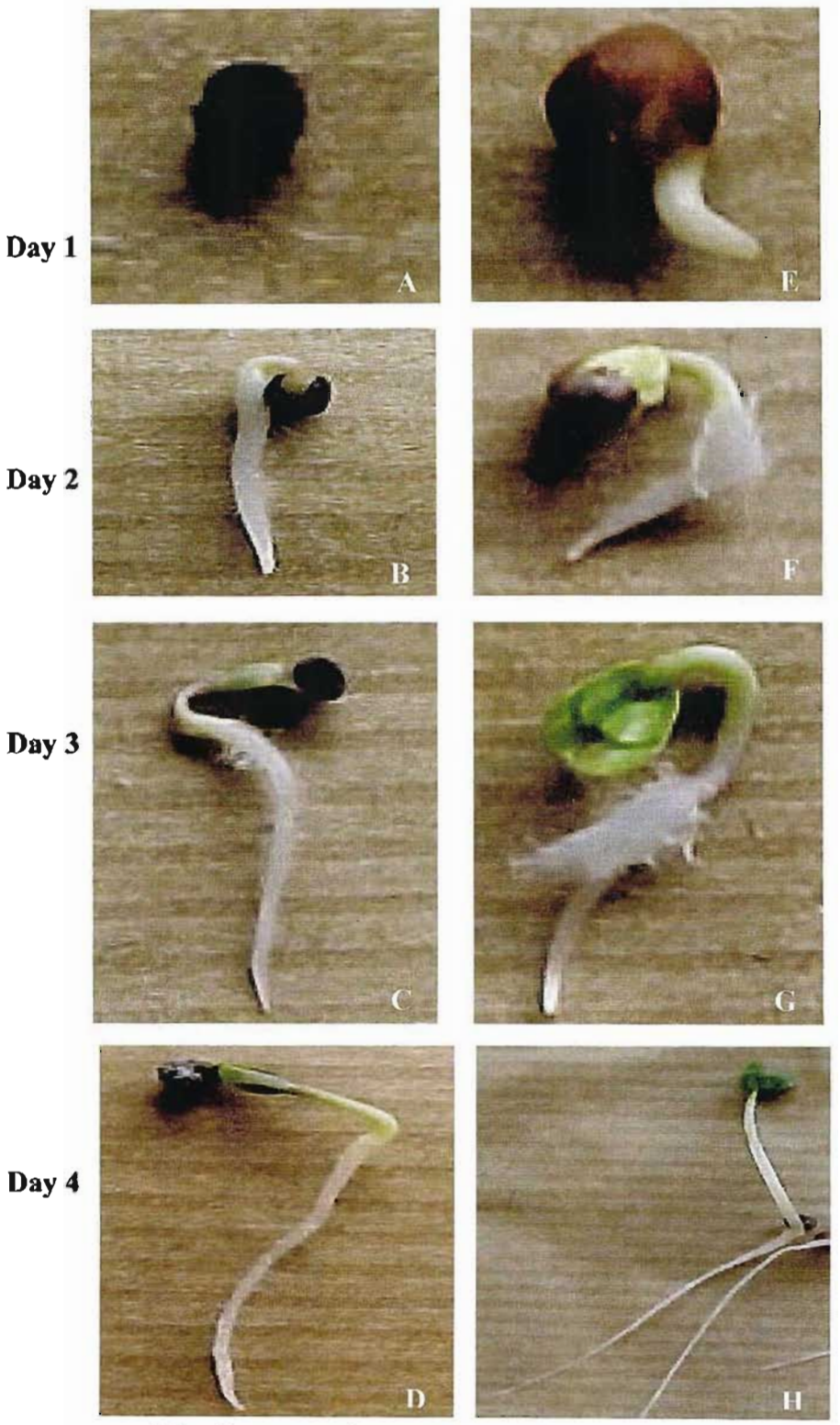

Figure 6.4. Stages of development of essential seedling structures during germination of the Brown seed lot of *Cleome* (A, B, C, D) and cauliflower (E, F, G, H). The seeds were germinated in alternating temperature [30/20 °C day/night (8/16 h)].

6.4 Discussion

Cumulative germination curves derived from germination percentage scores are shown in Figure 6.1 and indicated that most seeds germinated within four days. Cumulative germination of cauliflower and broccoli seed lots fitted the exponential curve because there was no time lag before germination started. This may be explained by the long time interval of 24 h (one day) used on the scale for incubation period. The ARC, KSC and brown seed lots started germinating on the second day and hence they were explained well by the Gompertz curve, which is a non-linear growth curve similar to the logistic regression curve (Draper and Smith, 1998). It was shown on Figure 6.2 that germination counts during the first three days of incubation were so varied that the SD >10. This was due to the small size of the radicle protruding, which made it difficult to see and to assess. The mean germination scores before the fourth day showed standard deviations greater than the maximum tolerance values of 8% recommended by ISTA (2004) for average germinations between 42-90% and could not be acceptable as test results. The relatively low and stable SD by the fourth day meant that different analysts and different laboratories could obtain similar germination counts by the fourth day. This is important for any recommendation of a test method for new species because of the possibility of obtaining uniform results from the test.

Analysis of variance has been used to compare seed treatments but physiologists are often cautious of decisions based on it due to the rule of 'normal distribution' of germination responses. This suspicion was reduced through angular transformation of the data. The analysis of variance supported the earlier observation that germination was varied during the first three days of incubation. Also, the observation that there was no significant germination after the fourth day was true in all the seed lots used. However, the analysis of variance does not give any indications as to when to stop the test.

Broken-stick regression modelling has been used to determine species abundance in ecological studies (Smart, 1976) and points of change in amino acid composition (sequence) in proteins (Itoh, Ueda and Hasegawa, 1980). Broken stick procedure was used again by Triboï, Martre and Triboï-Blondel (2003) and it efficiently partitioned the total quantity of N per grain in wheat between the different protein fractions. It was used in this study to determine the point of inflection on the germination curve. The cumulative germination of *Cleome* fitted a non-linear curve as observed by Böhringer *et al.* (1999) and the point of inflection coincided with the first germination count. When modelling with the fourth day, the point of inflection occurred late on the third day in the various seed lots of *Cleome* as follows: ARC, 3.74; KSC, 3.77; brown, 3.69. The fourth day was the end of the first curve and beginning of the second curve, and hence an appropriate time to assess the seedlings for the first time. The first count of the standard germination test was considered to be a measure of seed vigour (TeKrony and Egli (1977), and Kolasinska, Szyrmer and Dul (2000) found it better correlated with field emergence than the final count. Therefore, the first count being a good indicator of vigour can be used as a tool to separate seed lots, in addition to the purposes recommended by ISTA (2004). However, the broken stick method failed to break the germination curves of broccoli and cauliflower and this implies that the germination of the seed lots used did not follow a non-linear curve (Table 6.2 and Appendix 6.2). These observations could be due to cultivar differences between the *Brassicas* and *Cleome.* This is supported by the fact that each cultivar was represented by more than one seed lot (Larsen and Bibby, 2004).

Survival analysis was recommended for seed germination responses because it computes many parameters and germination responses need not be 'normally' distributed (Scott and Jones, 1982; Scott *et al.*, 1984). The calculation of the probability density and hazard rate was made possible through the construction of a life table analysis (Figure 6.3).

The two parameters showed that there was very low probability of seed germination after the fourth day of incubation. To pass the benefit of doubt to the seed producer, the test could be extended for a few more days to give a chance to slow germinating seed lots whose quality is at the border for sale. However, after the seventh day the probability of a seed germinating decreased to zero and it was not worth the labour and time spent to sustain the test to 10 days. The seventh day was, therefore, the most appropriate time to terminate the test. The probability density and hazard rate provided adequate indications on when to perform first counts and when to stop the test (Figure 6.3 and Appendix 6.3). These parameters gave good predictions regarding the possible germination of the seeds in the seed lots, as Pimpini *et al.* (2002) showed in radicchio.

The development of essential seedling structures determines when the first and final counts are performed in a germination test (ISTA, 1999). It is essential in *Brassica* and *Raphanus* species that the seedling is evaluated after it has shed off the seed coat and the primary root is normal (ISTA, 1979). Most seedlings of cauliflower and broccoli shed off the seed coat by the third day while *Cleome gynandra* seedlings did so on the fourth day. Primary roots had developed adequately by the third day and were ready for evaluation. On the basis of ISTA recommendations, the first count would be on or before the fourth day and the final count after the fourth day (Figure 6.4) and these observations are in agreement with the conclusions from statistical analyses.

6.5 Conclusions

Analysis of variance and standard deviations showed that germination counts were stable by the fourth day and it would be possible for different analysts and laboratories to obtain similar results. Broken-stick regression analyses showed that there were two significantly different curves with the point of inflection around the fourth day when modelling with the

third or fourth day. This implies that the first count may be done on the fourth day. Life table analysis confirmed that uniform germination scores were obtained by the fourth day. The probability density and hazard rate showed that germination occurred within four days, and that the speed and probability of seed germination after 7 days was close to zero or was zero. It is, therefore, recommended that first count for *Cleome gynandra* be performed on the fourth day and the germination test be terminated on the seventh day. The germination characteristics of broccoli and cauliflower were different from those shown by *Cleome*, even though they are closely related. The 'broken stick', probability density and hazard rate gave clear indications of when to assess the germination process and were good in determining the unknown.

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CHAPTER 7. GENERAL DISCUSSION AND CONCLUSSIONS

Cleome gynandra is classified as a member of the family *Capparaceae* in the order *Capparales* together with cabbage and wild mustard (Cronquist, 1981). Cronquist (1988) showed that the family *Capparaceae* was positioned at a lower level in the family tree compared to the *Brassicaceae*. The two families have many common phenotypic characteristics (Rendle, 1925; Porter, 1967) suggesting that *Capparaceae* is ancestral to *Brassicaceae*. The findings reported in Chapter 3 of this study support this line of thinking and indicate that *Cleome* has some similarities with *Brassica kaber* (wild mustard). *Raphanus sativus* (radish) seeds were shown to possess 12 S cruciferins similar to those of *Brassica napus* (rapeseed) (Laroche *et al.* 1984) and seed proteins of *C. gynandra* were shown to be similar to those of *Brassica kaber* (Figure 3.1-3.2). The seed proteins of *Cleome* were sequenced and by inference were shown to have homology with those of *Arabidopsis* in the proteome library (Table 3.1). It can be stated, therefore, that these observations support the classification of *Cleome* as a close relative of the *Brassicas*. The proteins are 12 S globulins of molecular weight of about 50 kDa, which is reduced by dithiothreitol (DTT) to yield sub-units of molecular weight 28-30 kDa and 18-22 kDa joined by disulphide bonds. The accumulation of these proteins diminished during seed germination (Figures 4.4–4.9).

