Effect of the environment during seed development on brassica seed quality

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosphy.

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High quality seed is essential for the establishment of a good crop. New Zealand grown brassica seeds usually have high germination but often have variable seed vigour. The latter can result in poor crop establishment and storability. High temperature stress during seed development is known to reduce seed vigour in some species, but whether temperature stress is responsible for seed vigour loss in brassica species was not known. The effects of high temperature during seed development on forage rape (*Brassica napus*) seed quality were determined by assessing seed mass, germination and vigour using a sowing date trial and field and controlled environment experiments.

A time of sowing trial was conducted in the 2011-12 season. A late flowering forage rape cultivar "Greenland" was sown on 25 March and 13 April, 2011 with sowings replicated four times in a randomized complete block design. Seed quality was assessed at three seed development stages (determined by seed moisture content (SMC)): at physiological maturity (PM) (≈50% SMC), pre-desiccation final stage (≈25% SMC) and harvest maturity (≈14% SMC). Seed had attained PM at between 47-52% SMC which was similar to other brassica species. The seed quality testing results demonstrated that sowing time had no effect on seed germination in the prevailing environmental conditions in that season, and at PM there were no differences in seed vigour. However, seed vigour was significantly reduced in seeds harvested at the pre-desiccation (≈25% SMC) and harvest maturity (HM) (≈14% SMC) stages for the early sowing. This was explained by a longer time of exposure to conditions which caused weathering during maturation for the March sowing.

In a controlled growth room, set at 30/25 ˚C (day/night, 12 hours each, R.H 70%), plants received heat stress for four days (240 ˚Ch) at (i) seed filling ii) PM and iii) seed filling plus PM before being returned to the field until seed harvest for two consecutive seasons, 2011-12 and 2012-13. Heat stress decreased seed quality in all three treatments. In both years seed vigour was adversely affected by the heat stress, but seed germination was not. High temperature stress during seed filling produced

smaller seeds but this did not occur with heat stress at PM. Seed developed at the top of the raceme was smaller and had lower germination compared with seed developed at the middle and basal raceme positions. This difference in seed quality between raceme positions became greater after heat stress.

A field trial was conducted in the same two seasons with artificially created high field temperature conditions (using plastic sheet cages) during forage rape seed development. The heat stress was imposed during phase-I (seed filling to PM) and phase-II (PM to HM) and at both Phase-I+II. Heat stress during phase-I significantly reduced seed germination, vigour and seed mass, confirming the results of the controlled environment experiment. Imposition of heat stress during phase-II (after PM), however, significantly reduced seed germination and vigour but did not affect seed mass. Hourly thermal time (HTT) at a base temperature (Tb) of 25 ˚C and the number of hours that temperature remained above 25 ˚C during phase-II (from PM to HM) were significantly correlated with germination and vigour, but not seed mass. The data suggested that for a Tb of 25 ˚C, at least 100 ˚Ch before PM and 300 ˚Ch after PM were required before vigour loss occurred.

The effects of high temperature during seed development were further studied at a physiological and ultrastructural level using heat stressed and non-stressed seeds from the controlled environment experiment. Both reactive oxygen species (ROS) (H_2O_2) and lipid peroxidation were measured. H_2O_2 and malondialdehyde (MDA) were both significantly higher in heat stressed seeds than in non- stressed seeds. Loss of seed vigour was associated with an accumulation of H_2O_2 and lipid peroxidation. H_2O_2 in heat stressed seeds was strongly correlated with seed vigour loss, suggesting that lipid peroxidation was not the only cause of seed deterioration. Seed vigour loss was also characterized by a marked decrease in the ROS scavenging antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities following heat stress. A significant negative effect of heat stress on the adenine nucleotides pool and adenylate energy charge (AEC) was recorded which indicated the altered metabolic system. This was mainly due to a decrease in cellular adenosine triphosphate (ATP), resulting in a decrease of AEC. Electron microscopy revealed significant cellular damage in heat stressed seeds, particularly in the cell membranes and mitochondria. The decreased level of nucleotides and energy levels, and higher electrolyte leakage recorded in heat stressed seeds was associated with this structural damage. Mitochondrial ATP synthesis provides an important source of energy to complete the germination process. The mitochondrial damage in this study as a result of heat stress suggests that the mitochondria were unable to synthesize sufficient energy for the active oxidative phosphorylation required to complete successful germination

Keywords: Forage rape, heat stress, raceme position, malondialdehyde (MDA), lipid peroxidation, sowing date, reactive oxygen species (ROS), electrolyte leakage, electron micropscopy, seed moisture content (SMC), mitochondrial ATP, structural damage

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1.1 Introduction

The genus Brassica is a large family of angiosperm plants with more than 300 genera and 3500 species distributed worldwide (Al-Shehbaz et al., 2006). It includes many important crop plants with economic value, including approximately 10% of the world's vegetable crops and 12% of the edible oilseeds crops (Economic Research Service, 2008). Worldwide, production of brassica including cauliflower, broccoli, rapeseed, swedes, mustard and cabbage exceeded 205 million tonnes in 2013-14, an increase of about 45 million tonnes from 2003-04 (http://faostat3.fao.org/browse/Q/QC/E). Depending on species (i.e. vegetable or forage brassica), the seed, leaves, stems and roots are eaten by humans and by animals (Desai, 2004; Hampton & Hill, 2002; Vaughan, 1977). Brassicas have a tap root system (Langer & Hill, 1991) but are generally grown for their above ground parts.

The oldest known plants are among the Cruciferae family of brassica species. According to old Sanskrit scriptures (ca. 1500 BC), *B. juncea* seeds were found during exploration of archaeological sites of the Indus valley civilization in India, which is dated back to at least ca. 2300 BC (Prakash & Hinata, 1980). China has a long history of rapeseed production with the oldest evidence found in archaeological discoveries dating back to ca. 5000 BC (Yan, 1990). It is known historically, that *B. rapa* was among the first domesticated brassica species, and for over 4000 years it has been cultivated from the Mediterranean region to northern Europe, Germany and central Europe to the Himalayan region of central Asia (Bonnema et al., 2011). Curly kale, cabbage and kohlrabi were the first cultivated brassica vegetable species, with other species such as cauliflowers and brussels sprouts established in the 18th and 19th century respectively (Nieuwhof, 1969).

Brassica plants are not only important from commercial aspects, but due to their high genetic resemblance with the model species *Arabidopsis thaliana*, this has made them an attractive model system to study the plant system and evolution. According to Lukens et al. (2004), the brassica genus consists of six species with high economic values that involve the natural interspecific hybrids *B. carinata* (2n = 34, BBCC), *B. rapa* (2n, 36, AABB) and *B. napus* (2n = 38, AACC) and also the diplod species *B. oleracea* (2n= 18, CC), *B. rapa* (2n = 20, AA) and *B. nigra* (2n = 16 BB). The cytogenetic relationships between the genomes of brassica crop species are referred to as U's triangle (U, 1935) (Figure 1.1).

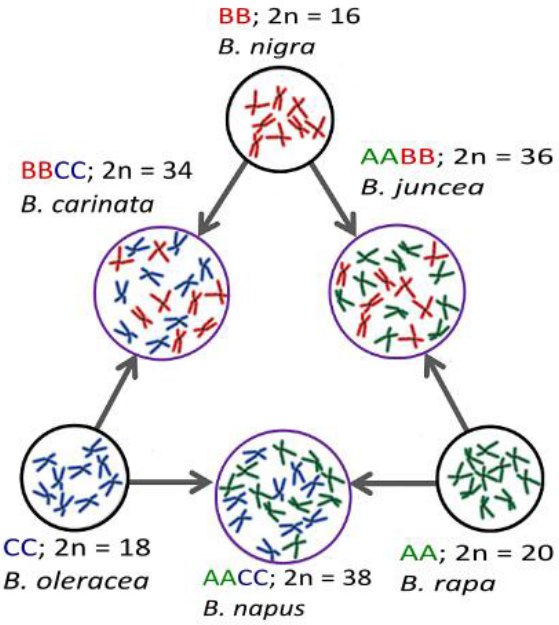


Figure 1.1 Genetic relationship between six brassica species described by the triangle of U (U, 1935).

Currently, both forage and vegetable brassicas are cultivated all over the world in a range of environments from cool temperate to hot tropical climates. Cabbage (*Brassica oleracea* L.) is the first known brassica crop introduced to New Zealand, being brought here by Captain James Cook in 1773 during his second voyage (Thomson, 2011). Now in New Zealand brassica crops are grown widely as forage for grazing animals, for vegetable production and for seed production.

1.2 Seed development in brassica

Seeds of brassica are non-endospermic as the mature seed does not retain the endosperm and the embryo is surrounded by the seed coat. Brassica seed development begins with embryogenesis followed by seed desiccation (Angelovici et al., 2010; Yu et al., 2010). During embryogenesis two overlapping stages of seed development are distinguished: morphogenesis and seed filling (Sabelli, 2012). Baud et al. (2002) provided a complete description of the brassica seed development process using *Arabidopsis thaliana* (Figure 1.2). Brassica seed development processes were grouped into three stages: (1) early embryo morphogenesis (2) maturation (3) late maturation. Early embryogenesis is initiated by a double fertilization process which yields the zygote (2n) and endosperm (3n), followed by a series of cellular division and differentiation during which the developing embryo changes its shape from pre-globular to a torpedo shape (Baud et al., 2002; Jenik et al., 2007). At the end of this phase, around 6 days after flowering (DAF), the torpedo shaped embryo enters into the early

maturation phase and forms a bent-cotyledon. Between 7 to 10 DAF, seed filling occur (Angelovici et al., 2009; Le et al., 2010).During this phase the endosperm degrades and is resorbed by the elongate

ing embryo through synthesis and accumulation of storage compounds (Figure 1.2) (Baud et al., 2008). Once the seed filling ends, further growth of the embryo is stopped by terminating the synthesis of storage compounds and its becomes metabolically quiescent. During the second phase of maturation (11 to 16 DAF), the endospermic starch level declines as it is used by the embryo to synthesise storage products, proteins and triacylglycerol (TAG) (Borisjuk et al., 2013).

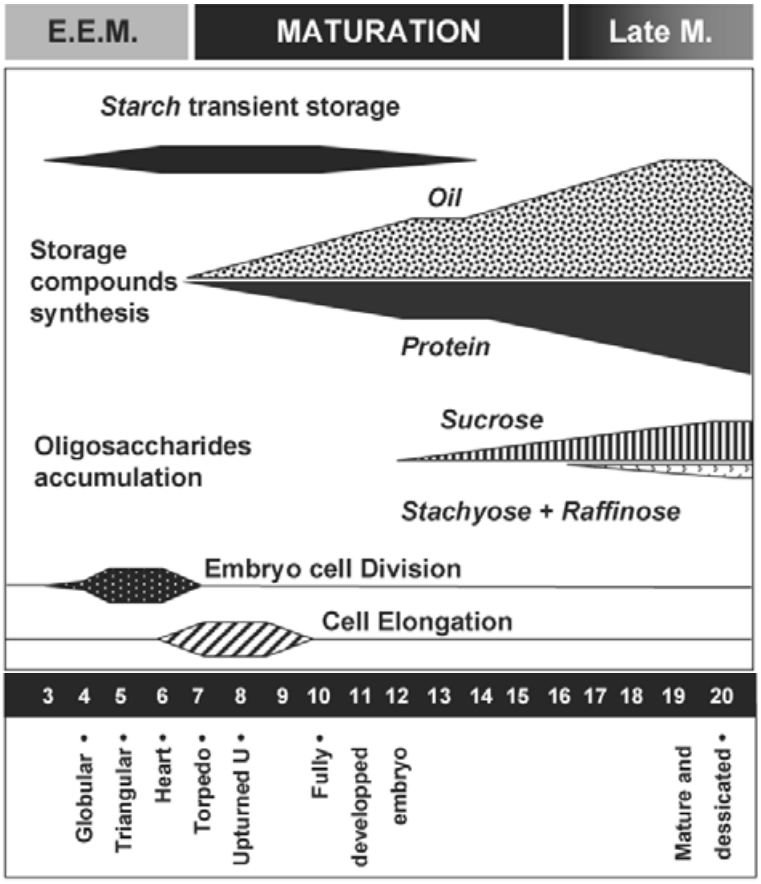


Figure 1.2 Schematic diagram of physiological and metabolic events during brassica seed development (Baud et al., 2002)

Despite the rapid loss of seed moisture content during late maturation, synthesis and accumulation of oligosaccharides (raffinose, stachyose and verbascose) occurs (Baud et al., 2008; Fait et al., 2006). The stored oligosaccharides, especially raffinose and stachyose, play a key role for the protection of membranes and proteins, contributing to making the seed desiccation tolerant (Bailly et al., 2001; Bentsink et al., 2000; Leprince et al., 1990).