Like *Brassica kaber*, *Cleome* does not show dormancy when harvested at physiological maturity. The seeds of *Cleome* did not exhibit primary dormancy and readily germinated at physiological maturity, when seed colour changed to black (Figure

4.1). Black seeds of *Cleome* selected from green pods showed > 90% germination after the first day of incubation at 20/30 °C in darkness.

One of the differences between *C. gynandra* and *B. kaber* is that the seeds of *Cleome,* unlike those of wild mustard, developed dormancy when harvesting was delayed to the time when the pods turned brown. The seeds from yellow and brown pods had a lag period of one day to radicle protrusion and germination percentages below 80%. This seemed to suggest that there was induction of "secondary dormancy" after physiological maturity in *Cleome,* as explained by Baskin and Baskin (1998). While dormancy breaking pre-treatments of KNO_3, GA_3, prechilling and hydration failed to improve germination percentage of seeds from yellow/brown pods of *Cleome*, it was observed that seed germination improved with storage (after-ripening).

Seed germination of *Cleome* was negatively photosensitive. Photoinhibition was more evident at suboptimal temperature of 20 °C and, was modulated by light exposures beyond 12 h day $^{-1}$ and delayed harvesting after physiological maturity (Figures 4.1, 4.2 and 5.2). In this study it was also shown that the effect of photoinhibition was minimal at high (optimum) germination temperatures (Figure 5.2). Black seeds from the brown pods showed that they were more affected by photoinhibition, while black seeds from the green pods were not. It is interesting to note that the black seeds selected from brown pods accumulated a low molecular weight polypeptide at about 13 kDa, which was absent from the physiologically mature black seeds from green pods (Figure 3.1). Photoinhibition is a result of light/temperature interaction that involves phytochromes, and phytochrome receptors are found in the seed coat. Photoinhibition induces pseudo-dormancy, a 'conditional dormancy', whereby exposure to white light at low

temperatures drives the Pfr ↔ Pr reaction towards high concentration of Pr. These reactions result in the blocking of GA_3 biosynthesis. However, the role of phytochromes on photoinhibition in *Cleome* was not investigated in this study.

When photoinhibited seeds were transferred to optimum conditions, they germinated to the level of the untreated control and further improvement was observed when the seeds were treated with GA_3 (Figure 5.3). The accumulation of GA_3 does not appear to be the direct cause of the loss of dormancy but appears to be related to improved germination (Jacobsen *et al.*, 2002; Gonai *et al.*, 2004). This action of GA_3 on dormant seeds suggests that photoinhibition, a pseudo-dormancy status, has similarities with secondary dormancy. The involvement of GA_3 in photoinhibition was indirectly shown in Figure 5.3 when photoinhibited seeds treated with the hormone regained their germination capability.

It has been established that successful priming allows all the physiological processes up to the end of Phase II of the germination process to proceed (McDonald, 2000), but not radicle protrusion, which is the result of radicle cell growth and elongation. It can be postulated that the physiological processes of Phase II of *Cleome* germination in this study did occur during photoinhibition, but the radicle failed to emerge. That there was no radicle protrusion could mean that either the effect of exogenous GA_3 was blocked, or the embryonic tissue became insensitive to it, or both. Long exposure to white light that resulted in photoinhibition of *Cleome* seeds at 20 °C may have interfered with the ABA/ GA_3 balance to the extent of stopping seeds from proceeding to Phase III of the germination process. This may have been indicated by the observation that the mobilization of storage proteins was not evident during photoinhibition (Figure 4.9).

Bewley and Black (1994) stated that storage proteins are mobilized during the final phase of germination (Phase III), when proteins are utilized for embryo enlargement and growth.

The seeds of *Cleome* that were in a pseudo-dormancy state germinated once the inhibitory effect of the stimulus was removed (Figure 5.3). The photoinhibited seeds did not respond to the dormancy-breaking treatment by KNO_3, but the germination of seeds treated with GA_3 was improved. White light inhibits the biosynthesis and/or tissue sensitivity to GA_3, which promotes cell expansion and hence radicle protrusion (Hays *et al.*, 2002; Kato-Noguchi, 2002; Yamaguchi and Kamiya, 2002). Further addition of GA_3 to the germination medium may have mitigated the inhibitory effects of ABA during photoinhibition, thereby increasing seed germination.

Seeds of *Cleome gynandra* germinated best in darkness at alternating temperatures of 20/30 °C or constant 30 °C (Figure 5.1). The observation that *Cleome* seeds germinate best in darkness is further confirmation that the seeds do not have primary dormancy as noted by Salisbury and Ross (1992). Seeds of *Cleome* showed improved germination under elevated temperatures in both continuous light and darkness (Chapter 5).

Various statistical methods have been used to describe the germination process and analyse seed quality (Böhringer *et al.*, 1999; McDonald *et al.*, 2001; Dell'Aquila, 2004). However, none of these methods have been used to indicate the appropriate times during germination when intermediate and final counts can be determined. Analysis of variance showed that there was no significant difference in daily germination percentages after the fourth day when the deviation measurements (SD) had stabilized. Broken-stick modeling showed that the point of inflection consistently occurred on the fourth day and probability

density predicted that there was zero germination on the fourth day. The seedlings of *Cleome* shed the seed coat on the fourth day. These observations provided a good indication that the best time to determine the first count was the fourth day of germination in *Cleome* (Figure 6.1 and Table 6.2). The statistics cited above corresponded well with seedling evaluation requirements and maximum tolerance of variation around mean percent germination as recommended by ISTA (2004). The probabilities calculated through the life table analysis confirmed that the first count should be done on the fourth day and provided an indication that the final count should be determined on the seventh day (Figure 6.2). These observations are in line with provisions given by international seed testing guidelines (ISTA, 2004) for similar plant species, especially the *Brassicas*. Broken-stick regression analysis, probability density and hazard rate are recommended for use when analyzing seed germination for unknown species and deciding on the first and final counts.

From the findings of this study it is recommended that seed germination of *Cleome* should be determined on the fourth and seventh days of incubation. And that seeds of *Cleome* germinate best in darkness at alternating temperatures of 20/30 °C and the seeds attain physiological maturity before the pods turn yellow. This information will be useful to seed analysts who seek to produce protocols for *Cleome* seed germination tests, or to understand *Cleome* seed preservation in genebanks.

APPENDICES

Appendix 1. Protocol for preparation of material for examination by Scanning Electron Microscopy (Hitachi S-570). This SEM was used to scan transverse and longitudinal cross-sections of *Cleome* seed coat (Practical Handbook for postgraduate students, Centre for Electron Microscopy, University of Natal, Pietermaritzburg, 2002).

CRITICAL POINT DRYING

Fixation in 3% buffered Glutaraldehyde		overnight (min. 8 hours)
Wash in 0.05 M Cacodylate buffer (plant material)		2 x 30 min.
Post fixation in 2% Osmium tetroxide (optional step)		1-4 hours
Wash in 0.05 M buffer		30 min.
Dehydrate	30% Alcohol	10 min.
"	50% "	10 min.
"	70% "	10 min.
"	80% "	10 min.
"	90% "	10 min.
"	100% "(2 changes)	2 x 10 min.

Transfer specimens to the CPD basket under Alcohol. The CPD apparatus (Hitachi HPC-2) must be pre-cooled before the baskets are placed in the chamber. If the specimens are 'Beached' or allowed to evaporate to dryness at any stage during the procedure they will be useless.

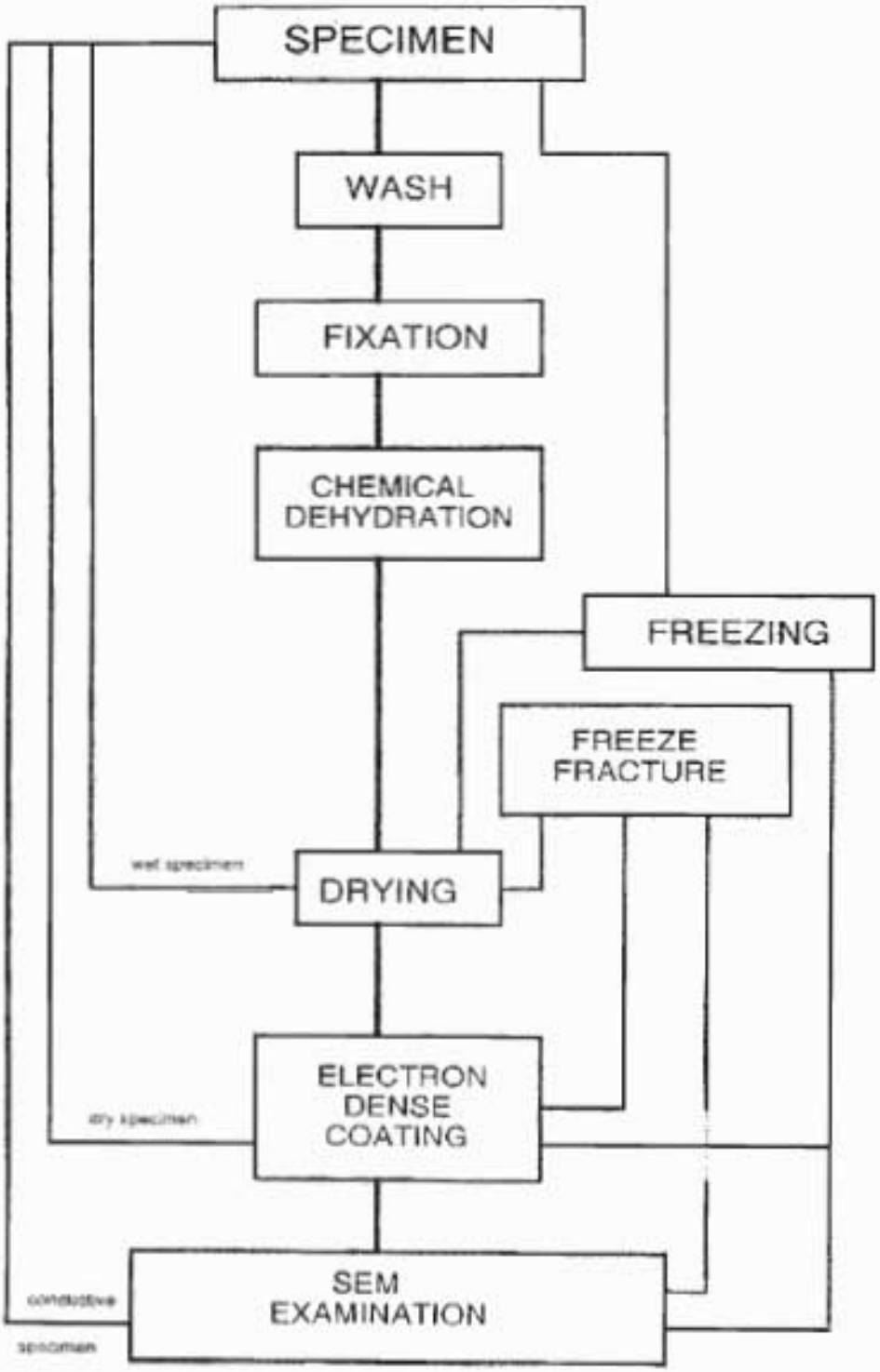
All procedures with the possible exception of the alcohol dehydration are to be carried out in a fume cupboard. Waste must be discarded into appropriate waste bottles in the fume cupboard. Note that waste containing Osmium must be placed in a special bottle.