Brassica seed comprises mainly storage compounds including carbohydrates (mostly in the form of starch), specialized storage proteins (SSPs) and lipids (mainly in the form of triacylglycerols (TAGs))

(Baud et al., 2008; Baud & Lepiniec, 2010). These components contribute 90% of the total seed weight. In rape seed (*Brassica napus*), cabbage (*Brassica oleracea)* and *Arabidopsis thaliana*, TAGs contribute about 35-40% ,70% and 35 to 54% respectively of the total seed dry weight (Baud et al., 2008; Murphy & Cummins, 1989; Qouta et al., 1991; Rahman et al., 2013). In germinating seeds TAG is oxidized and converted into free fatty acids (FAs) and glycerol, by TAG lipases through a chain of enzymatic reactions. FAs are later converted into sugars to provide energy for seed germination and seedling growth (Baud & Lepiniec, 2010; Chia et al., 2005; Quettier & Eastmond, 2009).

Starch is accumulated earlier during seed development and degraded later during seed maturation. It has been proposed that starch accumulation at the earlier stages is to strengthen the main sink before the synthesis of storage compounds (Baud et al., 2002; Periappuram et al., 2000). Generally a large seed size and higher seed mass is considered to better support seedling growth during the heterotrophic stage before photosynthesis begins (Pracharoenwattana et al., 2010). Elliott et al. (2008) associated better seedling establishment of *Brassica rapa* with large seed size and weight. Seed weight is highly correlated with seed size (Bagheri et al., 2013). During seed development, the composition and quantity of seed storage reserves are controlled by biosynthetic processes (Baud et al., 2008), and the later seed storage reserves mobilization during imbibition determines the germination potential (Cheng et al., 2013; Fait et al., 2006).

1.3 Forage brassica species: Production and use

1.3.1 Forage brassica species and their use

Forage brassicas are grown in a wide range of climates and soils throughout the year as supplementary feed in grazing systems. These include those that mainly produce root biomass e.g. turnip (*Brassica. rapa* L.), swede (*B napus* L.), and others that produce mainly leaf and stem e.g. rape (*B. napus* L.) and kale (*B. oleracea* L.) (Wilson et al., 2004). These crops are high quality, high yielding and fast growing. The livestock consume all parts (stem, leaves and roots) of turnip and swedes and all above ground parts (stem & leaves) of rape and kale (www.uwex.edu/ces/forage/brassica.html).

In New Zealand's husbandry of grazing animals, forage brassicas are widely cultivated both as a supplement and as an alternative to pastures because they can be fed '*in situ*' from early summer through to late winter. During pasture renewal, forage brassicas are also used as break crops. However, during the period of pasture feed shortage, they produce high quality feed and also help to avoid pasture related health problems like ryegrass staggers and eczema. Forage break crops provide an advantage for pasture renovation because they create better soil conditions and a clean seed bed by reducing weeds, pests and diseases (De Ruiter et al., 2009).

Four species of brassica are traditionally used as a forage crop in New Zealand: they are forage rape (*Brassica napus*), kale (*Brassica oleracea* var. acephala), swede (*Brassica napus* var. napobrassica) and

turnip (*Brassica rapa*) (White et al., 1999), but leaf turnip hybrid (*B. rapa* x compestris) is now also used in grazing systems as a summer/autumn feed option. (Stewart, 2002) (Figure 1.3). All forage brassica are biennials and species and cultivars have different characteristics that also suit different farming systems (De Ruiter et al., 2009).

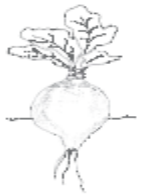
Common name	Latin name	Growth habit	Vegetative description
Leaf turnip (or turnip cross)	*Brassica rapa*; syn. B. *campestris*		• Non-bulb producing • Swollen tap root provides multiple growing points • Able to regrow after grazing • Leafy
Bulb turnips	*Brassica rapa*; syn. B. *campestris*		• Fleshy bulb • No neck • Yellow fleshed (hard) • White fleshed (soft)
Swedes	*Brassica napus* spp. *napobrassica*		• Fleshy bulb • Obvious neck • White or yellow fleshed
Rape	*Brassica napus* spp. *biennis*		• Numerous leaves • Fibrous stem • No bulb or fleshy stem • Grows to various heights
Kale (Chou Moellier)	*Brassica oleracea* spp. *acephala*		• Large swollen stem with varying leaf percentage • Stem – woody outer layer, soft fleshy marrow • Grows to various heights

Figure 1.3. Forage brassicas most commonly used for animal feeding in New Zealand. Adapted from Stewart and Charlton (2003).

(i) Forage rape

Forage rape (*Brassica napus* L.) is an allotetraploid species with a chromosome number of 2n=38 and is a derivative of *B. oleracea* L. (2n = 18, cc) and *B. campestris* L. (2n = 20, aa) (Nagaharu, 1935; Stewart, 2002). Forage rape is multi stemmed, has numerous leaves and fibrous roots with no bulb (White et al., 1999). However, different cultivars vary in stem height, diameter and palatability. Due to its rapid growth in autumn, forage rape can be used for rotational grazing in pasture systems and it also has high water and nitrogen (N) use efficiencies, high potential yield and low establishment costs (Garg & Manchanda, 2009). It has a late flowering period and longer growing season and can be grown in spring for summer/autumn feed with additional re-growth in winter (De Ruiter et al., 2009).

(ii) Kale

Kale (*Brassica oleracea* L. var. *acephala* DC.) comes from an ancient ancestor of cabbage, and was either domesticated in the eastern Mediterranean or Asia (Nieuwhof, 1969). It has a deep root system with better tolerance against drought (De Ruiter et al., 2009).It does not produce a solid head like the normal cabbage plant but produces large quantities of leaf and stem which are utilized mostly by cattle. It is used as a supplementary fodder in many parts of the world during the winter season (Decoteau, 2000; White et al., 1999). It is typically sown in spring for high yield and grazed between June and August. Cultivars with taller plants are more suitable for cattle grazing while cultivars with shorter plants are most suited to sheep and deer (De Ruiter et al., 2009).

(iii) Swede

Swede (*Brassica napus* L. var. *napobrassica*) is grown for winter and autumn forage for cattle and sheep (Bradshaw et al., 2009). It thrives best in cool and moist environments because it has low tolerance against drought. Its maturity process is slow but it is considered a high yielding winter crop. It is usually sown in late spring or summer (usually from 20th of November until the end of December) to produce high yields of high quality winter feed (leaf yield and edible roots (bulbs)) (De Ruiter et al., 2009).

(vi) Turnip

Turnip (*Brassica rapa* L.) grows fast and can be grazed 70 days after sowing. Its leaves and bulb can both be utilized for grazing. It is well adapted to almost all locations throughout New Zealand, being sown from October to February and grazed from summer to late winter (De Ruiter et al., 2009).In autumn and winter, the turnip plants develop quickly and produce a high forage yield with high protein content and digestibility (Undersander et al., 1991).Early maturing cultivars reach maximum production level by 60-90 days and late maturing ones reach grazing maturity in 90-120 days (De Ruiter et al., 2009). The turnip bulb serves as a large storage organ and contains around 60% of the dry matter produced by the plant (Ayres & Clements, 2002).

(v) Leaf turnip (Turnip Hybrid)

Leafy turnip originated from Poland and is usually a hybrid cross of *B. rapa x compestris* (cv. Pasja) (Kimber & McGregor, 1995). From mid-summer to early winter, it is grown widely in New Zealand for supplementary feed. The plants have a shallow root system and are therefore susceptible to drought and low fertile soils. Usually, it is sown from October to early autumn and matures quickly (ready to graze in 50-70 days) with high quality leaf and little stem and bulbs (De Ruiter et al., 2009).

1.3.2 Forage brassica seed production in New Zealand

In New Zealand, forage brassicas are the main animal feed other than pasture and pastoral products (White et al., 1999). Seed production in New Zealand is now largely centred in Canterbury, but historically other regions were also important, especially Southland. Until 1940, seeds of forage brassicas such as turnip, kale, rape, and swede were imported from Europe (Rolston et al., 2006) and no New Zealand bred brassica cultivars were available at that time. Brassica seed production in New Zealand was not given real importance until the outbreak of the second world war (1939-1945), when importing of the seed from United Kingdom(UK) was stopped (Claridge & Hadfield, 1972; Palmer, 1983). As a war time measure, a programme was arranged by DSIR (now Plant & Food Research, New Zealand) to produce forage brassica seed in sufficient quantity and quality to meet New Zealand's needs in close relationship with seed companies. Various forage brassica cultivars were released with various agronomic and profitability improvements by crossing European lines to develop distinctive New Zealand cultivars (Palmer, 1983; Rolston et al., 2006). This resulted in the establishment of a wide range of forage brassica cultivars. By the end of the war, brassica seed production was established in New Zealand. Now the New Zealand seed industry produces and processes forage brassica seeds for both domestic use and for export to all over the world (Hampton et al., 2012; Millner & Roskruge, 2013; Pyke et al., 2004).

1.4 Crop agronomy

Brassica crops can be sown on a wide range of soil types but require a well-drained soil with good fertility and depth. The optimum pH for brassica crops is highly basic. They require a minimum pH of 5.6 but the ideal pH can be within 5.8 to 6.2. If the pH is low (highly acidic soils) lime ($CaCl_2$) needs to be applied at a rate of 1 tonne/ha (De Ruiter et al., 2009). As brassica seeds are very small, they must be sown into a firm, moist and warm, aerated and well-structured seed bed for good germination and growth. Sowing time for various brassicas can be chosen according to thermal time requirements for maturation which have a major effect on yield potential (De Ruiter et al., 2009; Kimber & McGregor, 1995). Brassica can be sown by various methods i.e. ridging, broadcast and drill method, but for seed production, the drill method is preferred. For drilling, rape and kale are sown at a rate of 3-5 kg/ha and turnip and swedes at 0.5- 1.0 kg/ha. Most brassicas are biennials and produce seed in the second year (Wilson et al., 2004) and require a low temperature stimulus (vernalisation) for flowering (Wien, 1997). Brassica have perfect flowers and require insects for pollination. Cultivars of the same species may cross pollinate with other cultivars, so the correct isolation distance must be maintained to avoid contamination and to maintain genetic purity. The recommended isolation distance for most of the brassica crops is 1000 m (George, 2009). The crop should be irrigated at the start of the 4^{th} week after sowing depending upon the availability of moisture. Supplementary nitrogen is usually applied as top dressing in the areas where rainfall is high and leaching affects are more, but care should be taken

because it can cause lodging when the seed crop is maturing (George, 2009). Brassica seed crops have a tendency to shatter readily, so to minimize unnecessary seed loss, the crop is usually cut at 14% seed moisture content (SMC) and left in a swath for 10-14 days for further drying so that the seed can easily be separated from the pods. The crop is then combined when SMC is around 7-8%. Brassica pods are easily shattered, and therefore threshing is conducted using a slow combine drum speed normally not exceeding 700 rpm (George, 2009).

1.5 Seed yields

Ripening of seeds takes place over an extended period and it is necessary to harvest the seed at correct time to secure the highest yield of good quality seed. The average seed yield of different forage brassicas is: turnip 1.5 t/ha, swede 1.5 t/ha, forage rape 1.7 t/ha (George, 2009; Leeks, 2006).
Brassicas are a major earner for New Zealand with an estimated $ 80.3 M domestic value (Nixon, 2015). New Zealand's current brassica seed production is around 17002 tonnes (including brassica vegetables and canola) with a value of $ 15.15 M (Hampton et al., 2012). In New Zealand , forage brassica such as kale, fodder rape and turnip retail from between$ 11.3 to $ 15.9 /kg (based on the bare seed price) (Leeks, 2006). Total certified forage brassica seed produced from 2007-2014 is presented Table 1.1.

Table 1.1 Quantities of certified forage brassica seed (tonnes) produced from 2007-14 (AsureQuality, 2016)

Crop	2007	2008	2009	2010	2011	2012	2013	2014
Fodder rape	300	287	206	190	483	772	1367	2426
Kale	403	664	256	104	501	1032	796	896
Swede	284	176	149	923	26	54	214	57
Turnip	216	298	291	222	201	251	692	3967

1.6 Forage brassica seed quality

1.6.1 Components of seed quality

In practice, the term "seed quality" is used to express the overall value of seed for its intended end use. It refers to the different properties of seed which may have different degrees of practical importance depending upon their species and end use (Hampton, 2002). According to Hampton (2002), "Seed quality is a degree of excellence in certain characters or attributes which determine the performance of the seed lot in the field and during storage". Seed quality is a multidimensional concept having several components (Thomson, 1979). Traditionally, farmers equate seed quality with its ability

to germinate and be free of undesirable weed and seeds (Hampton, 2000). However, there are several components of seed quality which were grouped into three categories by Coolbear and Hill (1988).

Description:	Analytical, species and cultivar purity; uniformity in seed weight
Hygiene:	Contamination of noxious weed seed, insect and mite contamination, storage fungi contamination, pathogenic fungal, bacterial and viral infection
Potential Performance:	Seed germination and vigour, uniformity and field emergence, moisture content and potential storability

Crop establishment, growth and yield is greatly influenced by seed quality which also has an influence on production economics of all species of crops (Finch-Savage, 1995). Good quality seeds are required in changing global conditions and food insecurity (Larinde, 2009). Seed quality parameters provide valuable information on the fitness of a seed lot for sowing, for its ability to establish healthy and uniform and vigorous seedlings, for rapid emergence from soil for vigorous growth and for high yield. A high quality seed lot will allow the desired population from a reasonable seeding rate in a range of field environments (Egli et al., 2005).

1.6.2 Seed germination

By definition, germination is a physiological process which has a series of events (hydration, subcellular structural changes, respiration, macromolecular synthesis and cell elongation) that starts with the imbibition of water by a quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley & Black, 1994). Seeds must have the ability to germinate and produce seedlings as effective reproductive units (McDonald & Copeland, 1997). To the seed physiologist, germination starts with the protuberance of the radical from the seed coat (Desai, 2004).

However, the International Seed Testing Association (ISTA, 2016) states that “germination of a seed in a laboratory test is the emergence and development of seedling to a stage where the aspect of its essential structures indicate whether or not it is able to develop further into satisfactory plant under favourable conditions in the soil. The features of essential structure include:

1. Root system: (the primary root is intact; acceptable defects include: discoloured or necrotic spots, healed cracks and splits, and superficial cracks and splits.)
2. Shoot system (the elongated hypocotyl is intact; acceptable defects include: discoloured or necrotic spots, healed cracks and splits, superficial cracks and splits and loose twists; the terminal bud is intact; the cotyledons are intact; acceptable defects include: up to 50% of tissue not functioning normally, only one intact cotyledon, and three cotyledons” (ISTA, 2016).

Seed companies would like germination (% normal seedlings) to be as close to 100% as is possible. However, germination may be negatively affected by adverse environmental conditions during seed development, harvesting, cleaning, drying and storage (Dornbos, 1995), and particularly temperature, rainfall and humidity (Egli et al., 2005). A normal brassica seedling must have intact essential structures which show the potential for continued development into satisfactory plant (ISTA, 2016). New Zealand, forage brassica seeds generally have a germination above 85% (Leeks, 2006) as shown in Table 1.2**.**

Table 1.2 Forage and vegetable brassica species: average germination data for the 2004 season (Leeks, 2006).

Forage species	Mean Germ. (%)	Vegetable species	Mean Germ. (%)
Kale	88	Kale	89
Rape	91	Rape	96
Turnip rape	94	Fodder radish	92
Swede	93	Choisum	N/A
Turnip	93	----------	-------

New Zealand does not have a seed law and thus there are no minimum germination standards, unlike most countries which do have such standards for the trading of seed (Leeks, 2006). For example, Utah, in the United States of America has a laboratory germination standard for commercial kale of 75% (Code, 2000). However, the field emergence of seed lots is often overestimated by the germination test (Copeland & McDonald, 2001).The germination test express results following testing under optimal conditions. These do not exist in the actual field which can be exposed to many environmental stresses. This was the reason for the development of vigour testing (ISTA, 2016).

1.6.3 Brassica seed vigour

Among the major components of seed quality, seed vigour is an important quality attribute (Heydecker, 1972). It is an internationally accepted parameter for ranking the potential field performance of high germinating seed lots (Hampton & TeKrony, 1995). Vigour information for a seed lot, which is not a single measurable property like germination, is sometime derived from several tests instead of a single test. Vigour is properties of seed associated with various aspects of seed performance under stress, both in field and storage conditions as described by Hampton and TeKrony (1995). Seed lots of the same age, cultivar, and certification class which all have high germination may perform differently in the field (Hampton & Coolbear, 1990). Leeks (2006) also found this problem in brassica species and reported significantly higher correlations between a vigour test (accelerated aging test) and field emergence, than for the laboratory germination test. Similarly Komba (2003) observed

that vigour was a better predictor of field emergence than the standard germination test for high germinating kale seed lots.

Brassica seed grown in New Zealand normally possess high germination i.e. above 90%, but may differ in vigour (Table 1.3). Hampton and Hill (1990) stated that a germination test result of less than 90 % usually indicates that seed physiological deterioration has started. However high germinating (> 90%) seed lots may have low vigour (Table 1.3).

Table 1.3 Mean germination (Pre-AA) and vigour (Post-AA) results for four forage brassica spp. over three harvest seasons (Leeks, 2006).

S #	Species	Season 2002-03		Season 2003-04		Season 2004-05	
		Pre-AA* (%)	Post-AA (%)	Pre-AA (%)	Post-AA (%)	Pre-AA (%)	Post-AA (%)
1	Turnip rape	95	79	97	71	97	58
2	Rape	90	78	96	64	98	69
3	Kale	89	53	95	56	94	80
4	Swede	93	62	98	89	97	81

* AA = Accelerated aging vigour test

In many studies it has been reported that brassica seed vigour is highly variable. Leeks (2006) sowed 24 seed lots of forage and vegetable brassica at Lincoln. All 24 seed lots had a germination of 90% or greater but their field emergence ranged from 8 to 82 % because of differences in the vigour of these high germinating seed lots of brassica.

Low vigour is a problem in New Zealand forage brassicas. The importance of environment is well recognized for seed production (Delouche, 1980), and brassicas requires a low temperature stimulus for flowering. However, the developing seeds exposed to high temperature stress during seed maturation, can cause a rapid loss of vigour (Dornbos, 1995). Whether this is the reason for poor vigour in New Zealand grown brassicas is not known.

1.7 Seed vigour

1.7.1 What is seed vigour?

Much is known about seed mass and germination but seed vigour is a more complex parameter (Hampton & TeKrony, 1995) and that is why the ISTA vigour test committee took 27 years to agree on the definition of seed vigour as the " sum of those properties that determine the activity and level of performance of a seed lot of acceptable germination in a wide range of environments". Hampton (1998) described seed vigour as ""an index of the extent of the physiological deterioration and/or mechanical integrity of a high germinating seed lot which governs its ability to perform in a wide range of environments".

Usually high vigour seed lots can emerge under a wide range of field environmental conditions and store well. Low vigour seed lots may perform well under non-stressed conditions, but fail to perform under adverse conditions and have a poor storage potential (Hampton, 2000, 2002; Komba, 2003).
Seed vigour records the extent of physiological deterioration that has occurred within the seed. The germination test does not essentially indicate this deterioration. For a high germinating seed lot, a small difference in germination may represent a large difference in the deterioration process because of the nature of the normal distribution on which the seed survival curve (Figure 1.4) is based (Ellis et al., 1985).

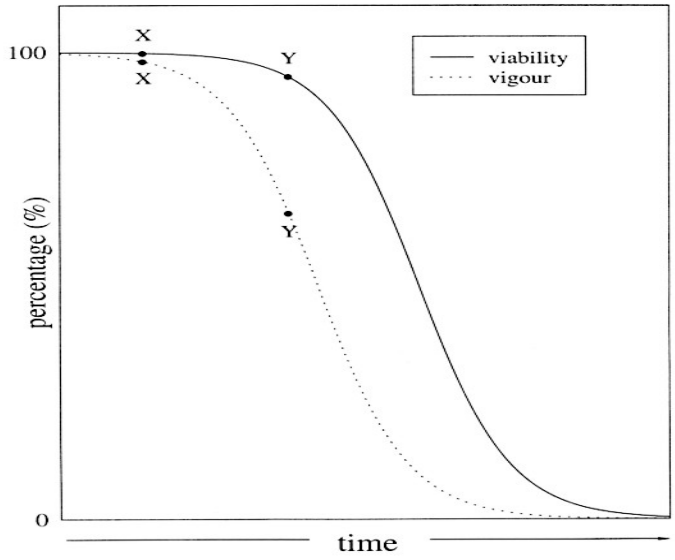


Figure 1.4 Relationship between seed viability and seed vigour over time.
X and Y represent two different seed lots.
Adapted from Delouche and Caldwell (1960).

Seed vigour is important for the seed the industry and for farmers. Seed vigour tests provide valuable information about seed quality not identified by the germination test. Companies are using vigour information for their in house quality standards for:-

i. identification of seed lots which meet the company standards

ii. ranking the seed lots for in house quality control.

iii. evaluating the suitability of a lot for storage.

iv. evaluating the seed lot potential for exporting so that the quality may not impaired during international transport.

Researchers use the vigour information:-

i. to increase their understanding of the concept of seed vigour and the causes of seed vigour differences among seed lots.

and ultimately the farmer (consumer) needs vigour information:-

i. to know the vigour status of each high germinating seed lot before making a decision about which one to buy (ISTA, 2016).

Seed vigour is now an internationally recognized seed quality parameter. In 2010 over 1 million vigour tests were conducted by member laboratories of ISTA and AOSA/SCST. (Hampton, pers. comm, 2016).

1.7.2 Testing for seed vigour

The germination test has long been used to evaluate the planting value of a seed lot (ISTA, 2016). It determines the percentage of normal and abnormal seedlings and dead/ non germinated seeds in the seed lot (Hampton & Coolbear, 1990). However, germination results often overestimate seed lot field performance, because of its inability to detect subtle quality difference among high germinating seed lots. Therefore, seed vigour testing is required as an important tool to detect quantifiable parameters associated with seed deterioration to rank high germinating seed lots in terms of potential performance (Hampton & TeKrony, 1995).

According to the established requirement of the vigour test (ISTA, 2016; McDonald, 2002) a vigour test must be able to :-

i. provide a more sensitive index of seed quality than the germination test.

ii. provide a consistent ranking of seed lots in terms of potential performance.

iii. be objective, rapid, simple and economically practical

iv. be reproducible and interpretable.

Over the last 40 years, many vigour test have been produced but three of them are readily distinguished (Hampton & Coolbear, 1990)

i. Single test based on some aspect of germination behaviour (e.g. Cold test and Accelerated Aging test)

ii. Physiological and biological test (e.g. Conductivity test)

iii. Multiple testing procedures.

The International Seed Testing Association (ISTA) currently recommends four vigour tests:-

i. Conductivity test
ii. Accelerated Aging (AA) test
iii. Controlled deterioration (CD) test.
iv. Tetrazolium test

These tests are now placed in the ISTA Rules because they are internationally standardized, reproducible and interpretable (ISTA, 2016). In this study only conductivity and accelerated ageing test (AA) were used.

(i) Conductivity test

The conductivity test measures the electrical conductivity of water in which seeds have been soaking. Amino acids, sugars and ions leak from seeds when they are placed in water (Halmer, 2000). The degree of leakage depends on the permeability of the cell walls. Any deterioration or rupture of the cell walls allows these electrolytes to move out of the seed into the soak water which increases its conductivity (Copeland & McDonald, 2001). The conductivity test was developed into a routine vigour test for the prediction of field emergence in garden peas (ISTA, 2016; Matthews & Bradnock, 1967; Matthews & Powell, 2006) and is now widely used for this purpose in Australia, New Zealand, Europe and North America (Hampton & TeKrony, 1995). This test has also been used on seed of many other species, especially soybean and other large grain legumes (Hampton & TeKrony, 1995). This procedure is simple, inexpensive and rapid. The extent of membrane deterioration is associated with the concentration of leachates in water (Copeland & McDonald, 2001). Seed lots with poor cell membrane integrity have a high conductivity and are deemed as low vigour (Powell et al., 1986).
During the seed re-hydration which occurs in early imbibition, the seed has the ability to reorganize or repair any damage to its cell membranes. The greater the ability of the seed or the speed to repair any damage and reorganize cell membrane, the higher will be its vigour (Hampton & TeKrony, 1995). As the seed deterioration progresses, the cell membranes become less rigid and more water permeable and allow the cell contents to escape into solution with water to increase the electrical conductivity (Coolbear, 1995)

(ii) Accelerated aging test (AA test)

A vigour test provides information about the physiological quality of seed (Elias & Copeland, 1997). One of the most important vigour tests for quality control programmes in the seed industry is the accelerated aging test (Jianhua & McDonald, 1997). This test was first developed to assess the longevity of soybean seed in commercial storage (Delouche & Baskin, 1973). The AA test has since been used to predict the life span/potential storage of different species including soybean (Egli, 1979), radish (Delouche & Baskin, 1973), corn (Scott, 1981) and red clover (Wang & Hampton, 1991) and subsequently has been used as an indicator of seed vigour and correlated successfully with field emergence in soybean(Egli et al., 1990), French bean (Hampton et al., 1992b) and ryegrass (Happ et al., 1993). In general this test is well correlated with field emergence (Mihailovi et al., 2009).

The accelerated aging test is one of the most important for testing vigour of soybean in North America (Ferguson, 1990). ISTA has also standardized this test for soybean and incorporated it into the ISTA rules (ISTA, 2016).

In this test seeds are exposed to a double stress provided by two environmental variables i.e. high temperature and high relative humidity which are the two main factors involved in seed deterioration or aging (Hampton & TeKrony, 1995). During the exposure of seeds to these conditions, the seeds imbibe moisture from the humid environment which raises moisture content and this combined with high temperature accelerates seed aging or deterioration. The seeds are then re-tested for germination. High vigour seed will better withstand these conditions and will deteriorate at a lower rate than low vigour seed and therefore, high vigour seed lots have a high germination after accelerated aging compared with low vigour seed lots which have a poor germination.

This test is rapid, inexpensive and simple. Leeks (2006) in a study using seed lots of brassica species (i.e. *Brassica rapa x compestris, B. compestris, B. napus, B. oleracea, B. rapa var. pekinensis*) found a high correlation between germination recorded from the accelerated aging test and field emergence. The suggested temperature during aging for brassica species is 41 ˚C for 72 hours (Hampton & TeKrony, 1995) but whether this is correct for all species of brassica is unknown.