Experience in the E.M. Unit laboratory in Pietermaritzburg has shown that, for a wide range of specimens, the Amyl Acetate steps may be left out without disadvantaging the dehydration process. Such changes to the procedure described above should however, be pursued with caution.

SPUTTER COATING

Materials that do not naturally conduct electricity and heat will not yield satisfactory images in the SEM unless they are made conductive in some way. This is done by coating specimens with a very thin layer of electrically conductive metal. The best coating material should form a continuous film on the specimen surface and should not readily react with specimen surface or oxidize over time. Gold and 60:40 Gold:Palladium are particularly used in this respect. Sputter coating, using a Polaron E5100, is the procedure used at the E.M. laboratory in Pietermaritzburg.

Below is the diagrammatic representation of the procedure followed before the sample is scanned through the SEM.



APPENDIX 2. Solutions used during protein extraction and SDS-PAGE analyses, and elecro-blotting in readiness for protein sequencing.

Thiourea/urea lysis buffer (2 M thiourea, 7 M Urea, 18 mM Tris, 0.2 % v/v Triton X-100)

7.61 g Thiourea

21.02 g Urea

0.11 g Tris

100 µl Triton X-100

Add ddH_2O to make up 50 ml

Extraction buffer (0.2 % v/v CHAPS, 1% v/v Ampholytes pH 3-10, 1% w/v DTT)

50 ml Lysis buffer

100 µl CHAPS

500 µl Ampholytes

Proteinase inhibitor complete mini

53 U/ml Dnase 1

4.9 U/ml Rnase A

0.5 g DTT

4X Running (Resolving) Gel Buffer (1.5 M Tris-Cl, pH 8.8)

36.3 g Tris (FW 121.1)

Add 150 ml ddH_2O

Adjust to pH 8.8 with HCl

Add ddH_2O to make 200 ml (Store up to 3 months at 4°C in the dark)

4X Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)

3.0 g Tris (FW 121.1)

Add 40 ml ddH_2O

Adjust to pH 6.8 with HCl

Add ddH_2O to make 50 ml (Store up to 3 months at 4°C in the dark)

10% SDS

10 g SDS

Add ddH_2O to make 100 ml (Store up to 6 months at room temperature)

10% Ammonuim persulphate (APS)

0.1 g Ammonium persulphate

Add ddH_2O to make 1.0 ml (Store in fridge)

Glycerol 50%

50 ml Glycerol

Add ddH_2O to make 100 ml (Store at room temperature for up to 6 months)

Tank buffer (0.025M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3)

30.28 g Tris (FW 121.1)

144.13 g Glycine

10 g SDS

Add ddH_2O to make 10L (Stored at room temperature for up to 1 month)

SDS-PAGE- resolving and stacking gels; 10 and 15%

	Resolving gel		Stacking gel
	10%	15%	
Acrylamide	3.15 ml	4.72 ml	650 µl
Water (ddH_2O)	5.40 ml	3.82 ml	3.0 ml
Tris pH 6.8	0	0	1.25 ml
Glycerol 50%	200 µl	200 µl	0
Tris pH 8.8	1.25 ml	1.25 ml	0
SDS 20%	50µl	50µl	25 µl
APS 10%	36 µl	36 µl	25 µl
Temed	15µl	15µl	5 µl

2X Loading/Sample buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v Glycerol, 0.2 M DTT, 0.02% Bromophenol Blue, pH 6.8)

2.5 ml 4X stacking gel buffer

4.0 ml 10% SDS

2.0 ml glycerol

2.0 mg bromophenol blue

0.31 g dithiothreitol (DTT; FW 154.2)

Add ddH_2O to make 10.0 ml (Store 0.5 ml aliquots at -20°C for up to 6 months)

Standard staining solution (0.025% Coomassie brilliant blue R 250, 40% methanol, 7% acetic acid)

0.5 g Coomassie Brilliant blue R 250

800 ml methanol

Stir until dissolved

Add 140 ml acetic acid

Add ddH_2O to make 2 L (Store at room temperature for up to 6 months)

Destaining solution I (40% methanol, 7% acetic acid)

400 ml methanol

70 ml acetic acid

Add ddH_2O to make 1 L (Store at room temperature indefinitely)

Destaining solution II (7% acetic acid, 5% methanol)

700 ml acetic acid

500 ml methanol

Add ddH_2O to make 10 L (Store at room temperature indefinitely)

Rehydration buffer (8 M Urea, 2% CHAPS, 0.3% DTT, 2.0% IPG buffer)

2.4 g Urea

0.1 g CHAPS

100 µl IPG buffer (pH 3-10)

15 mg DTT

Trace Bromophenol blue

Add ddH_2O to make 5 ml (store in 2.5 ml aliquots at -20 °C)

Equilibration solution (6 M Urea, 30% glycerol, 2% SDS, pH 8.8)

180 g urea

150 g glycerol

10 g SDS in 0.05 M Tris-HCl buffer, pH 8.8

16.7 ml Resolving gel buffer

Add ddH_2O to make 500 ml

Equilibration buffer I

Dissolve 100 mg DTT in 10 ml Equilibration solution

Use 10 ml Equilibration buffer I with 50 µl Bromophenol blue solution.

Equilibration buffer II

Dissolve 400 mg Iodoacetamide in 10 ml Equilibration solution

Use 10 ml Equilibration buffer II with 50 µl Bromophenol blue solution.

Transfer buffer – CAPS buffer (10 mM CAPS, 10% v/v methanol, pH 9.0)

4.426 g CAPS (FW 221.3)

200 ml methanol

Add ddH_2O to make 2 L (Pre-chill buffer before use)

APPENDIX 3.1 Unedited Sequences found in the proteome library that showed high homology with the four protein bands from *Cleome*.

Q9SRM9/T19F11.8 PROTEIN (488 AA; 54.400)

MDLNAQEKKA GFFQRLQDFP SKLKDDVTKR VKNVQKFAKD DPRRIIHSMK VGVALTLVSL
LYYVRPLYIS FGVTGMWAIL TVVVVFEFTV GGTLSKGLNR GFATLIAGAL GVGAVHLARF
FGHQGEPIVL GILVFSLGAA ATFSRFFPRI KQRYDYGALI FILTFSFVAI SGYRTDEILI
MAYQRLSTIL IGGTICILVS IFICPVWAGE DLHKMIANNI NKLAKYLEGF EGEYFQPEKI
SKETSSCVRE YKSILTSKST EDSLANLARW EPGHGRFRLR HPWKKYLKIA GLVRQCAVHL
EILNGYVLSN DKAPQEFESK IQEPITTMSR EVGEALKAIA KSIKTMRNDS ACVNAHIDNS
KKAIKNLKIA LKSSYPETYK DLLEIIPGVT MASILIEVVN CVEKIYEAVE EFSGLAHFKE
TLDSKLSAEI GQHQLLHRGC VKPVLDGDNE KEDNSSCHVL ITVHDEGYLP TATAKNVLGA
EKTRVDIV

Q8H7H9 HYPOTHETICAL PROTEIN (464 AA; 49.401)

MSIMRNFLSM IIMLCVCLNW CFAEGAEKSD SGKVLDSYTI QVSSLFPSSS SCVPSSKASN
TKSSLRVVHM HGACSHLSSD ARVDHDEIIR RDQARVESIY SKLSKNSANE VSEAKSTELP
AKSGITLGSG NYIVTIGIGT PKHDLSLVFD TGSDLTWTQC EPCLGSCYSQ KEPKFNPSSS
STYQNVSCSS PMCEDAESCS ASNCVYSIGY GDKSFTQGFL AKEKFTLTNS DVLEDVYFGC
GENNQGLFDG VAGLLGLGPG KLSLPAQTTT TYNNIFSYCL PSFTSNSTGH LTFGSAGISE
SVKFTPISSF PSAFNYGIDI IGISVGDKEL AITPNSFSTE GAIIDSGTVF TRLPTKVYAE
LRSVFKEKMS SYKSTSGYGL FDTCYDFTGL DTVTYPTIAF SFAGGTVVEL DGSGISLPIK
ISQVCLAFAG NDDLPAIFGN VQQTTLDVVY DVAGGRVGFA PNGC

Q9SMU4/T2J13.90 HYPOTHETICAL PROTEIN (416 AA; 47.272)

MGIKVSSKIK RILASTGYIP IFIFKYRYYF YFFYCKFARK FVTKKIIILK LSSIGTASGS
SNPKDGADVD VREEYANAFR TESYNHFWTR VVQLSRKKSA VSSSSSSPPI ESSSTSARLM
SYRLFAHNLL DPDLNTITRI LDVSRVGRHT RTLLSDYFLE TANAFLLCTQ LLKNIHHLRS
KYESLKPKFH SENHNSLALI DQFTEISKWF DPFISSGSRI QLIRSGCLYL LKRLESRRDK
TRAKLKLING LTHSSGLLVL ALTTTLIVTI ASHAFALFLA APTLLASQFK PAGLRNKLTK
TAARLDVAAK GTYILSRDLD TISRLVTRIN DEVNHVRAMA EFWVGRGSGR VRGSEEVARE
LKRCEESFSE ELDELEEHIY LCFMTINRAR NLLVKEILDS DDPPNCSFAP KSKKKL

O23088/BAC TM018A10 (AT4G00890 PROTEIN) A TM018A10.5 (431 AA; 47.066)

MFNNRQSRRR FRLGNLARLV QLRQSVSSQP PRRTVQETLS QSPPQSPLFH PQSPPEPKSL
PLSPLSPKSK EEPESQTMTP LMSKHVKTHR NLLIQSPPKS PPESTESQQQ FLASVPLRPL
TTEPKTPLSP SSTLKATEES QSQPSPPLES IVETWFRPSP PTSTETGDET QLPIPPPQEA
KTPPSSPSMM LNATEEFESQ PKPPLLPSKS IDETRLRSPL MSQASSPPPL PSKSIDENET
RSQSPPISPP KSDKQARSQT HSSPSPPPLL SPKASENHQS KSPMPPPSPT AQISLSSLKS
PIPSPATITA PPPPFSSPLS QTTPSPKPSL PQIEPNQIKS PSPKLTNTES HASPEQNLVK
PDANLMNKIP AKNEVPKNRG TLEKKTEPMI MMFINSNVQG FNTSLSLDSS SIDHKPGVHL
TIDYDQSCDF S

Q9ZQL4/AT2G07280 HYPOTHETICAL PROTEIN(401 AA; 46.021)