1.8 Factors affecting seed vigour

During the seed development process from fertilization to nutrient accumulation, a series of morphological and physiological changes occur and these may affect seed performance potential (Copeland & McDonald, 2001). Physical charactristics were , intially, considered to be a cause of differences in seed vigour (Perry, 1980). However, other factors, particulerly physiological changes associated with seed vigour have been examined in later invesitgations and Powell (1988) concluded that physical and physiological damage to cell membranes was the fundamental cause of the seed detrioration that leads to a loss of vigour. Physiological maturity is considered to be the stage at which the seed attains its maximum dry weight and has maximum germination and vigour (TeKrony & Egli, 1997). At this point seed deterioration starts and continues as the seed desicates on the plant, during harvesting, processing and storage (Hampton & TeKrony, 1995). However, Shinohora et al. (2006a) showed that deterioration can begin before PM. Diffrences in seed vigour may be influenced by factors such as genetic makeup, the environment during seed production, seed size and maturity, pod position and seed storage environments (Castillo et al., 1994).

1.8.1 Genetic effects

Seed vigour may be influenced by the genetic constitution of the seed (Wang et al., 2010) Seed characteristics such as hard seededness, susceptibility to mechanical damage and chemical composition of seed are under genetic control. These factors affect the expression of seed quality.

Many centuries ago, brassica plants were developed by cultivation and unconscious selection from plants simpler than the present wild turnip, to produce an enlarged storage organ suitable for human consumption (Stewart, 2002). Through plant breeders working on improving seed yield, seed characteristics and disease resistance, they unintentionally selected for increased seed vigour (Copeland & McDonald, 2001). Dornbos (1995) established that genetic differences among cultivars to attain and maintain good seed quality exist, but for seed vigour this appears to be smaller than the effects of environmental stress during seed production. It is possible to select characters which would delay a loss in seed vigour. For example, Kuo (1989) found that in a small number of soybean cultivars imbibition was delayed for least one hour after soaking due to a lower seed coat permeability. This character allows the seed to become less susceptible to wetting and drying and can delay seed deterioration until harvest.

Hybrid vigour is a component of heterosis. Hybrid seed has superiority often greater under stress conditions than under optimal conditions. Hybrid corn (*Zea mays*) and barley seeds, for example germinate more rapidly and grow more rapidly than their inbred parents (McDaniel, 1969), which is attributed to improved efficiency of mitochondria and the carbon assimilation enzyme system (McDaniel & Sarkissian, 1968).

Susceptibility to mechanical damage has also been shown to be under genetic control whether induced by harvesting or conditioning equipment. The study of Barriga (1961) on navy beans indicated that 41 strains possessed different tolerances to mechanical damage. Studies on snap beans also show that coloured cultivars are more resistant to mechanical damage than white seeded cultivars (Atkin, 1958; Wester, 1970). Such resistance means less nutrient leakage from seed during germination (York et al., 1977). Soybeans with a black seed coat imbibe water more slowly than unpigmented soybean seed and this resulted in less imbibition injury (Tully & Leopold, 1981).

Improving nutritional quality through breeding has also resulted in creating problems in seed physiological quality, for example high lysine corn which resulted in seed quality problems. This process often gives rise to shrunken, small and low vigour seed. Plant breeders are now trying to find a gene(s) that control nutritional quality but do not affect seed vigour (Copeland & McDonald, 2001).

1.8.2 Mother plant nutrition

Seed physiological quality may be affected by conditions prevailing during seed development and hence the crop establishment in the next growing season. Padrit et al. (1996) listed “mother plant nutrition” as one of the factors affecting seed vigour and indicated that elemental deficiencies and nutritional imbalances can affect the cell wall integrity and ultimately seed vigour.

Generally, stresses caused by mineral deficiency during seed development are indirect, resulting from their direct effects on plant development and subsequently on seed quality (Powell, 1988; Welch, 1995). Welch (1995) reported that cell wall damage is the fundamental cause of seed deterioration at physiological maturity. Amjad et al. (2004) stated that electrical conductivity of seed leachates in pea decreased (low leachates indicate high seed vigour) with an increasing rate of phosphorous applied to the mother plant. Shukla et al. (1993) also reported that phosphorous application to the pea crop increased the protein contents of seed and thereby, enhanced the seed vigour. Saraswathy and Dharmalingam (1992) also concluded that seed vigour index and protein contents of *Brassica juncea* seed was significantly increased when high concentrations of N and K were applied to the maternal plant. A positive interaction of potassium with nitrogen and other nutrients also affects seed quality because it promotes synthesis of photosynthates and their transportation to fruits, grain and storage organs to enhance their conversion into starch, protein, vitamins and oil (Usherwood, 1985). Nutrient deficiency may affect seed filling rates and the seed hormone concentration necessary for the germination process and vigour (Gray & Thomas, 1982). Thus, nutrient deficiencies can indirectly affect seed vigour and viability through their effects on seed constituents. No research investigating brassica seed quality in relation to mother plant nutrition has been published.

1.8.3 Seed/pod position on plant

Seed weight and seed quality in relation to pod position may be influenced by temperature. Modi and Asanzi (2008) reported that a low production temperature (20/10 °C day/night) gave heavier seed of maize while high temperature (30/20 °C day/night) produced lighter seeds. Gbikpi and Crookston (1981) measured greater rates of dry matter accumulation in seeds from pods located in the upper canopy of indeterminate soybean cultivars compared with pods located in the lower position of the canopy. Keigley and Mullen (1986) concluded that when soybean plants were exposed to high temperature throughout seed development both germination and vigour declined by 28% and 38% respectively. They also found that the seed from the middle stem region of soybean produced heavier seedlings compared with seeds from the other regions of the stem. Hampton (1991) indicated that pod position can affect seed vigour in large-seeded legumes. Studies on soybeans showed seed from the top pod position gave higher germination and vigour than those from a lower pod position (Adam et al., 1989; McDonald et al., 1983), but Rahman (2002) found that soybean seeds from top pods had inferior vigour compared to seed from middle and bottom pods. Komba (2003) demonstrated that brassica seeds from pods at the middle position of the raceme had higher vigour than those from top pods. Khalil et al. (2010) also concluded that seed harvested from the middle location of the canopy gave better germination than seeds from the top position, but these results contradict with the results of McDonald et al. (1983) who reported that top position seeds had a higher germination compared

with the seed produced at the bottom position. Interestingly, Ghassemi-Golezani et al. (2010) indicated that seeds of Chickpea *(Cicer arietinum* L.) harvested from the lower position had a higher seed quality (vigour) compared with middle and upper plant seed positions and attributed this to early formation and a longer duration of pod filling lower in the canopy.

1.8.4 Seed mass/size

Generally, seed size is expressed as the term "thousand seed weight", within the industry. However, seed size and seed mass are different terms. Seed size refers to volume of seed and the term seed weight and seed mass refers to density (Castro et al., 2006). Seed size plays an important role in plant life. It partly determines the number of seeds that can be produced and also may affect germination, vigour and seedling performance (Basra, 2006). Seed size is one of the factors which can cause differences in seed vigour (Hampton & TeKrony, 1995) and is influenced by stage of seed development, environmental conditions and genetic constitution (Copeland & McDonald, 2001).

A relationship exists between seed size/weight, germination and vigour in many species (Austin, 1972). However, the relationship is a complex one depending upon the crop species and can be applied only for different seed sizes within a seed lot. Leeks (2006) found that within a seed lot, large size seed of brassica had lower conductivity readings and hence higher vigour than small and medium seeds. However, Komba (2003) found that medium seeds (retained on 2.00 mm screen) and small seeds (retained on 1.5 mm screen) had higher vigour than very small and large seeds of kale.

Large sized seeds have more reserves and metabolic activity, and in theory should have better germination and vigour than small sized seeds (Halmer & Bewley, 1984). However, as noted by Komba (2003), large seeds are possibly more susceptible to mechanical damage than smaller seeds, and if this damage results in loss of membrane integrity, seed vigour may be reduced. The relationship between seed size and seed vigour is not established, and reports are conflicting. Copeland and McDonald (2001) explained that large seeds produce, generally, larger seedlings with more competitive advantage over small ones. Farahani et al. (2011) observed that wheat seed size had a significant effect on germination and vigour as large seeds had higher germination and vigour.

On the contrary, however, studies on soybean seed quality by Vyas et al. (1990) showed that larger seeds of soybean had a lower seed germination and vigour than small seeds after storage in ambient temperature for 180 days. They suggested that small seeds had a thicker seed coat that may retard imbibition and low gas exchange to slow the embryo growth rate (Basra, 2006; Susko & Lovett-Doust, 2000).

Dubey et al. (1989) also stated that larger seeds of mustard had a greater germination and field stand as compared with smaller ones. Lang and Holmes (1964) observed that seedling emergence was greatly

affected by seed size in swede and found that medium seed (1.2-1.77 mm) had a greater emergence than large (> 1.7 mm) and small (< 1.2 mm) seeds, a result that supported that of Komba (2003).

Seed weight/mass expresses the amount of food storage in the seed and it is an important factor for both germination and vigour (Padrit et al., 1996; Taweekul, 1999). Increasing temperature during reproductive growth also affects seed mass. Spears et al. (1997) and Hampton et al. (2013) reported that with increasing temperature, seed mass may reduce because of an acceleration in seed growth and a reduced seed filling period duration (Young et al., 2004). However, Peltonen-Sainio et al. (2011) reported no increase in seed mass in response to elevated temperature. Reduction in seed mass does not necessarily means loss or decrease in other seed quality attributes like germination and vigour (Castro et al., 2006).

1.8.5 Seed deterioration/aging

The physiological changes in seed that lead to loss of viability are termed seed deterioration. Deterioration of seed has been defined as "an irreversible degenerative change in the quality of a seed after it has reached its maximum quality level" (Abdul-Baki & Anderson, 1972). It is an inexorable, irreversible process that progressively impairs the capabilities and performance of the seed and culminates in its death or in practical terms loss of the germinative capacity (Delouche, 1973).

Germination and vigour are at a maximum when seed attains is highest dry weight, at physiological maturity (Basra, 2006; TeKrony & Egli, 1997). While this is correct for germination, it may not be so for vigour (Shinohara et al., 2006b). Seed is seldom harvested at this point due to high moisture content and remains on the mother plant from a few days to weeks before it reaches harvest maturity (TeKrony, 1977) and for months and possibly years in storage during which seed will gradually deteriorate and eventually die (Gregg et al., 1994).

Seed vigour is the first quality component lost as seed deteriorates, followed by germination (Trawatha et al., 1995). The most important factors associated with seed deterioration are reported as loss of cell wall integrity and enzyme degradation (Priestley, 1986), genetic degradation (Sen & Osborne, 1977) and reduced respiration (Ferguson, 1990) before the death of seed (Basra, 1995). Wilson and McDonald (1986) attributed vigour loss in deteriorating seed to a reduction in superoxide dismutase (SOD) activity in seed during the first few hours of imbibition. Basra (2006) concluded that seed deterioration of cotton seed was due to both lipid peroxidation and the activity of free fatty acid which causes disruption of the membranes.

1.9 Seed physiological deterioration at the cellular level, enzyme inactivation and membrane degradation

1.9.1 Mechanism of seed deterioration

Seed deterioration, once it begins, is an irreversible process (McDonald, 1999). It is a sequence of progressive events starting with a chain of biochemical events, especially membrane degradation and biosynthetic activities, leading to the loss of various seed quality attributes which are indicated by an increased number of abnormal seedlings and finally seed mortality (Walters et al., 2010).The biochemical and structural changes are interrelated. Oxidative stress causes structural changes through degradation of membranes and proteins, damage to DNA and RNA, modification of DNA folding and increased fragility of the cell matrix (Chen et al., 2012; Chen et al., 2013; Dimitrov, 1994; Halliwell & Gutteridge, 2015; Lee et al., 2010; McDonald, 1999; Sen & Osborne, 1977), reduced respiration (Ferguson, 1990; Grass & Burris, 1995b; Priestley, 1986), loss of membrane integrity and degradation and inactivation of enzymes (Bailly, 2004; Demirkaya, 2013; Kibinza et al., 2006; Lee et al., 2010). Some of the physiological and biochemical mechanisms of seeds deterioration are discussed below:

1.9.2 Cellular membrane functions and degradation

Loss of membrane integrity and their functions has been cited as the most likely cause of seed deterioration (McDonald, 1999). Loss of membrane integrity leads to increased leakage of amino acids, sugars and ions so that the cellular membranes become unable to maintain their osmotic turgor due to loss of barrier properties. Any stress during seed deterioration can alter lipid bilayer fluidity and integrity of the cellular membrane, affecting transport of ions between cellular compartments (Coolbear, 1995; Copeland, 1995; Parrish et al., 1982).

Membrane damage is now known to be not the only cause of seed deterioration, but is an early indicator. However, increased leakage which is the symptoms of seed deterioration, should be treated very cautiously because the increased leakage from seed might either be due to cell damage by mechanical means, increased concentrations of some specific solutes or due to loss of membrane integrity (Coolbear, 1995).Considering this argument, Powell and Matthews (1977) demonstrated that higher electrolyte leakage occurred before the loss of pea seed viability as determined by the tetrazolium test and associated higher electrolyte leakage with areas of dead or deteriorated tissues in the cotyledons of pea seeds (Powell, 1985). Nilsen et al. (1996) reported that the environmental stress increased seed cell membrane damage and increased its leakiness. Powell (2006) related low vigour and production of abnormal seedlings with seeds with higher electrolyte leakage due to seed membrane damage. Similarly, Shinohara et al. (2006b) demonstrated that higher pea seed leachates were associated with deteriorated seed tissues on the adaxial regions of the cotyledons.

Disorganization of cell membranes has also been reported to reduce mitochondrial functions which may allow the release of peroxidative enzymes which cause further damage to cellular membranes (McDonald, 1999).

1.9.3 Lipid peroxidation and ROS generation during seed deterioration

Reactive oxygen species (ROS) are known to be produced during seed development, and have a dual role, being either cytotoxic or as a signal transduction mediator in various metabolic pathways which regulate seed development, germination and response to various biotic and abiotic stresses (Apel & Hirt, 2004; Bailly et al., 2008).The important factor for seed deterioration is the continuous production of ROS during aerobic metabolism as a by-product and/or as a consequence of any abiotic stress such as heat stress (Chen et al., 2012; Lee et al., 2010). ROS include both free radicals i.e. superoxide radicals (O^{-}_{2}), hydroxyl radicals (OH^{-}) and molecules i.e. hydrogen peroxide (H_2O_2) and single oxygen (O_2^{-}). Over production of ROS, as a consequence of various abiotic stresses causes, oxidative stress by damaging proteins, carbohydrates, lipids and DNA strands. ROS are localized in the different compartments of the cells such as the chloroplast, mitochondria, peroxisomes (glyoxysomes in oily seeds)(Bailly, 2004; Reumann, 2000). Glyoxysomes are the major site of ROS production in oil seeds and have a role in lipid reserve mobilization by the enzymes of the β-oxidation and glyoxylate cycle which convert the lipid reserves into sugars during the early seedling development period(Huang et al., 1983; Miller et al., 2010). H_2O_2, a ROS in molecular form is generally produced during fatty acid β-oxidation by the activity of enzymes such as such as glycolate oxidase (Corpas et al., 2001). H_2O_2 can be beneficial or detrimental depending upon the level of its accumulation. H_2O_2 play an important role in the completion of germination and considered is as a messenger or transmitter of environmental signals during seed germination (Bailly et al., 2008).Though H_2O_2 is a stable molecule it can freely diffuse through membranes from its site of production to surrounding areas and can target other molecules such as lipids, proteins and nucleic acids due to its oxidizing power (Halliwell, 2006; Mittler, 2002).H_2O_2 causes breakage of DNA and reacts with thiol containing enzymes to inactivate them, especially those important for the Calvin cycle (Charles & Halliwell, 1980).

Superoxide radicals (O^{-}_{2}) and hydroxyl radicals (OH^{-}), both possess an unpaired electron. As free radicals they cause lipid peroxidation. ROS mediated lipid peroxidation is a free radical chain process leading the to the deterioration of polyunsaturated fatty acids (Bailly, 2004; Repetto et al., 2012). It is initiated by free radical attack when the hydrogen atom from the methylene group is removed to produce a lipid radical. In aerobic conditions, the carbon atom of the lipid radical stabilizes with oxygen produced by the ROS process to yield peroxyl radical (RCOO.), but the resulting chain reaction is capable of removing another hydrogen atom from another fatty acid to form lipid hydroxide. This is unstable and fragments to form shorter chain aldehydes including malondialdehyde (Figure 1.5) (Halliwell & Gutteridge, 2015; Jairam et al., 2012; Repetto et al., 2012).

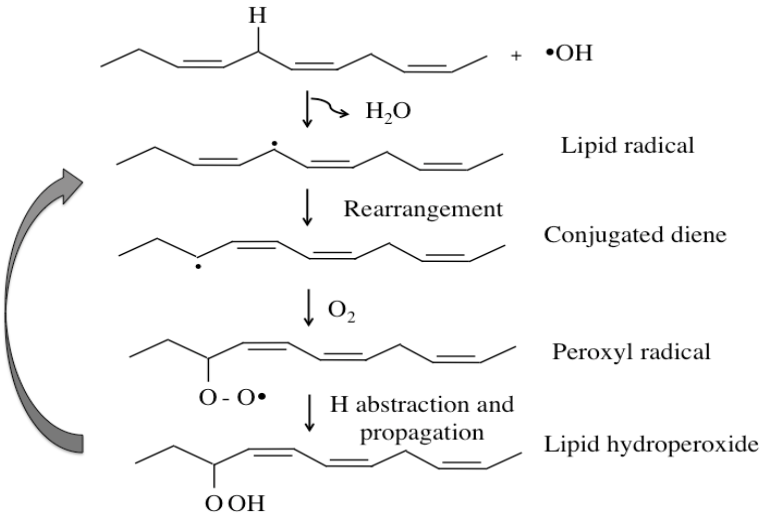


Figure 1.5. Stepwise schematic diagram of lipid peroxidation process (Jairam et al., 2012).

Lipid peroxidation is the most important event which occurs following oxidative stress, and is likely to degrade the membranes of those tissues which are rich in polyunsaturated fatty acids (PUFA) (Dix & Aikens, 1993). High oil content seeds are more prone to lipid peroxidation due to their high percentage of PUFAs such as linoleic and linolenic acid. These PUFAs are very susceptible to attack by ROS, particularly by O_2^- and $OH^•$, and yield mixtures of lipid hydro peroxides. Increased peroxidation reduces membrane fluidity and increased leakage (Møller et al., 2007). McDonald (1999) proposed lipid peroxidation as a primary cause of seed deterioration.

1.9.4 Role of antioxidant enzymes during seed deterioration

Any abiotic stress, especially heat stress, results in an increase of ROS induced oxidative stress, from optimum to the level where it disrupts the redox cellular homeostasis (Azooz et al., 2011; Mittler, 2002). ROS such as $^1O_2$, H_2O_2, $O^•_2$ and $^•OH$ can damage surrounding biomolecules (Snider et al., 2010). These ROS in excess, are very deleterious for cellular metabolism due to their highly reactive properties (Ahmad et al., 2010). A already noted, H_2O_2 can freely diffuse through membranes from the site of production to the surrounding areas, while others cannot cross the biological membrane (Bailly, 2004; Bailly et al., 2008). To counteract the toxic effects of ROS, cells are equipped with efficient enzymatic and non-enzymatic antioxidant defence systems to protect them from oxidative stress. This defence system includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and low molecular weight non-enzymatic antioxidants such as ascorbic acid (AsA) tocopherol, glutathione and phenolic compounds (Ashraf, 2009; Caverzan et al., 2016; Jaleel et al., 2008).Each subcellular component normally contains more than one enzymatic defence system for the efficient removal of ROS at their generation site. For example, peroxisomes contain at least two

enzymatic activities: CAT and SOD to quench or detoxify H_2O_2 and superoxide radicals (O^{-2}) respectively (Gill et al., 2015; Gill & Tuteja, 2010)

SOD is a key and very effective intracellular metalloenzyme in aerobic cells. SOD removes the superoxide radical (O^{-2}) by converting it into H_2O_2 by dismutation, which decreases the risk of hydroxyl radical (OH•) production (a very oxidizing and deleterious compound), through the Haber-Weiss reaction (Bowler et al., 1992; Gill et al., 2015; Gill & Tuteja, 2010). Other enzymatic activity in the cellular compartment e.g. either APX, CAT and GR etc. then eliminate or detoxify H_2O_2 by reducing it into water and oxygen through the ascorbate-glutathione cycle in chloroplasts, cytosol mitochondria and peroxisomes (Mittler, 2002; Paula et al., 1996).

The antioxidant system is therefore a natural defence against abiotic stress, and has a role in in any living system to detoxify excessive ROS generated during oxygen reduction in the electron transport respiratory pathway (Bailly, 2004). Failure to detoxify ROS during any abiotic stress (such as heat, cold, drought, salinity and light) can result in damage to cellular membranes and macromolecules (Apel & Hirt, 2004). Heat stress can decrease the activities of ROS scavenging enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase and other antioxidant enzymes (Chaitanya et al., 2002)

1.9.5 Ultrastructure changes during seed deterioration

The mechanism of seed deterioration is complex as it involves biochemical, physiological, molecular and structural changes (McDonald, 1999). Various cellular alterations have been associated with seed deterioration following seed storage. For example, seeds of rye (*Secale cereale*) expressed damaged organelles and leakage of contents from organelles into cytoplasm (Hallam et al., 1973).

Mitochondria are the most sensitive organelles during seed deterioration. Mitochondria have been identified as a primary target during seed deterioration in several ultrastructural studies, as reviewed by Smith and Berjak (1995). Abdul-Baki and Baker (1973) demonstrated that mitochondria in aged seeds were damaged and swollen. Morphological changes in mitochondria and the resulting mitochondrial dysfunction induce metabolic changes due to slow or restricted ATP production (Daum et al., 2013; Yin et al., 2009). Li and Xing (2010) reported that cell ageing is associated with significant alterations in mitochondrial morphology.

Wang et al. (2012) reported ultrastructural changes in cellular organelles and structurers during the pre-harvest seed deterioration of soybean seeds and found a thinner cell wall, an asymmetrical nucleus, vacuolization, bigger lipid bodies and smaller protein bodies and chloroplast.

Kong et al. (2014) found serious cellular alteration of oat embryo cells in transmission electron micrograph images, including disintegration of plasma membranes and inflated mitochondria of seeds heat stressed at 22% SMC. Cellular membranes also play an important role in metabolic activities by

regulating cellular material transportation and exchange within the cell. Priestley (1986) in ultrastructural studies, found swollen cellular membranes during seed deterioration

Wang et al. (2015) confirmed in a transmission electron microscopic study after controlled deterioration of *Ulmus pumila* seeds that cotyledonary mitochondria swelled as an early sign of cellular alteration and suggested that changes in mitochondrial structure may be a precursor of the seed deterioration process. Yan et al. (2015) found serious damage to embryo cells in ultrastructural studies of aged *Elymus sibiricus* seeds and reported shrunken cells, an injured nucleus, and abnormal and irregular cell membranes.

1.10 Factors affecting seed deterioration

Environmental factors experienced by the mother plant are recognized as major determinants of seed quality. Ideal field conditions such as a good soil, adequate solar radiation and rainfall, an optimum temperature and low humidity during maturation and harvest have been determined for selection of specialized seed production areas (Delouche, 1980). However seed can not always be produced in ideal areas. Environmental factors may influence seed size, seed composition and seed quality. Both germination and vigour can be reduced by unfavourable environmental conditions (temperature, rainfall, relative humidity) during seed growth and development (Dornbos, 1995).

1.10.1 Environment during reproductive growth (pre-harvest)

Environmental conditions during reproductive growth may affect the production and the quality of seed.Adverse climatic conditions reduce seed quality. For example environmental stresses, particularly high temperature before and after seed physiological maturity(PM), negatively affect seed physiological quality (vigour) in soybean (Egli et al., 2005). High temperature stress is one of the most important but less studied abiotic stresses affecting plant productivity. There have been only few reports on the effects of high temperature stresses in brassica species. Gusta et al. (2004) reported that canola seed quality and vigour were reduced by high temperature during seed embryogenesis and seed maturation. Polowick and Sawhney (1988) observed that when canola was grown under high temperature conditions (32° C/26° C; day/night) male and female sterility was induced. Angadi et al. (2000) studied high temperature stress effects on *Brassica juncea*, *B. napus* and *B. rapa* and concluded that all the species were adversely effected by heat stress of 35/15 ˚C at early flowering because they produced abnormal pods and seeds.

(i) Before physiological maturity (PM)

Environmental conditions, particularly high temperature before PM may cause seed deterioration. High temperature during seed fill led to the production of small and poor quality seeds in *Phaseolus vulgaris* (Abdus Siddique & Goodwin, 1980). Shinohara et al. (2006a) in a field study on garden pea

found that high temperature before physiological maturity significantly affected seed quality when seed moisture content was 70-80% by impairing cell membrane integrity, possibly by reducing assimilate transport into seeds. In a follow up study, under control environmental conditions when pea plants were exposed to 30 ˚C day/25 ˚C night for four days (240 ˚Ch, Tb= 25˚ C), they demonstrated that high temperature both before physiological maturity (PM) when SMC was 70-80% and at PM (55-65% SMC) significantly increased conductivity in peas, but did not do so when the stress was applied after PM (Shinohara et al., 2006b). Shinohara et al. (2008) also examined the relationship between vigour test results for 262 garden pea seed lots produced in New Zealand and the climate data of five regions over four consecutive production seasons and concluded that variation over season and regions occurs for vigour and these variations were significantly associated with temperature during seed development. They proposed that regions of production may be selected for producing seed lots with high vigour by avoiding temperature stress. Seed vigour has previously been generally considered to be at maximum at physiological maturity (Hampton & TeKrony, 1995). However, Dornbos (1995) reported that seed quality may be significantly reduced by the environment before PM, a result confirmed by Shinohara et al. (2006b). Pieta Filho and Ellis (1991) concluded that seed quality in spring barley was reduced by a wide range of environmental factors both before and after PM. High temperature during soybean seed filling disturbed normal seed development and resulted in abnormal, shrivelled and low quality seed (Spears et al., 1997). Generally, the mean germination of seeds decreased as the mean maximum temperature increased during seed filling (Egli et al., 2005; Hampton & TeKrony, 1995; Khalil et al., 2001; Khalil et al., 2010; Thomas et al., 2009). High temperature before physiological maturity (PM) probably impairs assimilate supply to synthesise the storage compounds necessary for the later germination process (Dornbos & McDonald, 1986), and seeds can suffer physiological deterioration to the extent that they are unable to germinate (Coolbear, 1995; Powell, 2006).