MAEITEDPNI VPPVATLTDP SDDIVILDWD NVWIPRQSRL LADVHAVEQD RDDDEVRDKK
VSYMLEKIQS GYGFAKYEWP GGVMSLDLID IVKTRSNKKK KMKPLLNSPK GSVKRRRSTH
LNPDLPPSLS QDLAARVDSL ENKIGVLKAQ LKKVSDRNTL IQNRFRDSRF LHATRRAKPS
MFRFKKLVRN SASETTSRFM HARWRAKPTF LRELFGKDTG EQDWTHFEHP LGGLDRNSEP
QLSSRLDDIQ MEETIESFNT ALTEHQSENT DKESEEVGLD VPTVISQPLK DKPQEFPSVD
INIGLLNDGT LNREIEFPES DLVMDPFIFP VPYHVSQYQP NGEQVFKTPI HNQVHHTIPN
TYTLFLHCFC ISIINTPQCK HPFQISLMEH GMKCCPSVQL L

Q8GWE9/AT3G60930/T27I15 20 HYPOTHETICAL PROTEIN (413 AA; 44.967)

MQSTKLTLRE VYAKKKEDKE RRLAEERRLV NTGLISPRAD PEATQDGNVI PDATAPQTAP
EASRDGVNPY TAGPVDAAPA EAQGAEPSAA APEAVLALPA IDKAAGKRIR VDDVSSKKKK
KKKKASGSEV EKILPIFEDR TASANLLGGC VGPLLPPPDT LLESRKYAET ASHFLRAVAS
MNRMVHSYDS AMRSNMEVAG KLAEAESRIQ AIEREKNEAL SEAAAAKLEK EEVERTAHVN
KENAIKMAEQ NLKANSEIVR LKRMLSEARG LRDSEVARAV QTTRREVSET FIAKMKNAEH
KVSLLDEVND RFMYLSQARA NAQLIEALEG GGVLESEKEQ VDEWLKDFAD AEVNLNRFMS
ELKDDLKAPA PEPAPLSPGG HRSVESLADE AGITDQAGSL LPAKDNRPSE DLD

Q8GYQ8/AT1G64080/F22C12 2 HYPOTHETICAL PROTEIN (411 AA; 44.541)

MEAFSLLNYW KNSGGAVTFL PHSSESSSSS LCRYSAGESI GTTTIVTSVT ETEDEEADEN
DDEGPFFDLE FALPTEEEDT DGGVGREGDS DCTDGGCEFK FTLSSCSGGE DRVDPDSLDD
VFFKETIVEE VEPPTSSEQQ TCSVKAPAAQ LSASILKTAT KLRVFMLGMK KSKLIQAKSE
ANFGSEDLDK QTHPPPPLSL SPESQQKSTV TVNLKVEEVP IISLFSRENS SKNSSSSSSS
SSSSSSSLKK QNGNESVVSD EKRFVMMQKY LKKVKPLYIR VSRRYGEKLR HSGQLTAPSS
ARSTAEKADS PVATKTNNKP GNNINIPAGL KVVRKHLGKS RSSSSTATTP SSTTATVTTQ
SESRRRDDSL LQQQDSIQSA ILHCKRSFNS SRDKDPSVLP RSVSEPSSYE K

AT2G42710: 286 AA; 31.601

MAAMKLLLSQ ARRQGLIKPF SSPFQQIPRL FSSSSSSSSS SSSSSDSNPS PDSNESRKKA
VTIEPVSYAA KPKDQKSEPK NVESTENLQS PESANWTREE IRYVKDSPSI NPVSYAQRVA
PLPEDRVAGE NEGDRTPEEM ERERKRIELE NRARRRFLRA NAVEEDTSSL PLPTLLKPEL
KHGKKPIFDL MEAIREIKGN AKAKFDETLE AHVRLGIEKG RSELIVRGTL ALPHSVKKDV
KVAFFAEGAD AEDAKAAGAD VVGGLELIEE ILSNEPMHST SIHSDI

AT2G22890: 279 AA; 31.505

2[MATSLQTKYT LNPITNNIPR SHRPSFLRVT STTNSQPNHE MKLVVEQRLV NPPLSNDPTL
QSTWTHRLWV AAGCTTVFVS FSKSIIGAFG SHLWLEPSLA GFAGYILADL GSGVYHWATD
NYGDESTPLV GIHIEDSQDH HKCPWTITKR QFANNLHFMA RGTTLIVLPL DLAFDDHVVH
GFVSMFAFCV LFCQLFHAWA HGTKSKLPPL VVGLQDIGLL VSRIHHMNHH] RAPYNNNYCV
VSGVWNKVLD ESNVFKAMEM VLYIQLGVRP RSWTEPNYE

Q8L9R0/AT4G31150 HYPOTHETICAL PROTEIN (277 AA; 30.881)

MDSEGASSES SSSGDQLEKW TEEQDELKKK LIAYDDFTWK LSSSTELSQG SEILKYVGGV
DMSFCKEDSS VACACLVVLE LPSLRVVHHD FSLLRLHVPY VPGFLAFREA PVLLQILQKM
RDEKHPFYPQ VLMVDGNGIL HPRGFGLACH LGVLAHLPTI GVGKNLHHVD GLNQSEVKQS
LQLQINEHEQ TITLVGNSGI TWGVGFRPTL SSLKPIYVSV GHRISLDSAV EVVKITCKYR
VPEPIRQADI RSRAYLQKHQ TESFKELAVR PPDQTEP

Q9SKV3/F5J5.17 (259 AA; 30.797)

MANEKKMLPK RIILMRHGEI SVYYIIYIYI YIYLCEVNKL DSIYIYIYII LLYIFCMSTN
TIRININFYI ESNLFIYLPF SMEIYSPPYI YEHNIPRVLE EGSWYFMENI HVIKVSVGSP
PFTDFKFKIK FLPNTKMSLV DAYDVVVRVE NITRPDYVPQ DFENPRLSQF LEFELRNTWG
KSLTCVASGY TCDLFVHLWR DLGLGLCYKW TFGMNPTTCV LQFWKISDHE GRQCLISHNG
ASRIVFNQNY PEINMTHIS

Q9C870/T8E3.4 HYPOTHETICAL PROTEIN (268 AA; 30.717)

MEDQYEKKIT WTIKNFSFVQ SQAIDSDIFV VGDSKWHLVA YPKGNGESTN KCLSLYLNVA
DFQSLPNGWK RHIKYRLTVV NQMSEKLSEQ EVIQGWFYKN FHISGFQTML PLSKLLDKNG
GFLVNGDVKI VVEVGVLEVV GKSDVLEETL LVHESIDING FQVLPSQVES VNNLFEKHPD
IVSEFRSKNP LMRTTYLNDL LCLTEILCES SEELSTGDMA NAYSTLTCLK KAGFKLDWLE
KKLKEVCEAR VQEIDEEWKD LTDLKEKF

Q9SHT3/AT2G05410 PROTEIN (265 AA; 30.214)

MAYPKGINKA HDSFSLFLNV PDNESLPTGW RRHAKVSFSL VNQGSEKLSQ RKVTQHWFVQ
KAFTWGFPVM ITHTELNAKM GFLVNGELKV VAKIEVLEVV GKLDVSKESS PIMKTIDVNG
FQVLPSQVDS VKRLFEKNLD IVSKFRLKNP YLKTACMNLL LSLTETLCQS PQELSNDGLS
DAGVALAYLI ETGLKLDWLE KKLDELKEKK KKEESCLVRL REMDEQLQPF KKRCLDIEDQ
ISKEKEELLA AREPLSLYDD IDNNV

Q9SVE5 (265 AA; 28.642) : Expansin like 2 protein

MLQGFLFLLS VVLLFSSSAA ACDRCLHSSK AAYFSSASAL SSGACAYGSM ATGFFAGHIA
AALPSIYKDG SGCGACFQVR CKNPTLCSSK GTTVIVTDLN KTNQTDLVLS SRAFRAMAKP
VVGADRDLLK QGIVDIEYRR VPCDYGNKKM NVRVEESSKN PNYLAIKLLY QGGQTEVVAI
YIAQVGSSHW SYMTRSHGAV WVTDKVPNGA LQFRFVVTAG YDGKMVWSQR VLPANWEAGK
SYDAGVQITD IAQEGCDPCD DHIWN Belongs to the expansin family)

AT4G05220 (226 AA; 26.035)

MTPDRTTIPI RTSPVPRAQP MKRHHSASYY AHRVRESLST RISKFICAMF LLVLFFVGVI
AFILWLSLRP HRPRFHIQDF VVQGLDQPTG VENARIAFNV TILNPNQHMG VYFDSMEGSI
YYKDQRVGLI PLLNPFFQQP TNTTIVTGTL TGASLTVNSN RWTEFSNDRA QGTVGFRLDI
VSTIRFKLHR WISKHHRMHA NCNIVVGRDG LILPKFNHKR CPVYFT

AT2G45740 (236 AA; 25.943)

MGTTLDVSRA ELALVVMYLN KAEARDKLCR AIQYGSKFLS GGQPGTAQNV DKSTSLARKV
FRLFKFVNDL HGLISPVPKG TPLPLVLLGK SKNALLSTFL FLDQIVWLGR SGIYKNKERA
ELLGRISLFC W 3[MGSSVCTTL VEVGEMGRLS SSMKKIEKGL KNGNKYQDED YRAKLKKSNE
RSLALIKSAM DIVVAAGLLQ LAPTKITPRV TGAFGFITSI ISCYQLLPTR PKIKTP]

AT2G45740/F4118.28 (105 AA; 11.433)

3[MGSSVCTTLV EVGEMGRLSS SMKKIEKGLK NGNKYQDEDY RAKLKKSNER SLALIKSAMD
IVVAAGLLQL APTKITPRVT GAFGFITSII SCYQLLPTRP KIKTP]

AT2G22890 (230 AA; 25.741)

2[MATSLQTKYT LNPITNNIPR SHRPSFLRVT STTNSQPNHE MKLVVEQRLV NPPLSNDPTL
QSTWTHRLWV AAGCTTVFVS FSKSIIGAFG SHLWLEPSLA GFAGYILADL GSGVYHWATD
NYGDESTPLV GIHIEDSQDH HKCPWTITKR QFANNLHFMA RGTTLIVLPL DLAFDDHVVH
GFVSMFAFCV LFCQLFHAWA HGTKSKLPPL VVGLQDIGLL VSRIHHMNHH]

T16H5.210 (194 AA; 22.480) Lectin-like protein

MRVKRRKTVS CCTREISQLH GQSLKQINIG VGSLILTKHQ GYEFYCKKVT FVFFCFFKIS
LNSAYLYTLY SDVRTEVAKM ERVAWLEVVG KFETEKLTPN SLYEVVFVVK LIDSAKGWDF
RVNFKLVLPT GETKERRENV NLLERNKWVE IPAGEFMISP EHLSGKIEFS MLEVKSDQWK
SGLIVKGVAI RPKN

F14D16.29 (197 AA; 22.094)