(ii) After physiological maturity

High temperature after PM can reduce germination sometimes but more often reduces vigour (Green et al., 1965). Weathering, temperature and humidity are considered to be the major factors influencing seed aging during this phase (Matthews et al., 1980).In areas of high rainfall or humidity weathering is a serious problem, which when coupled with high temperature, results in the rapid loss of seed vigour (Delouche, 1980). Green et al. (1965) and TeKrony et al. (1980) reported that warm and humid weather which delayed the time for desiccation to harvest maturity also caused seed deterioration or vigour loss through the continuous respiration and consumption of metabolites i.e. sugars. In addition, warm and humid weather also favours pathogen attack which may contribute to seed deterioration through physical damage.

During the desiccation period, Green et al. (1965) found that soybean seed for an early sowing had lower laboratory germination and field emergence when the crop matured during hot dry conditions, than for a late sowing which reached harvest maturity after the hot dry conditions. Reduction in seed vigour at high temperature has also been recorded in controlled environment studies (Egli et al., 2005; Zanakis et al., 1994). High temperature causes disruption of seed cell membranes (Coolbear, 1995), which is usually the first indicator of seed deterioration (Powell, 1985).

1.10.2 Environmental factors (at harvest)

Seed physiological maturity is achieved at a SMC of 40-60%, depending on species, after which seeds continue to lose moisture until they reach harvest maturity (Hampton, 1991). Harvest time and harvest method can have a strong effect on seed germination and vigour. Gray (1987) stated that some vegetable seeds are combine harvested at PM to reduce the yield losses through seed shedding but these can result in seed deterioration following the mechanical impaction due to physical damage to cell contents (Hampton, 1991). Bruggink et al. (1991) indicated electrolyte leakage increased during early imbibition following mechanical damage to seed at harvest.

According to McDonald (1999), two types of seed deterioration exist: deterioration of seeds on the mother plant prior to harvest and processing (short-term deterioration) and seed deterioration during storage (long term deterioration). Coolbear (1995), noted that the consequences of this short term deterioration is quite similar to the deterioration of seed which occurs during storage at high seed moisture contents.

1.10.3 Environmental factors (post-harvest)

Copeland and McDonald (2001) stated that temperature and relative humidity (which increase seed moisture content) are highly interdependent factors during storage of seed and influence the life span of seed. The seed is usually dried to a moisture content of 8-14% (varies with species) after harvest for safe storage. The post-harvest process of drying may also affect seed vigour. During drying, heated air is often used and this may increase deterioration or even kill seeds, if used by inexperienced operators (Hampton et al., 1992a). Hill (1999) indicated that high temperature during artificial drying of moist seed is harmful and injurious. Gray et al. (1985) reported that artificial drying at 28 ˚C with 60 % R.H result in the reduction of seed viability and vigour if the moisture loss was 10% per day. High seed moisture contents also hasten seed deterioration during seed storage. At high SMC, seeds respire and produce both heat and moisture which creates favourable conditions for growth and multiplication of insects and storage fungi (Hill, 1999). Coolbear (1995) observed that this fungal invasion results in the production of extracellular hydrolytic enzymes which damage cell membranes and hence increase the leakage of ions from seed (Delouche, 1963).

Seed production practices such as cleaning, harvesting and handling can all result in mechanical damage. As seed size increases and moisture content decreases, the seeds become more susceptible to mechanical damage (Elias & Basra, 2006). Copeland and McDonald (2001) stated that small mechanical damaged areas cause few problems initially, but later they may cause deterioration of vital embryonic tissues and hence reduce seed quality.

1.11 Mechanism of heat stress during seed deterioration

Several hypotheses have been proposed for the mechanism by which heat stress affects seed deterioration. Deteriorative changes in seeds occurs when they are exposed to adverse environmental conditions, and the deterioration rate is enhanced with the increase in either seed moisture content or storage temperature resulting in loss of seed quality (Ellis et al., 1985; Kapoor et al., 2010). High temperature during seed development can reduce seed vigour without affecting seed germination (Hampton et al., 2013). High temperature causes several morphological, anatomical, and physiological and biochemical changes. Heat stress can cause either direct or indirect damage to cells. Direct injury involves degradation and denaturation of both lipids and protein which results in membrane damage by increasing fluidity. Indirect damage includes inactivation of enzymes in membranes, mitochondria and chloroplasts, impaired protein synthesis and disintegration and loss of membrane integrity (Howarth, 2005; Wahid et al., 2007).

Seed deterioration following heat stress has been reported in many crops including garden pea (Shinohara et al., 2006a), soybean (Ren et al., 2009; Wang et al., 2012), sunflower (Bailly et al., 2003), lentil (Bhandari et al., 2016), brassica (Angadi et al., 2000; Brunel-Muguet et al., 2015; Morrison, 1993; Young et al., 2004), wheat (Farooq et al., 2011) and mung bean (Kumar et al., 2011).

Several studies have demonstrated that environmental stress during reproductive development and seed maturation significantly affects membrane integrity by altering its chemical composition (Basavarajappa et al., 1991). Dornbos et al. (1989) in a study on soybean seeds concluded that high temperature stress during seed filling significantly altered fatty acid composition of the membranes and phospholipids, and proposed this as one of the mechanisms of seed deterioration.

Other hypotheses regarding effects of heat stress on seed quality involve alterations in seed metabolism. Grass and Burris (1995a) reported a decline in seed vigour due to heat stress. This reduction in seed vigour due to heat stress was associated with mitochondrial degeneration and impaired ATP production which reduced energy levels and respiratory activity (Grass & Burris, 1995b).

Shinohara et al. (2006a;b) in field and controlled environment experiments with garden peas demonstrated that temperature stress during the seed filling stage (SMC ≈70-80%) significantly

reduced seed vigour and associated this seed quality deterioration with a starch deficiency of the cells in the adaxial region of the cotyledons.

Wang et al. (2012) conducted a proteomic analysis of the pre-harvest seed deterioration of soybean under high temperature and humidity stress and found 42 protein spots differentially expressed to match 31 different proteins involved in 13 cellular responses and metabolic processes. They proposed a pre-harvest seed deterioration mechanism (Figure 1.6), which suggests that heat related damage to proteins and altered metabolism pathways (resulting from increased chloroplast damage, a lowered photosynthetic rate, an increased tricarboxylic acid rate, reduced protein synthesis and reduced nitrogen assimilation) lead to a shortage of storage compounds, coupled with membrane damage, which all resulted in reduced resistance to seed deterioration.

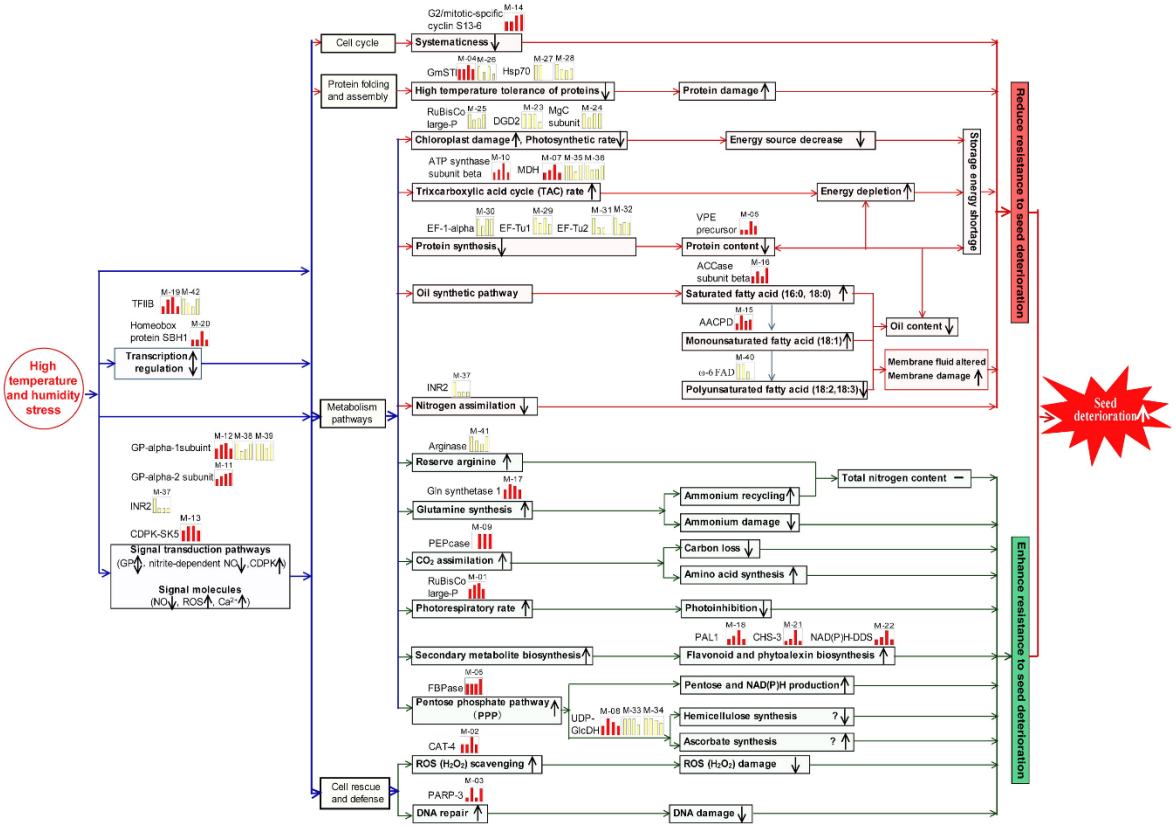


Figure 1.6 Mechanism of heat stress seed deterioration of developing soybean. Up-regulated processes and pathways by Wang et al. (2012).
Up-regulated processes and pathways and substances are marked by "↑", and down-regulated processes and pathways were indicated by "↓", those changed marked by "↕".those unaffected by "–". The processes and pathways, which needs to be further investigated are marked by "?".

1.12 Conclusions

High quality seed is necessary for successful crop establishment. However, adverse climatic conditions, especially during seed development, can negatively affect seed quality. Forage rape seed production is mainly centred in the Canterbury region of New Zealand, where, while harvest conditions are usually dry, temperature can exceed 30 ˚C due to the föhn effect of northwest winds (Hampton, 2004). Additionally, the expected rise in temperature due to global climate change may create further problems for seed quality (Hampton et al., 2013).

New Zealand forage brassica seed lots generally have a high germination but may differ in seed vigour. Previous data suggest that seed vigour is highly variable among seasons (Leeks, 2006). However, why some seed lots have low vigour is not known. However, it was hypothesised that adverse climatic conditions during seed development and maturation would explain this variability.

High temperature during seed development is known to reduced seed vigour in some species, but whether high temperature stress during forage rape seed development causes seed vigour loss is not known. Little information is available on the effects of temperature stress during reproductive development on brassica seed vigour, or of the effect of seed position on the raceme on seed quality.

Most of the research studies reported to date have been on large seeded crops, particularly soybean, to determine effects of temperature on seed vigour. Brassica species are small seeded crops and seed size and weight are influenced by the stage of development, environmental factors during seed development and genetic constitution (Copeland & McDonald, 2001).

Research on the direct effects of high temperature stress on brassica species is limited. Hence there is an urgent need for evaluating high temperature stress effects on brassica species during seed development to determine the effect on seed quality. The hypotheses of the current study are that high temperature stress during seed development, and the position of the seed on the raceme, will negatively affect seed vigour.

Chapter 2
Effect of sowing date on brassica seed quality

2.1 Introduction

Forage brassica crops are widely grown in New Zealand pastoral systems as supplementary forages and an alternative to pastures. They are traditionally used to supply forage during the winters, when poor pasture growth does not meet the grazing animals' demand. Forage brassicas are frost tolerant and have the potential to produce high yields of forage from early summer to late winter (De Ruiter et al., 2009). Forage brassica seed is produced mostly in Canterbury (Hampton et al., 2012).

High quality seed is a key component for achieving an adequate plant population in a range of field conditions (Egli et al., 2005). Seeds attain physiological quality gradually during seed development, and this quality can be affected by both biotic and abiotic factors during this phase. Seeds accumulate and store protein, lipid and carbohydrates during their development and maturation and require an adequate and continuous supply of assimilates for synthesis of these storage compounds to acquire germination characteristics (Dornbos & McDonald, 1986). The climate events prevailing during seed development can affect seed quality (Coolbear, 1995; Dornbos, 1995), with adverse climatic conditions (temperature, rainfall and relative humidity) in the field reducing seed germination and vigour (Feaster, 1949; Keigley & Mullen, 1986). Some researchers have suggested that weather at the end of the growing season can also negatively influence seed quality (Cheng et al., 2014; Fenner, 1991; Gutterman, 2000). Seed yield potential in Brassica crops depends on the events occurring prior to and during the flowering stage (Mendham & Salisbury, 1995), while the reproductive stage is the most susceptible stage for temperature stress in most crops in which a temperature response has been studied (Hall, 1992).