5[MSSKMVKSSE IVGKLNLRAH QEGGFFAETF RDSSVFLSTS QLPPTCSSLP LKVDRAVSTS
IYFLLPSGSV SRLHRIPMAE TWHFYLGEPL TV]6[VELYDDGK LKFTCLGPDL FEGDQKPQYT
VPPNVWFGSF PTKDVHFSQD GALLKAEARD SENHFSLVGC TCAPAFQFED FELAKRSDLL
SRFPQHESLI TMLSYPE]

Q8L7J1/AT2G05400/F16J10.5 HYPOTHETICAL PROTEIN (194 AA; 21.782)

MISLTELHAE EGLLRNGELT VVAKVEVLEV VGKLDVSEES SPVIETIDVN GFQVLPSQVE
YAKSLFERHL DIASKFRPKN PYLKTAYMNV LLSLTQTICQ SPQELSNDDL SDAGAALAYL
REAGFELDWL EKKLNEVKEK KKKEEACLAE IQDMDEHVKP LKKKYLDLEA QIDKKKAELL
AARAPLSLND DNVV

Q8GYZ3/AT1G19130/F14D16_18 (192 AA; 21.640)

5[MSSKMVKSSE IVGKLNLRAH QEGGFFAETF RDSSVFLSTS QLPPTFKVDR AVSTSIYFLL
PSGSVSRLHR IPMAETWHFY LGEPLTV]6[VEL YDDGKLKFTC LGPDLFEGDQ KPQYTVPPNV
WFGSFPTKDV HFSQDGALLK AEARDSENHF SLVGCTCAPA FQFEDFELAK RSDLLSRFPQ
HESLITMLSY PE]

Q8LDX4 (187 AA; 21.254)

5[MSSKMVKSSE IVGKLNLRAH QEGGFFAETF RDSSVFLSTS QLPPTFKVDR AVSTSIYFLL
PSGSVSRLHR IPMAETWHFY LGEPLTV]IRS LSTRSLQTSG LVRFLQRMFI FLKTGLCLKL
RLETLRTISL LLAAPALLLS SSRTLSSRNA LISYQGFRNT SHSSQCYLTR SERAYLEETP
YWLNGFW

QLJ85/F16J14.17/AT3G22620, GB:AAC80588.1 HYPOTHETICAL PROTEIN (203 AA; 20.779)

MSKIISLVVA MIAVLALPIR GQQQPLSQCT PSMMTTVSPC MGFITNSSSN GTSPSSDCCN
SLRSLTTGGM GCLCLIVTGT VPFNIPINRT TAVSLPRACN MPRVPLQCQA NIAPAAAPGP
AATFGPSMSP GPETDPIVPE PTPAAQTPQS DTTRPFTPSV DGGAPTSDDG GSTSRPSETP
SSAYALSPSL LFFSIALVAL KFY

Q9C871/T8E3.3 HYPOTHETICAL PROTEIN (175 AA; 19.861)

MVPLTKLLDK NGGFMVNGEV KIVAEVGVLE VVGKSDVLEE TSLVMESMYV NGFQVLPSQV
EFVKSLFEKH PDIESKLHLK NPHMRITYLN SLLTLTEILS QSPENISNDD LANAYSTLSY
VTKAGFKLDW LEKDLKDVGE TRIQEIEEEL KDIKQKCVEM KHKCVDMEAL LEFLL

Q9LZ13/F9G14 30 HYPOTHETICAL PROTEIN (138 AA; 15.399)

MTGIGGEKCK RCGIYEQGSL VSDKEFDVWE VCPTDFSASQ VYMHFKEKEI NATFVCHGCA
KFHSAVAASS PQEEGYNGLT FMIAIIAGVL CTTLVVVGGV FMFKHTQRMK KQRDQARFMQ
LFEESDEPED ELGLDPVI

Q98E65/MLR4389 PROTEIN (137 AA; 14.567)

MTGETDLKTL LASMTPELLD GIHVFATLAP GVSQPKNLDP VMLFREREGT TLIVTEDAAE
AAGLSASFRC RMITLNVHSS LEAVGFLAAI TTRLAAAGMG VNPVSAFYHD HLFVPAERAE
EAMVLLRGLA AENGQVE

Q9LUN1/EMB07858.1 PROTEIN (247 AA; 28.045)

MNRKASVSKE LNAKHSKILE ALLKHPDNRE CADCRSKAPR WASVNLGIFI CMQCSGIHRS LGVHISQVRS ITLDTWLPDQ VAFMKSTGNA KGNEYWESEL PQHFERSSSD TFIRAKYSEK RWVSPGAIQP APIVSQLSCK VSHLVESGYK PETPKKARTL SLDEEILLHH VLQVTPPETR TRAGSVDMKE NVYVVPLPEF KKPNQKNENF SSEEEKRLYE DEEDGGFGKT RIIMWALILV KRLAKMG

Q9M2I4/F9D24.230 HYPOTHETICAL PROTEIN (224; 25.712)

MKCNNVIVSE ADTDDTAKED LFEDGTVEEY PDDDDASSLH QWKSMVYTSK TVENFGTECN NKESFYESWW ISDFLETIDV NGFQVLASQV QSVSQIFKRH PDTAIGFRPK NQQIRKAYMD ALLSLIETLC QSPDKLSDDD LSNADETLVD LIDVGFKLDW LKTKLNDVSE KKKLGESSVV RLETMEEQLQ KLKHMVLDLE SQMQKEKEKV LAARAPLSFK DIFY

Q9M2I6/F9D24.210 HYPOTHETICAL PROTEIN (181; 20.661)

MARNTEGSES SDDEQRYSSS SEEWSEESSE DAHEIVEVNG FQILDSQVSQ VNAIFEKHPD IASDFNLKNQ QLKNAYMGVL LDLIMTLCRP PKELTMDDLN KADRTVLDLT KAGLKLQWLR QKLDEAFLKK EKKRDIGARI RELEEQVKKR KLNLSDLESD LKKQKAAALA AETPFDFSDV V

Q9M2I3/F9D24.240 HYPOTHETICAL PROTEIN (173; 19.724)

MLKENSCEAN DSDDGDFSPS QDSSEDEETV EVNGFHVLPS QENLVSQMLK KHPDLTSNFD LKNQQLKNAY MDVLVDISET LSQSTKALSM EDLDKAESTL FDLTKAGLKV GWLRQKLDEA YLKKEKQRIS GVRIRELEEQ VNKRKLTLSD LESDLKKEKA NQLSMAQLKK RMV

Q9M2J3/F9D24.140 HYPOTHETICAL PROTEIN (153; 17.831)

MWNGDGTVEV NGFRVFYSEV DCVRRIFEKH PETATNIRPK NQMVKNAYMN NLLDLIDIIC LAPQELTEEE IRNAENTLLE LVEVGFKLDW LKNRLEELCV KKKKMEARGA RMRELDGMIV EQRRVLWALE TELKNEENEA VSDSARLGFD DVV

Q8S898/AT2G05430 HYPOTHETICAL PROTEIN (188; 22.001)

MSKISESKST LSHHGTAYDE DLDNDEIVNI KGFWIFRSKL GLAKRVFEKH PETTTKFCLK SEFAKETYLT ALLDLIDMIN MLPQQSLFEA ELKEAENTIL DLEAAGFKLD WLKRKLEEIR VTKKKAKNRT TRMRELDRKI QKHLEELSVL QEEMKKEQFE AMCDKPEYYL DSLCLNEVTC DSSEPITK

Q84RE3/AT2G05430/F16J10.2 HYPOTHETICAL PROTEIN (188; 21.974)

MSKISESKST LSHHGTAYDE DLDNDEIVNI KGFWIFRSKL GLAKRVLEKH PETTTKFCLK SEFAKETYLT ALLDLIDMIN MLPQQSFFEA ELKEAENTIL DLEAAGFKLD WLKRKLEEIR VTKKKAKNRT TRMRELDRKI QKHLEELSVL QEEMKKEQFE AMCDKPEYYL DSLCLSEVTC DSSEPITK

Appendix 3.2 Analysis of variance for variation of bands (Figure 3.2) between the species *Cleome* and *B. kaber*

(a) Analysis of original data

```
 47  "General Analysis of Variance."
 48  BLOCK rep
 49  TREATMENTS Species*Band
 50  COVARIATE "No Covariate"
 51  ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] presence
```

Analysis of variance, Variate: presence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep	2	0.0370	0.0185	0.12	
Species	1	0.4630	0.4630	2.97	0.094
Band	8	4.1481	0.5185	3.33	0.006
Species x Band	8	1.7037	0.2130	1.37	0.246
Residual	34	5.2963	0.1558		
Total	53	11.6481			

Table	Species	Band	Species Band
rep.	27	6	3
d.f.	34	34	34
s.e.d.	0.1074	0.2279	0.3223
l.s.d.	0.2183	0.4631	0.6549

Stratum	d.f.	s.e.	cv%
rep	2	0.0321	4.7
rep.*Units*	34	0.3947	57.6

Comment:
The species only differed at 10% probability level, while the bands are significantly different.

(b) Analysis for the transformed data: $(x + 0.5)^{1/2}$

```
 103  "General Analysis of Variance."
 104  BLOCK rep
 105  TREATMENTS Species*Band
 106  COVARIATE "No Covariate"
 107  ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] trans

Analysis of variance, Variate: transformed presence
Source of variation     d.f.       s.s.       m.s.    v.r.  F pr.

rep                        2    0.03970    0.01985    0.56

Species                    1    0.17863    0.17863    5.02  0.032
Band                       8    1.05195    0.13149    3.69  0.003
Species x Band             8    0.53590    0.06699    1.88  0.096
Residual                  34    1.21073    0.03561

Total                     53    3.01691


Table              Species        Band     Species
                                             Band
rep.                    27           6         3
d.f.                    34          34        34
s.e.d.              0.0514       0.1089    0.1541

l.s.d.              0.1044       0.2214    0.3131

Stratum                  d.f.          s.e.         cv%

rep                         2        0.0332         3.1
rep.*Units*                34        0.1887        17.6
```

Comments: The ANOVA is consistent with that before transformation. However, the CV% was reduced significantly after data transformation, meaning that this transformation was good for the data.

(c) Analysis with SDS-PAGE showed that some protein bands appeared (red), while the average intensity of other bands increased when the proteins were reduced with DTT (green). Proteins were extracted with or without and average band intensity was determined with Quantity One computer package (2004). The average intensity of protein bands of various molecular weights are shown for (i) *C. gynandra* and (ii) *B. kaber*

(i)

MWM		Non reduced		Reduced	
Mol wt	Intensity	Mol wt	Intensity	Mol wt	Intensity
75	119.14				
				62.56	144.32
50	120.54	48.44	170.77		
37	102.17	41.34	155.28		
				29.797	168.949
25	120.15			27.49	184.94
20	102.06	25.61	155.31		
15	104.81	17.77	163.99	18.85	186.52
13				13.73	231.76

(ii)

	MWM	Non reduced		Reduced	
Mol wt	Intensity	Mol wt	Intensity	Mol wt	Intensity
75	119.97			72.64	166.55
		64.59	162.49	65.98	182.81
50	119.06	49.6	206.85	51.63	161.58
37	100.37				
		30.14	171.92	30.14	221.53
		27.37	182.92	26.15	232.573
25	139.21	24.88	213.38		
20	94.81				
		17	204.83	18.04	245.69
15	97.98				
13		13.13	248.76		

Appendix 4 Analysis of variance for the germination of *Cleome* seeds and protein band intensity

(a) Germination of ARC seed lot for 96 h

```
  54  "General Analysis of Variance."
  55  BLOCK reps
  56  TREATMENTS Temp*POL(days;2)
  57  COVARIATE "No Covariate"
  58  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]%germ

Analysis of variance, Variate: %germ

Source of variation     d.f.       s.s.       m.s.     v.r.  F pr.

reps                       3     165.67      55.22     1.13
Temp                       3   12341.67    4113.89    83.89  <.001
days                       2    3385.50    1692.75    34.52  <.001
  Lin                      1    2926.12    2926.12    59.67  <.001
  Quad                     1     459.37     459.37     9.37  0.004
Temp.days                  6    1397.83     232.97     4.75  0.001
  Temp.Lin                 3    1162.38     387.46     7.90  <.001
  Temp.Quad                3     235.46      78.49     1.60  0.208
Residual                  33    1618.33      49.04

Total                     47   18909.00


Table                Temp         days         Temp
                                                days
s.e.d.               2.86         2.48         4.95

l.s.d.               5.82         5.04        10.07

cv% = 24.4
```

(b) Germination of KSC seed lot for 96 h

```
Analysis of variance, Variate: %germ

Source of variation     d.f.       s.s.       m.s.     v.r.  F pr.

rep                        3     426.92     142.31     2.98
temp                       3   33372.92   11124.31   232.63  <.001
day                        2   10510.17    5255.08   109.89  <.001
  Lin                      1    9522.00    9522.00   199.12  <.001
  Quad                     1     988.17     988.17    20.66  <.001
temp.day                   6    7761.83    1293.64    27.05  <.001
  temp.Lin                 3    7063.00    2354.33    49.23  <.001
  temp.Quad                3     698.83     232.94     4.87  0.007
Residual                  33    1578.08      47.82

Total                     47   53649.92
```

Table	temp	day	temp day
s.e.d.	2.823	2.445	4.890
l.s.d.	5.744	4.974	9.948

cv% = 17.2

(c) Germination of black seed from green, yellow and brown pods for 96 h

```
 77  "General Analysis of Variance."
  78  BLOCK REP
  79  TREATMENTS exposure*lots*POL(days ; 2)
  80  COVARIATE "No Covariate"
  81  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]%germ
```

Analysis of variance, Variate: %germ

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP	3	251.46	83.82	1.78	
exposure	1	3528.38	3528.38	75.11	<.001
lots	2	58827.00	29413.50	626.10	<.001
days	3	38893.46	12964.49	275.96	<.001
Lin	1	31137.41	31137.41	662.80	<.001
Quad	1	7385.04	7385.04	157.20	<.001
Deviations	1	371.01	371.01	7.90	0.006
exposure.lots	2	237.00	118.50	2.52	0.088
exposure.days	3	565.46	188.49	4.01	0.011
exposure.Lin	1	484.01	484.01	10.30	0.002
exposure.Quad	1	9.37	9.37	0.20	0.656
Deviations	1	72.08	72.08	1.53	0.220
lots.days	6	9124.67	1520.78	32.37	<.001
lots.Lin	2	4516.47	2258.23	48.07	<.001
lots.Quad	2	3265.33	1632.67	34.75	<.001
Deviations	2	1342.87	671.43	14.29	<.001
exposure.lots.days	6	2228.67	371.44	7.91	<.001
exposure.lots.Lin	2	1404.47	702.23	14.95	<.001
exposure.lots.Quad	2	469.00	234.50	4.99	0.009
Deviations	2	355.20	177.60	3.78	0.028
Residual	69	3241.54	46.98		
Total	95	116897.62			

Table	exposure	lots	days	exposure lots
s.e.d.	1.399	1.714	1.979	2.423

Table	exposure days	lots days	exposure lots
s.e.d.	2.798	3.427	4.847

```
Table              exposure        lots        days    exposure
                                                            lots
l.s.d.                2.791       3.418       3.947       4.834

Table              exposure        lots    exposure
                       days        days        lots
                                                days
l.s.d.                5.582       6.837       9.669

cv% = 16.5
```

(d) Band intensity of KSC seeds germinated in light and darkness (Figure 4.4)

```
  56  "General Analysis of Variance."
  57  BLOCK reps
  58  TREATMENTS COMP(treat;3;Cont)
  59  COVARIATE "No Covariate"
  60  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5] band intensity

Analysis of variance, Variate: band intensity (picxels)

Source of variation     d.f.       s.s.       m.s.    v.r.  F pr.

reps                       3    220.380     73.460   22.76

treat                      6   4666.074    777.679  240.99  <.001
   Contrast (dry vs D24)   1      0.456      0.456    0.14  0.711
   Contrast (L24 vs D24)   1     81.664     81.664   25.31  <.001
   Contrast (D24 vs L48)   1     19.034     19.034    5.90  0.026
Residual                  18     58.087      3.227

Total                     27   4944.541

Table               treat
s.e.d.              1.270

l.s.d.              2.669

cv% = 1.8
```

Note: dry vs D1 = contrast between band 1 in dry seed vs band 1 in seeds germinated in darkness.

L1 vs D1 = contrast of band 1 in seeds germinated in light with band 1 in seeds germinated in darkness.

D1 vs L2 = contrast of band 1 in darkness with band 2 in light.

(e) Band intensity of ARC and KSC seed lots germinated in darkness (Figure 4.6)

```
 117  "General Analysis of Variance."
 118  BLOCK rep
 119  TREATMENTS COMP(Treat;4;Cont_1)
 120  COVARIATE "No Covariate"
 121  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; CONTRASTS=5;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] band intensity


Analysis of variance, Variate: band intensity (picxels)

Source of variation      d.f.       s.s.       m.s.    v.r.  F pr.

rep                         3     286.67      95.56    2.01

Treat                       5   21526.08    4305.22   90.72  <.001
   Contrast (dry A vs K)    1     359.92     359.92    7.58  0.015
   Contrast (dry A vs A24)  1     142.47     142.47    3.00  0.104
   Contrast (A24 vs A48)    1    4209.03    4209.03   88.69  <.001
   Contrast (A48 vs K48)    1     330.50     330.50    6.96  0.019
Residual                   15     711.88      47.46

Total                      23   22524.63


s.e.d.                 4.87

l.s.d.                10.38

cv% = 11.9
```

dry A vs K = Bands from ungerminated seeds of KSC showed significantly higher intensity compared to those from ARC seed lot.

A24 vs A48 = contrast of bands from seeds of ARC germinated for 24 h with those germinated for 48 h.

Appendix 5.1 Analysis of variance of the germination percentages obtained when ARC and KSC seed lots were germinated under different light and temperature combinations.

(a) ARC seed lot

```
 256  "General Analysis of Variance."
 257  BLOCK REPS
 258  TREATMENTS LIGHT*POL(TEMP ; 2)
 259  COVARIATE "No Covariate"
 260  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]GERM%

Analysis of variance, Variate: GERM%
```

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
REPS	5	434.82	86.96	1.34	
LIGHT	1	2613.73	2613.73	40.39	<.001
TEMP	2	2332.90	1166.45	18.02	<.001
Lin	1	1257.24	1257.24	19.43	<.001
Quad	1	1075.66	1075.66	16.62	<.001
LIGHT.TEMP	2	406.45	203.23	3.14	0.064
LIGHT.Lin	1	223.58	223.58	3.45	0.077
LIGHT.Quad	1	182.88	182.88	2.83	0.108
Residual	21(4)	1358.97	64.71		
Total	31(4)	6890.97			

Table	LIGHT	TEMP	LIGHT TEMP
s.e.d.	2.68	3.28	4.64

Table	LIGHT	TEMP	LIGHT TEMP
l.s.d.	5.58	6.83	9.66

cv% = 17.3

(b) KSC seed lot

Analysis of variance, Variate: germ%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
light	1	5340.17	5340.17	483.03	<.001
temp	2	9710.33	4855.17	439.16	<.001
Lin	1	7656.25	7656.25	692.53	<.001
Quad	1	2054.08	2054.08	185.80	<.001
light.temp	2	8814.33	4407.17	398.64	<.001
light.Lin	1	6320.25	6320.25	571.68	<.001
light.Quad	1	2494.08	2494.08	225.60	<.001
Residual	18	199.00	11.06		
Total	23	24063.83			

Table	light	temp	light temp
s.e.d.	1.357	1.662	2.351
l.s.d.	2.852	3.493	4.940

cv% = 4.7

Appendix 5.2 Analysis of variance for the germination percentages obtained when ARC and KSC seed lots were germinated at varying light exposure levels at 20 °C and alternating 20/30 °C.

(a) Germination of KSC seed lot at 20 °C

```
  42  "General Analysis of Variance."
  43  BLOCK reps
  44  TREATMENTS COMP(Exposure;5;Cont)
  45  COVARIATE "No Covariate"
  46  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; CONTRASTS=5;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] germination%

Analysis of variance, Variate: germination%
```

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Reps	3	11.35	3.78	0.18	
Exposure	4	31587.80	7896.95	365.32	<.001
Contrast A (0 vs 8)	1	24.50	24.50	1.13	0.308
Contrast B (8 vs 12)	1	10082.00	10082.00	466.40	<.001
Contrast C (0 vs 12)	1	9112.50	9112.50	421.55	<.001
Contrast D (12 vs 16)	1	528.13	528.13	24.43	<.001
Contrast E (16 vs 24)	1	0.12	0.12	0.01	0.941
Residual	12	259.40	21.62		
Total	19	31858.55			

```
s.e.d.              3.29
l.s.d.              7.16
cv% = 12.3
```

(b) Germination of ARC seed lot at 20 °C

```
Analysis of variance, Variate: Germination%
```

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication	3	33.34	11.11	0.19	
Expose	4	7870.25	1967.56	34.20	<.001
Contrast (0 vs 8 h)	1	4.50	4.50	0.08	0.784
Contrast (8 vs 12 h)	1	1458.00	1458.00	25.34	<.001
Contrast (12 vs 16 h)	1	810.03	810.03	14.08	0.003
Contrast (16 vs 24 h)	1	42.78	42.78	0.74	0.405
Residual	12	690.35	57.53		
Total	19	8593.94			

```
s.e.d.              5.36
l.s.d.             11.69
cv% = 30.5
```