Seed reaches its maximum seed quality, including seed vigour, prior to, at or just after physiological maturity (when seeds acquire their maximum seed mass/dry weight) (Samarah et al., 2004). Once full physiological maturity has been attained, the association between mother plant and seed becomes disrupted, and movement of all nutrients and water essentially stops. From this point until harvest, seeds are also susceptible to environmental stress, and continuous exposure to adverse environmental conditions can lead to a rapid decline in germination and vigour (Ghassemi-Golezani & Hosseinzadeh-Mahootchy, 2009; Rasyad et al., 1990; TeKrony & Egli, 1997).

Variability in environmental conditions as a function of sowing time is an important factor which can significantly affect the time and length of the vegetative and reproductive period, thereby affecting yield, its components, and seed quality (Bhuiyan et al., 2008). Sowing time can influence the crop's

phenological development depending on temperature and heat units accumulated during the critical stages of growth (Greven, 2000; Miralles et al., 2001; Morrison et al., 1989).The stage of plant development at which the plant is exposed to high temperature determines the severity of damage to the crop. High day temperatures alter the leaf exchange properties, cause closure of stomata and reduction in cell water contents, a decrease in dry matter and reduced relative growth due to the reduction in net assimilation rate (Ashraf & Hafeez, 2004; Wahid & Close, 2007; Young et al., 2004). Heat stress during the seed filling phase lead to a reduction in density and weight of wheat seeds and a reduction in starch, protein and oil content in maize seeds (Guilioni et al., 2003; Wilhelm et al., 1999). Increasing temperature causes a reduction in the number of seeds per ear and seed weight in wheat and open bud and flower bud abortion increases following short episodes of high temperature (Ferris et al., 1998; Gulioni et al., 1997). These results suggest that sowing time/date or its manipulation is an important factor to minimize high temperature effects on plant growth.

Seed production and quality are significantly affected by sowing time (Yadav & Dhankhar, 2001). The temperature optimum is one which allows maximum benefit from favourable environmental conditions during the growing season, particularly during the reproductive process. Early sowing usually allows increased vegetative and reproductive growth, and an increased number of reproductive units per plant, which consequently increases seed yield but not always seed quality. Delays in sowing can cause a rapid decline in seed quality depending on the environment (Thakur & Singh, 1998). Seed development under warm, wet conditions can reduce germination and vigour (Kmetz et al., 1978; TeKrony et al., 1987). Yield potential of *Brassica napus* is proportional to the length of the vegetative and stem elongation periods. Delayed sowing of brassica species results in delayed growth and development under a reduced photoperiod (Nanda et al., 1996) and often higher temperature during flowering and pod formation which consequently lowers seed yield and physiological quality (Richards & Thurling, 1978). Yield losses were also found through water stress during flowering and seed filling due to late sowing. However, most of the New Zealand grown forage rape for seed production is irrigated.

Thermal time accumulation or calendar days are specifc to any location and results of one site may not be used for other locations and years. Various models have been suggested to explain the phenological development of a plant in order to forecast crop development (Morrison & Stewart, 2002). Manipulation of environmental conditions during seed development and maturation can be achieved by modifying the sowing time for a specifc location and cultivar (Scarisbrick & Daniels, 1981). Therefore, determination of optimum sowing time for a specific cultivar is necessary to harvest good quality seed.

Conflicting reports have been published in the literature on the impact of sowing time on growth and yield of oilseed rape. Some indicate that delayed sowing increased yield in Great Britain (Jenkins & Leitch, 1986; Leach et al., 1999), whereas, in Australia higher yield was observed with early sowing regardless of site and cultivar (Walton et al., 1999).These different response to sowing time can be attributed to the use of different cultivars, and to the different environments; more variability is observed over the years in a maritime climate than in a continental climate (Mendham et al., 1981; Van der Molen et al., 2006).

Most of the New Zealand production guidelines for forage rape are derived from overseas data for European and Australian oilseed rape. Little information is available on forage rape production practices in New Zealand, particularly for seed production. Oilseed rape is cultivated for production of edible oil but forage rape is bred for its vegetative dry matter production which may possibly compromise seed production (Chakwizira et al., 2010). However, variable responses in forage rape crop establishment have been observed due to different climatic conditions and soil types. Chakwizira et al. (2010) reported a forage rape field trial in Canterbury, New Zealand which had four sowing dates (February, March, April and May).They found that delaying autumn sowing reduced dry matter and seed yield and that the May sowing had poor emergence because of cold and wet conditions leading to lower plant establishment and therefore population. The effect of sowing date on forage rape seed quality in New Zealand conditions has not yet been reported.

For forage rape therefore, crop management practices i.e. seed bed preparation, soil fertility, sowing time and rate are not well defined for New Zealand conditions and little is known of the effects of sowing time on forage rape seed quality in the New Zealand environment. So there is scope to study the effect of sowing date on forage rape seed quality, and particularly seed vigour, in New Zealand conditions.

Canterbury is the main centre of New Zealand forage brassica seed production. Short periods of high temperature are very common in Canterbury because of the föhn effect of northwest winds, (Hampton, 2004). These occur during reproductive development and seed maturation of forage brassica from September to early January (e.g. Tables 2.1-2.3). It is possible that in these conditions, brassica seeds can suffer physiological deterioration at a rate determined by both environmental and management factors, as the plant has multi-age reproductive structures that flower and set seeds over an extended period of time.

Table 2.1 Mean daily temperature (˚C)* during the reproductive growth of forage rape at Lincoln, Canterbury, New Zealand over seven growing seasons

Month	September	October	November	December	January
Season/GS†	GS 50-60	GS 61-65	GS 67-75	GS 75-88	GS 88-89
2004-5	15.2	15.9	19.4	17.2	21.7
2005-6	14.4	15.6	19	21.4	23.1
2006-7	16.8	16.5	18.4	17.4	20.2
2007-8	14.4	17.1	18.3	20.3	22.9
2008-9	15.5	17.3	19.6	19.8	24.5
2009-10	15.4	14.3	18.6	20	20.8
2010-11	16.2	16.4	19.7	22.1	21.5

***** Recorded from NIWA, Lincoln, Broadfield EWS station. †GS = Growth stage
GS 0-11 = Germination; 12-20 = Leaf development; 20-29 = Side shoot formation; 30-49 = Stem elongation (rosette stage); 50-59 = Stem elongation, flower buds present; 60-69 = Flowering; 70-79 = Seed development; 80-100 = Ripening (see Appendix-1 for complete detail).

Table 2.2 Mean daily maximum temperature (˚C)* during the reproductive growth of forage rape at Lincoln, Canterbury, New Zealand over seven growing seasons.

Month	September	October	November	December	January
Season/GS†	GS 50-60	GS 61-65	GS 67-75	GS 75-88	GS 88-89
2004-5	22.9	27.0	26.2	26.0	32.0
2005-6	22.0	25.1	30.0	29.3	29.9
2006-7	21.7	25.9	25.9	28.6	28.9
2007-8	21.9	24.3	28.4	30.9	32.7
2008-9	24.5	26.8	29.5	28.4	35.0
2009-10	23.0	22.1	29.3	27.7	31.2
2010-11	22.0	23.4	29.2	31.4	32.6

***** Recorded from NIWA, Lincoln, Broadfield EWS station. †GS = Growth stage
GS 0-11 = Germination; 12-20 = Leaf development; 20-29 = Side shoot formation; 30-49 = Stem elongation (rosette stage); 50-59 = Stem elongation, flower buds present; 60-69 = Flowering; 70-79 = Seed development; 80-100 = Ripening (see Appendix -1 for complete detail).

Table 2.3 Mean daily minimum temperature (˚C)* during the reproductive growth of forage rape at Lincoln, Canterbury, New Zealand over seven growing seasons.

Month	September	October	November	December	January
Season/GS†	GS 50-60	GS 61-65	GS 67-75	GS 75-88	GS 88-89
2004-5	3.7	6.4	8.8	7.9	11.8
2005-6	5.3	7	7.6	12.9	11.2
2006-7	5.5	6.5	7.9	8.5	10.6
2007-8	5	5.7	8	11.4	12.5
2008-9	5.8	5.6	8.9	10.7	12.5
2009-10	4.1	5	7.3	9.4	11.4
2010-11	5.2	6.1	9.5	12.3	11.6

***** Recorded from NIWA, Lincoln, Broadfield EWS station. †GS = Growth stage
GS 0-11 = Germination; 12-20 = Leaf development; 20-29 = Side shoot formation; 30-49 = Stem elongation (rosette stage); 50-59 = Stem elongation, flower buds present; 60-69 = Flowering; 70-79 = Seed development; 80-100 = Ripening (see Appendix -1 for complete detail).

2.2 Objective

The objective of this study was to determine the impact of two autumn sowing dates on forage rape seed quality. The hypothesis was that high temperature stress during seed development would reduce forage rape seed vigour but not germination. This was tested by a time of sowing trial with two sowing dates.

2.3 Materials and methods

A forage rape field trial was conducted in the 2011-12 season. Plots of forage rape (*Brassica napus*) cv. 'Greenland', a late flowering type grown for fodder production in New Zealand were established in 2011 at AgResearch Farm Lincoln, Canterbury (43° 38' S, 172° 28' E). The treatments were two sowing dates, 25 March (early) and 13 April (late), each replicated four times in a randomized complete block design. Seeds were sown at 3 kg ha^{-1} in a plot size of 10 m x 2.7 m, maintaining a 15 cm row spacing at a depth of 2 cm using a tractor drawn precision cone-seeder drill. Nitrogen (N), in the form of Urea was applied in two split equal doses of 150 kg/ha on 21 August 2011 (early) and 5 September 2011 (late). A level of 23 kg N ha^{-1} was available in the soil prior to sowing as indicated by a soil test (Hill Laboratories, Hamilton, New Zealand). The plots were irrigated when water deficit was approximately 100 mm, estimated by using Penman evapotranspiration data from a local weather station (43°37´S, 172° 28´S). Netting was erected to cover the entire trial site to minimize seed loss from birds. Seeds produced from these sowing dates were expected to reach physiological maturity (maximum seed mass) in late December 2011 (early) and January 2012 (late). Seeds were therefore, expected to be

developing under two different sets of environmental conditions. Plant growth reproductive development was monitored and the following dates were recorded.

1. Beginning of flowering
2. Peak flowering
3. First pod appearance
4. Seed physiological maturity (P.M.)
5. Seed harvest maturity (H.M)

The time of appearance of first flower/ beginning of flowering, peak flowering and pod development were recorded weekly for each plot. The beginning of flowering was assessed as the date when 5% of the plants in a plot had one open flower, and peak flowering as when there were no further increases in flower numbers (Kirkegaard et al., 2008). First pod appearance was when 5% of plants in a plot had one pod. PM is the seed developmental stage at which the seed ceases to accumulate food reserves, attains maximum dry weight and begins maturation. After the start of pod development, 10 randomly selected pods the from middle of the raceme were hand harvested, threshed and seed moisture content and seed dry weight were measured at weekly intervals to assess these maturity stages as described in Section 2.3.2. Previous literature indicated that brassica seed attains physiological and harvest maturity at around 50-55% and 14% SMC respectively (Kimber & McGregor, 1995).

2.3.1 Weather data

2.3.1.1 General weather conditions

The weather data for the trial site throughout the experiment and 30 years average normal data from March to January (growing period of forage rape seed) were collected from the Lincoln, Broadfield EWS weather station (NIWA), 1 km from the trial site. The climate data recorded were mean daily, daily maximum and minimum temperatures, humidity and daily and monthly rainfall.

2.3.1.2 Growing degree days

To evaluate the brassica growth stages, the growth stage scale BBCH was used. Growing degree days (GDD) as accumulated heat units to determine the phenological growth stages and length of growing season have been widely used for vegetables and other crops (McMaster & Wilhelm, 1997). To measure GDD, 2011-12 season data for daily maximum (T_{max}) and minimum (T_{min}) air temperatures at a base temperature (T_{base}) of 5 ˚C were used.

$$GDD= [(T_{max} +Tmin/2)-T_{base})]$$

2.3.2 Forage brassica seed development study

A seed development study was conducted measuring seed fresh weight, dry weight and seed moisture content (SMC). Three plants from each of the March and April sown plots, were selected and tagged. The plants were selected from middle rows of plots, avoiding border rows. From the start of seed development, at five day intervals, 20 pods from the racemes on the middle section of plants were hand harvested and seeds removed immediately. Fresh weight of 100 seeds (four replicates) was recorded immediately after separation from pods. The seeds were then dried in a low constant temperature oven at 103 ± 1˚C for 17 hours to determine dry weight and seed moisture content (SMC) on a fresh weight basis according to internationally agreed methodology (ISTA, 2016) as described in Section 2.3.3.1.