(c) Germination of KSC seed lot at alternating 20/30 °C

```
 109  "General Analysis of Variance."
 110  BLOCK replication
 111  TREATMENTS COMP(exposure;5;Cont)
 112  COVARIATE "No Covariate"
 113  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; CONTRASTS=5;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] germination%

Analysis of variance, Variate: germination%

Source of variation     d.f.       s.s.       m.s.    v.r.  F pr.

replication                3     127.75      42.58    0.73

exposure                   4     458.20     114.55    1.96  0.165
   Contrast (0 vs 8)       1     171.12     171.12    2.93  0.113
   Contrast (8 vs 12)      1       8.00       8.00    0.14  0.718
   Contrast (0 vs 12)      1     253.12     253.12    4.33  0.059
   Contrast (12 vs 16)     1       8.00       8.00    0.14  0.718
   Contrast (16 vs 24)     1       2.00       2.00    0.03  0.856
Residual                  12     701.00      58.42

Total                     19    1286.95

s.e.d.                 5.40
l.s.d.                11.78
cv% = 9.7
```

(d) Germination of ARC seed lot at 20/30 °C

```
Analysis of variance, Variate: germination%

Source of variation     d.f.       s.s.       m.s.    v.r.  F pr.

Replication                3      20.80       6.93    0.09
expose                     4     385.20      96.30    1.29  0.327
   Contrast (0 vs 8)       1       0.50       0.50    0.01  0.936
   Contrast (8 vs 12)      1     264.50     264.50    3.55  0.084
   Contrast (0 vs 12)      1     288.00     288.00    3.87  0.073
   Contrast (12 vs 16)     1      60.50      60.50    0.81  0.385
   Contrast (16 vs 24)     1       4.50       4.50    0.06  0.810
Residual                  12     893.20      74.43

Total                     19    1299.20

s.e.d.                 6.10

l.s.d.                13.29

cv% = 16.9
```

Appendix 5.3 Analysis of variance for the germination percentages obtained after seed pretreatments.

(a) Effect of Scarification with sulphuric acid and pricking on germination of ARC seed lot

```
 572  "General Analysis of Variance."
 573  BLOCK Rep
 574  TREATMENTS light* COMP(treat;5;Cont_4)
 575  COVARIATE "No Covariate"
 576  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; CONTRASTS=5;
FPROB=yes; PSE=diff, lsd; LSDLEVEL=5] %germ


Analysis of variance, Variate: %germ

Source of variation      d.f.       s.s.       m.s.    v.r.  F pr.

Rep                         2      46.67      23.33    0.58

light                       1     512.53     512.53   12.84  0.002
treat                       4    8828.80    2207.20   55.28  <.001

   Contrast (cont vs treat)1    1657.63    1657.63   41.52  <.001
   Contrast (Acid10 vs Pri)1     408.33     408.33   10.23  0.005
   Contrast (Cont vs Pri)  1      12.00      12.00    0.30  0.590
   Contrast (Acid05 vs 10) 1     456.33     456.33   11.43  0.003
   Contrast (Acid10 vs 20) 1    3675.00    3675.00   92.05  <.001
light.treat                 4     220.80      55.20    1.38  0.279
  light. Contrast 1         1       5.63       5.63    0.14  0.712
  light. Contrast 2         1       8.33       8.33    0.21  0.653
  light. Contrast 3         1      12.00      12.00    0.30  0.590
  light. Contrast 4         1     120.33     120.33    3.01  0.100
Residual                   18     718.67      39.93

Total                      29   10327.47


Table                 light       treat       light
                                                treat
s.e.d.                 2.31        3.65        5.16

l.s.d.                 4.85        7.66       10.84

cv% =  14.3
```

(b) Germination of ARC seed lot after pre-chilling, hydration, and incubating the seeds on filter papers soaked with KNO_3 and GA_3

```
122  "General Analysis of Variance."
 123  BLOCK Repli
 124  TREATMENTS COMP(treatment;5;Cont)
 125  COVARIATE "No Covariate"
 126  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]germination%

Analysis of variance, Variate: germination%

Source of variation      d.f.       s.s.       m.s.    v.r.  F pr.

Replications                3     117.72      39.24    0.70

treatment                   6    2082.28     347.05    6.19  0.001
   Contrast (con vs treat)  1      28.03      28.03    0.50  0.488
   Contrast (con vs chill5) 1      18.00      18.00    0.32  0.578
   Contrast (chill vs hydr) 1       2.67       2.67    0.05  0.830
   Contrast (KNO3 vs GA)    1      32.00      32.00    0.57  0.460
Residual                    18    1008.89      56.05

Total                       27    3208.89


s.e.d.                  5.29

l.s.d.                 11.12

cv% = 14.8
```

APPENDIX 6.1 Exponential and Gompertz regression analysis of the cumulative germination percentage of *Cleome* seed lots (ARC, Brown and KSC)

```
"Gompertz (asymmetrical s-shaped curve)" (A = lower asymptote (intercept),
      A+C = upper asymptote, B = slope and M = point of inflection)

1150  MODEL germ%
1151  TERMS day*Lot
1152  FITCURVE [PRINT=model,summary,estimates; CURVE=gompertz; SENSE=right;
      CONSTANT=estimate; FPROB=yes] day

 Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0

Summary of analysis ***

              d.f.         s.s.         m.s.      v.r.  F pr.
Regression       3      321480.     107160.0    536.65  <.001
Residual       584      116616.        199.7
Total          587      438096.        746.3

Percentage variance accounted for 73.2
Standard error of observations is estimated to be 14.1

Estimates of parameters ***

                 estimate         s.e.
B                   1.497        0.220
M                   1.699        0.121
C                   70.57         5.04
A                   -3.80         4.59

1154  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, constant parameters separate
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0

Summary of analysis ***

              d.f.         s.s.         m.s.      v.r.  F pr.
Regression       5      377016.      75403.2    718.48  <.001
Residual       582       61080.        104.9
Total          587      438096.        746.3

Change          -2      -55536.      27767.9    264.59  <.001
```

```
Percentage variance accounted for 85.9
Standard error of observations is estimated to be 10.2

Estimates of parameters ***

                          estimate         s.e.
B                            1.497        0.118
M                            1.6987       0.0638
C                           70.57
A  Lot ARC                 -15.90
A  Lot Brown               -3.280
A  Lot KSC                   7.893

1155  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot.day

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, all linear parameters separate
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0

Summary of analysis ***

             d.f.          s.s.          m.s.      v.r.  F pr.
Regression      7       389704.      55672.01    667.26  <.001
Residual      580        48391.         83.43
Total         587       438096.        746.33

Change         -2       -12688.       6344.10     76.04  <.001

Percentage variance accounted for 88.8
Standard error of observations is estimated to be 9.13

Estimates of parameters ***

                          estimate         s.e.
B                            1.511        0.103
M                            1.7086       0.0551
C  Lot ARC                  54.38
A  Lot ARC                 -3.362
C  Lot Brown                67.93
A  Lot Brown               -1.208
C  Lot KSC                  88.32
A  Lot KSC                 -5.788
1156  ADD [PRINT=model,summary,estimates; NONLINEAR=separate; FPROB=yes]

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, all parameters separate
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0
```

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	390834.	35530.41	433.03	<.001
Residual	576	47261.	82.05		
Total	587	438096.	746.33		
Change	-4	-1130.	282.60	3.44	0.009

Percentage variance accounted for 89.0
Standard error of observations is estimated to be 9.06

Estimates of parameters ***

		estimate	s.e.
B	Lot ARC	1.346	0.253
M	Lot ARC	1.770	0.167
C	Lot ARC	54.52	5.21
A	Lot ARC	-2.63	4.67
B	Lot Brown	1.759	0.380
M	Lot Brown	1.607	0.163
C	Lot Brown	68.91	6.81
A	Lot Brown	-3.47	6.34
B	Lot KSC	1.459	0.175
M	Lot KSC	1.7622	0.0923
C	Lot KSC	87.35	4.66
A	Lot KSC	-4.20	4.16

"Exponential (or asymptotic regression)" (A = intercept, B = slope and R = constant, a non-linear parameter)

```
1160  MODEL germ%
1161  TERMS day*Lot
1162  FITCURVE [PRINT=model,summary,estimates; CURVE=exponential; SENSE=right;
      CONSTANT=estimate; FPROB=yes] day
```

Nonlinear regression analysis *****

Response variate: germ%
Explanatory: day
Fitted Curve: A + B*(R**X)
Constraints: R < 1

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	2	319062.	159530.9	784.03	<.001
Residual	585	119034.	203.5		
Total	587	438096.	746.3		

Percentage variance accounted for 72.7
Standard error of observations is estimated to be 14.3

```
Estimates of parameters ***
                  estimate         s.e.
R                   0.4588       0.0236
B                  -151.79         8.18
A                    69.09         1.12
1164  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, constant parameters separate
     Fitted Curve: A + B*(R**X)
      Constraints: R < 1

Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      4       374598.      93649.5    859.84  <.001
Residual      583        63497.        108.9
Total         587       438096.        746.3

Change         -2       -55536.      27768.2    254.95  <.001

Percentage variance accounted for 85.4
Standard error of observations is estimated to be 10.4

Estimates of parameters ***

                         estimate         s.e.
R                          0.4588       0.0115
B                         -151.8
A  Lot ARC                  56.95
A  Lot Brown                69.57
A  Lot KSC                  80.75

1165  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot.day

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, all linear parameters separate
     Fitted Curve: A + B*(R**X)
      Constraints: R < 1

Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      6       387136.      64522.67   735.64  <.001
Residual      581        50960.        87.71
Total         587       438096.       746.33

Change         -2       -12538.      6268.92     71.47  <.001

Percentage variance accounted for 88.2
Standard error of observations is estimated to be 9.37
```

```
Estimates of parameters ***

                          estimate         s.e.
R                           0.4594       0.0102
B  Lot ARC                  -117.4
A  Lot ARC                   52.84
B  Lot Brown                -146.9
A  Lot Brown                 69.02
B  Lot KSC                  -190.6
A  Lot KSC                   85.47

1166  ADD [PRINT=model,summary,estimates; NONLINEAR=separate; FPROB=yes]

Nonlinear regression analysis *****

 Response variate: germ%
      Explanatory: day
  Grouping factor: Lot, all parameters separate
     Fitted Curve: A + B*(R**X)
      Constraints: R < 1

Summary of analysis ***

              d.f.          s.s.          m.s.      v.r.  F pr.
Regression       8       388170.      48521.26    562.71  <.001
Residual       579        49926.         86.23
Total          587       438096.        746.33

Change          -2        -1034.        517.02      6.00  0.003

Percentage variance accounted for 88.4
Standard error of observations is estimated to be 9.29
*** Estimates of parameters ***

                          estimate         s.e.
R  Lot ARC                  0.5090       0.0340
B  Lot ARC                 -107.20         7.40
A  Lot ARC                   54.38         1.45
R  Lot Brown                0.3851       0.0278
B  Lot Brown                -174.2         13.2
A  Lot Brown                 66.83         1.09
R  Lot KSC                  0.4840       0.0210
B  Lot KSC                 -181.76         8.25
A  Lot KSC                   86.64         1.35
```

Appendix 6.2 Exponential and Gompertz regression analyses of the cumulative germination percentage of broccoli (BROCC) and cauliflower (CAULI) seed lots.

"Exponential (or asymptotic regression)" (A = intercept, B = slope and R = constant, a non-linear parameter)

```
1489  MODEL germ
1490  TERMS Day*Lot
1491  FITCURVE [PRINT=model,summary,estimates; CURVE=exponential; SENSE=right;
      CONSTANT=estimate; FPROB=yes] Day

Nonlinear regression analysis *****

 Response variate: germ
      Explanatory: Day
     Fitted Curve: A + B*(R**X)
      Constraints: R < 1
Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      2      207869.    103934.40   1048.39  <.001
Residual      389       38564.        99.14
Total         391      246433.       630.26

Percentage variance accounted for 84.3
Standard error of observations is estimated to be 9.96

Estimates of parameters ***

                estimate         s.e.
R                 0.0588       0.0222
B                 -1129.         423.
A                 97.357        0.610

1493  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot

Nonlinear regression analysis *****

 Response variate: germ
      Explanatory: Day
  Grouping factor: Lot, constant parameters separate
     Fitted Curve: A + B*(R**X)
      Constraints: R < 1

Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      3      208241.     69413.69    705.19  <.001
Residual      388       38192.        98.43
Total         391      246433.       630.26

Change         -1        -372.       372.26      3.78  0.053

Percentage variance accounted for 84.4
Standard error of observations is estimated to be 9.92
```

Estimates of parameters ***

		estimate	s.e.
R		0.0588	0.0160
B		-1129.	
A	Lot BROCC	98.33	
A	Lot CAULI	96.38	

1494 ADD [PRINT=model,summary,estimates; FPROB=yes] Lot.Day

Nonlinear regression analysis *****

Response variate: germ
Explanatory: Day
Grouping factor: Lot, all linear parameters separate
Fitted Curve: A + B*(R**X)
Constraints: R < 1

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	4	208307.	52076.81	528.61	<.001
Residual	387	38126.	98.52		
Total	391	246433.	630.26		
Change	-1	-66.	66.19	0.67	0.413

Percentage variance accounted for 84.4
Standard error of observations is estimated to be 9.93

Estimates of parameters ***

		estimate	s.e.
R		0.0589	0.0160
B	Lot BROCC	-1107.	
A	Lot BROCC	98.15	
B	Lot CAULI	-1147.	
A	Lot CAULI	96.56	

1495 ADD [PRINT=model,summary,estimates; NONLINEAR=separate; FPROB=yes]

Nonlinear regression analysis *****

Response variate: germ
Explanatory: Day
Grouping factor: Lot, all parameters separate
Fitted Curve: A + B*(R**X)
Constraints: R < 1

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	5	208312.	41662.49	421.86	<.001
Residual	386	38121.	98.76		
Total	391	246433.	630.26		
Change	-1	-5.	5.18	0.05	0.819

```
Percentage variance accounted for 84.3
Standard error of observations is estimated to be 9.94

Estimates of parameters ***

                                 estimate        s.e.
R  Lot BROCC                      0.0536      0.0319
B  Lot BROCC                      -1217.       720.
A  Lot BROCC                      98.090      0.860
R  Lot CAULI                      0.0640      0.0307
B  Lot CAULI                      -1057.       505.
A  Lot CAULI                      96.626      0.864

"Gompertz (asymmetrical s-shaped curve)" (A = lower asymptote (intercept),
      A+C = upper asymptote, B = slope and M = point of inflection)
1499  MODEL germ
1500  TERMS Day*Lot
1501  FITCURVE [PRINT=model,summary,estimates; CURVE=gompertz; SENSE=right;
      CONSTANT=estimate; FPROB=yes] Day

Nonlinear regression analysis *****

 Response variate: germ
      Explanatory: Day
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0

Summary of analysis ***

              d.f.          s.s.          m.s.      v.r.  F pr.
Regression       3       207864.      69287.91    697.02  <.001
Residual       388        38569.         99.41
Total          391       246433.        630.26

Percentage variance accounted for 84.2
Standard error of observations is estimated to be 9.97
Estimates of parameters ***

                  estimate
B                    2.984
M                   0.6497
C                    224.0
A                   -126.6

1503  ADD [PRINT=model,summary,estimates; FPROB=yes] Lot

***** Nonlinear regression analysis *****

 Response variate: germ
      Explanatory: Day
  Grouping factor: Lot, constant parameters separate
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0
```

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	4	208602.	52150.41	533.48	<.001
Residual	387	37831.	97.76		
Total	391	246433.	630.26		
Change	-1	-738.	737.92	7.55	0.006

Percentage variance accounted for 84.5
Standard error of observations is estimated to be 9.89

Estimates of parameters ***

		estimate	s.e.
B		2.83096	0.00290
M		-9.7504	0.0131
C		1.096E+15	
A	Lot BROCC	-1.096E+15	
A	Lot CAULI	-1.096E+15	

1504 ADD [PRINT=model,summary,estimates; FPROB=yes] Lot.Day

Nonlinear regression analysis *****

Response variate: germ
Explanatory: Day
Grouping factor: Lot, all linear parameters separate
Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
Constraints: C > 0

Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	5	208307.	41661.45	421.80	<.001
Residual	386	38126.	98.77		
Total	391	246433.	630.26		
Change	-1	294.	-294.39	-2.98	<.001

Percentage variance accounted for 84.3
Standard error of observations is estimated to be 9.94

Estimates of parameters ***

		estimate	s.e.
B		2.8332	0.0436
M		-1.59	1.58
C	Lot BROCC	100688.	
A	Lot BROCC	-100590.	
C	Lot CAULI	104347.	
A	Lot CAULI	-104250.	

```
1505  ADD [PRINT=model,summary,estimates; NONLINEAR=separate; FPROB=yes]


Nonlinear regression analysis *****

 Response variate: germ
      Explanatory: Day
  Grouping factor: Lot, all parameters separate
     Fitted Curve: A + C*EXP(-EXP(-B*(X-M)))
      Constraints: C > 0

Summary of analysis ***

              d.f.         s.s.         m.s.      v.r.  F pr.
Regression       7      208307.     29758.11    299.72  <.001
Residual       384       38126.        99.29
Total          391      246433.       630.26

Change          -2           0.        -0.24      0.00  <.001

Percentage variance accounted for 84.2
Standard error of observations is estimated to be 9.96

Estimates of parameters ***

                                 estimate
B  Lot BROCC                        3.148
M  Lot BROCC                       0.8243
C  Lot BROCC                        149.0
A  Lot BROCC                       -50.89
B  Lot CAULI                        2.853
M  Lot CAULI                       0.4960
C  Lot CAULI                        319.9
A  Lot CAULI                       -223.3
```

APPENDIX 6.3 Life table analysis of the cumulative germination percentage of seed lots of *Cleome* (ARC, KSC and Brown) and cauliflower, which produced the probability density and hazard rate used in Figure 6.3.

(a) ARC

Time interval (h)	Numbers		Proportion			Probability	
	Entering	Terminal	Terminating (q)	Surviving (p=1-q)	Cumul. survival (s)	Prob. density (f)	Hazard rate (λ)
0	200	0	0	1	1	0	0
1	200	0	0	1	1	0	0
2	200	40	0.2000	0.8000	0.800	0.2000	0.2220
3	160	63	0.3937	0.6063	0.485	0.3150	0.4900
4	97	11	0.1134	0.8866	0.537	0.0687	0.1202
5	86	2	0.0232	0.9768	0.866	0.0206	0.0230
6	84	2	0.0238	0.9762	0.953	0.0232	0.0240
7	82	0	0	1	0.976	0	0
8	82	1	0.0122	0.9878	0.988	0.0122	0.0120
9	81	2	0.0247	0.9753	0.963	0.0244	0.0250
10	79	3	0.0380	0.9620	0.938	0.0371	0.0390

(b) KSC

Time interval (h)	Numbers		Proportion			Probability	
	Entering	Terminal	Terminating (q)	Surviving (p=1-q)	Cumul. survival (s)	Prob. density(f)	Hazard rate (λ)
0	200	0	0	1	1	0	0
1	200	0	0	1	1	0	0
2	200	77	0.3850	0.6150	0.6150	0.3850	0.4768
3	123	64	0.5221	0.4779	0.2939	0.3211	0.7066
4	59	16	0.2725	0.7275	0.3477	0.0801	0.3155
5	52	7	0.1353	0.8647	0.6291	0.0471	0.1452
6	49	2	0.0404	0.9595	0.8297	0.0254	0.0413
7	48.14	1.3	0.0270	0.9730	0.9336	0.0224	0.0274
8	46.58	1.56	0.0335	0.9665	0.9404	0.0313	0.0341
9	46.14	0.44	0.0095	0.9905	0.9573	0.0090	0.0096
10	46.14	0.86	0.0373	0.9627	0.9535	0.0357	0.0380

(c) Brown

Time interval (h)	Numbers		Proportion			Probability	
	Entering	Terminal	Terminating (q)	Surviving (p=1-q)	Cumul. survival (s)	Prob. density(f)	Hazard rate (λ)
0	200	0	0	1	1	0	0
1	200	3	0.0150	0.9850	0.9850	0.0150	0.0151
2	197	80	0.4061	0.5939	0.5850	0.4000	0.5096
3	117	22	0.1880	0.8120	0.4822	0.1100	0.2075
4	95	3	0.0316	0.9684	0.7863	0.0152	0.0321
5	92	1	0.0109	0.9891	0.9578	0.0086	0.0110
6	91	3	0.0330	0.9760	0.9654	0.0316	0.0334
7	88	0	0	0	0	0	0
8	88	0	0	0	0	0	0
9	88	0	0	0	0	0	0
10	88	0	0	0	0	0	0

(d) Cauliflower

Time interval (h)	Numbers		Proportion			Probability	
	Entering	Terminal	Terminating (q)	Surviving (p=1-q)	Cumul. survival (s)	Prob. density(f)	Hazard rate (λ)
0	200	0	0	1	1	0	0
1	200	58	0.2900	0.7100	0.7100	0.2900	0.3392
2	142	127	0.8944	0.1056	0.0750	0.6350	1.6178
3	15	1	0.0667	0.9333	0.0986	0.0050	0.0690
4	14	2	0.1429	0.8571	0.8000	0.0141	0.1538
5	12	2	0.1667	0.8333	0.7143	0.1333	0.1818
6	10	1	0.1000	0.9000	0.7500	0.0714	0.1053
7	9	1	0.1111	0.8889	0.8000	0.0833	0.1176
8	8	0	0	1	0.8889	0	0
9	8	0	0	1	1	0	0
10	8	0	0	1	1	0	0