2.3.3 Seed Quality tests

Twenty plants from each treatment were selected randomly (5 per plot) at the following seed development stages.

i. Physiological maturity (maximum seed mass, ≈ 50% SMC)
ii. Pre-final desiccation (≈ 25% SMC)
iii. Harvest maturity (≈ 14% SMC)

Seeds were hand harvested from the two sowing dates at the three above seed development stages to eliminate the effects of machine threshing on seed quality. All pods were removed from the plants, shelled and the seeds were mixed together to make a working sample (seed lot) for each development stage. They were then ambient air dried to between 8-10% SMC, placed in hermetically sealed zip lock bags and stored at 5 °C until quality testing was conducted.

All seed quality tests, viz. standard germination, vigour (conductivity test, accelerated aging test) and thousand seed weight (TSW) were performed according to internationally agreed methods prescribed by the International Seed Testing Association (ISTA, 2016) at the Lincoln University, Seed Research Centre laboratory.

2.3.3.1 Seed moisture content (SMC)

Seed Moisture Content (SMC) was determined by using a mechanically ventilated forced air oven (Sanyo, Model MOV-212). 5-6 g seed was taken at random from each sample and placed into an aluminium container which was covered with its lid. The container was then weighed to three decimal places and placed into the oven (with lid removed). The oven was set at 103 ± 1˚C and the container with seeds was left to dry for 17 hours (low constant temperature method; ISTA 2016). The containers were then removed from the oven and covered with their lid and then allowed to cool for 15 min in a

desiccator and re-weighed to determine the moisture loss. SMC percentage was calculated on a fresh weight basis using the following equation:

SMC (%) = (M2-M3) x 100 / (M2-M1)

Where:

M1 = moisture tin with cover pre oven
M2 = moisture tin with cover and seed pre-oven
M3 = moisture tin with cover and seed post-oven.

2.3.3.2 Thousand seed weight (TSW)

Thousand seed weight (TSW) was obtained by counting and weighing eight replicates of 100 randomly selected seeds from each plot. The average weight of the eight replicates of 100 seeds was multiplied by 10 to give 1000-seed weight. The weight was expressed to three decimal places (ISTA, 2016).

2.3.3.3 Germination test

Germination tests were carried out using the top of paper (TP) method as specified in the ISTA Rules for brassica species (ISTA, 2016). Four randomly selected replicates of 100 seeds from each seed lot were placed on a moist germination blotter and placed into a plastic sandwich box which was then covered with a lid (Figure 2.1). These sandwich boxes were placed in a germination room at 20 ±2 °C.

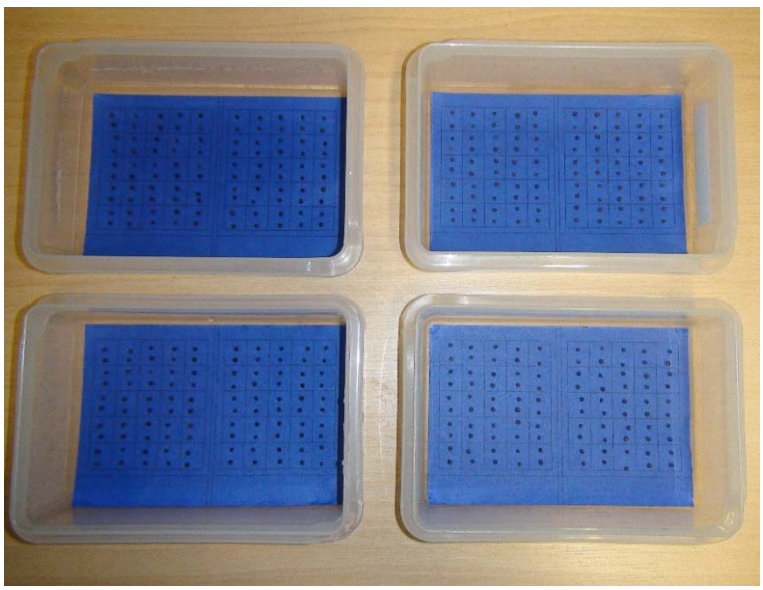


Figure 2.1 Four replicates of 100 seeds were placed on germination blotters placed into plastic sandwich boxes.

Germination was assessed by counting the number of seedlings emerged at 3, 5, 7 days (normal seedlings) and 10 days (normal and abnormal seedlings and remainder) (ISTA, 2016).During the assessment of normal and abnormal seedlings, the majority of normal seedlings were removed during the interim counts and the assessment of doubtful and abnormal seedlings was left until the end of test, to avoid the incorrect classification of slower growing but otherwise normal seedlings.

According to ISTA (2016), germinated seeds with intact seedlings having the essential structures (root and shoot systems) well developed, complete and healthy, indicating the potential for continued development when grown under favourable conditions of moisture, temperature and light were considered as normal seedlings. Abnormal seedlings were those which were damaged, deformed or unbalanced seedlings or failed to show the capacity to develop into a normal plant, under favourable conditions of moisture, temperature and light (Figure 2.2-2.4) (ISTA, 2013, 2016).

(a)Normal (b) Abnormal (c) Abnormal

Figure 2.2 (a) Normal Brassica seedlings with complete and intact primary root.
(b) and (c) Abnormal seedlings, primary root is stunted, even though secondary roots are present (Source: ISTA, Seedling Evaluation Handbook, ISTA 2013)

(a)Normal (b)Abnormal (c)Abnormal (d)Abnormal

Figure 2.3 (a) Well developed normal brassica seedling.
(b) Abnormal seedling; the seedling is deformed and cotyledons are necrotic (c) primary root is missing (d) the hypocotyl is too short.

(Source: ISTA, Seedling Evaluation Handbook, ISTA 2013)

(a) Normal (b)Abnormal (C)Abnormal (d)Abnormal

Figure 2.4 (a) Normal seedling; the primary root is visible.
(b), (c) & (d) Abnormal seedlings; the primary root is trapped in seed coat.

(Source: ISTA, Seedling Evaluation Handbook, ISTA 2013)

The un-germinated or remaining seeds were counted as either fresh un-germinated seeds or dead seeds by visual assessment or by dissecting the seed (ISTA, 2016). The variability among replicates in the germination test results was assessed using a tolerance table (ISTA, 2016). If the difference among the four replicates of 100 seeds (from lowest to highest) did not fall within the maximum range of tolerance allowed, the germination test was repeated (Appendix 2). Replicate data exceeding the tolerance indicate a problem with the test result in that the variation has exceeded that explained by random sampling variation alone (ISTA, 2016).

2.3.3.4 Vigour tests

(i) Conductivity test

A single cell electrical conductivity meter (CDM210, Radiometer, Copenhagen) was used to measure change or difference in conductivity as electrolytes leached in soaked water (Figure 2.5). Prior to the conductivity test, seed moisture content of each lot was determined by the method described in Section 2.3.3.1, as it is known that the initial SMC of seeds is source of variation in conductivity (Castillo et al., 1992). The seeds harvested at all three stages i.e. 50%, 25% and 14 % SMC were ambient air dried to 8-10% (as mentioned in Section 2.3.3) to minimize the variation between seed lots and replicates.

Four replicates of 100 seeds were randomly selected from each seed lot, weighed and each replicate soaked in 50 ml deionized water in plastic vials (ISTA, 2016) and kept in a controlled temperature room (20°±2 ˚C) for 16 hours. After 16 hours of imbibition, the seeds and steep water were stirred to mix briefly and the electrical conductivity of the leachates from imbibed seeds was measured by inserting the single electrode cell into the water. The conductivity of the water of a control (flask with deionised water at 20 C˚) was also measured and this value (background reading) was subtracted from the

conductivity reading of the steep water from each sample. The conductivity per gram of seed weight for each replicate was calculated after accounting for background conductivity of the original water and the average of four replicates provided the results. Thus for each replicate:

$$\text{Conductivity } (\mu S\ cm^{-1}\ g^{-1}) = \frac{\text{Conductivity reading – background reading}}{\text{Weight (g) of seed sample}}$$

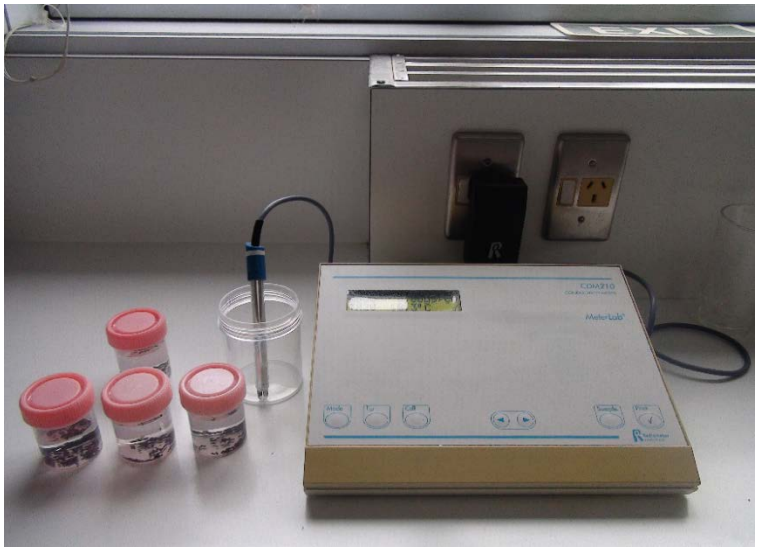


Figure 2.5 Electrical conductivity meter used for the conductivity test.

The samples were retested, if the mean conductivity of the four replicates differed (lowest to highest) between replicates by more than the maximum tolerance values allowed for the conductivity test (Appendix 3) (ISTA, 2016). For this vigour test, the higher the conductivity readings, the lower the vigour of seed.

(ii) Accelerated ageing (AA) test:

The accelerated ageing (AA) test subjects the seed to two environmental variables (high temperature and high relative humidity) for a short period of time as described by Hampton and TeKrony (1995) and ISTA (2016). This treatment accelerates natural seed deterioration.

Accelerated ageing was performed using an inner aging chamber i.e. plastic boxes (11.0 x 11.0 x 3.5 cm) (Figure 2.6) with a suspended wire tray of 10.0 x 10.0 x 0.3 cm (length x width x depth) with a mesh screen inside with a pore size of 1.16 ±0.01 x 1.63 ±0.01 mm. A minimum of 400 seeds per sample, after being weighed, were placed on the mesh screen so as to form a single layer. 40 ml deionized water was placed in each inner chamber and the mesh screen tray with seeds was then placed in the inner chamber so that the seeds did not come into contact with the water. The inner chamber boxes were covered with a tight lid and placed in an outer chamber (oven) for 72 hours (Figure 2.7), the outer

chamber (oven) door was closed to recover the temperature to 41 ± 0.3 ˚C and the aging temperature was continuously monitored during the aging period to be certain that temperature was constant at that level. After 72 hours of aging, seeds were removed from the inner chamber and planted for a standard germination test using the top of paper method within one hour after removal as described in Section 2.3.3.3.

Germination evaluations were performed at 5, 7 and 10 days and the results expressed as mean percentage of normal seedlings for each sample. Seed vigour for this test is determined by the extent of difference in the percentage normal seedlings from the standard germination test (control) and the germination after AA; the greater the difference, the lower the vigour.

Figure 2.6 Inner chamber plastic box with a wire tray for AA, and mesh screen placed on tray with single layer of forage rape seeds.

Figure 2.7 Outer ageing chamber (Oven) with inner chamber placed on shelves, maintaining constant temperature of 41± 0.3 ˚C is used.

2.3.4 Statistical analysis

The results of the quality tests were compared following an analysis of variance for the two sowing date treatments. A randomized complete block design with four replications was used for the TSW, germination, AA and conductivity test data. Comparisons of means for seed quality tests of each sowing dates at LSD (5%) were carried out using Genstat Software (16th Edition, VSN International ltd, Hemel Hempstead, UK).

An analysis of covariance (ANCOVA) was used to establish common relationship between GDD (accumulate during particular seed development stages) and seed quality attributes i.e. seed germination, AA-germination, conductivity and seed mass of two sowing dates. Common slope was used to test statistical significance of the relationship between GDD and seed quality attributes at LSD (5%).

2.4 Results

2.4.1 Environmental conditions during the growing season

Environmental variables were monitored during the experiment from March 2011 to January 2012 (Figure 2.8). In 2011-12, monthly mean temperature ranged from a low of 5.9 ˚C in July to a high of 15.8 ˚C in January , monthly maximum temperature ranged from 11.2 ˚C in July to 21.1˚ C in January and similarly, minimum temperature during this season ranged from 0.7 ˚C in July to 11.1 ˚C in December (Figure 2.8). Total rainfall received during the season was 590 mm and monthly rainfall received during this period ranged from 21 mm in July 2011 to 105 mm in October 2011. Of this 343.8 mm (58%) was received during the months from August 2011 to January 2012. Events of extreme air temperature during the day occurred mostly during the reproductive growth period in the months of November, December and January reaching between 26-27 ˚C. Monthly mean relative humidity (R.H %) ranged from 69.9 to 87.8 %, being lowest during November 2011 and highest in May 2011 (Figure 2.8). All environmental variables were lower than the 30 year average (Figure 2.9) except for total rainfall, which was higher with 105 mm during the month of October 2011 compared with the 30 years average of 50.6 mm (Figure 2.9).

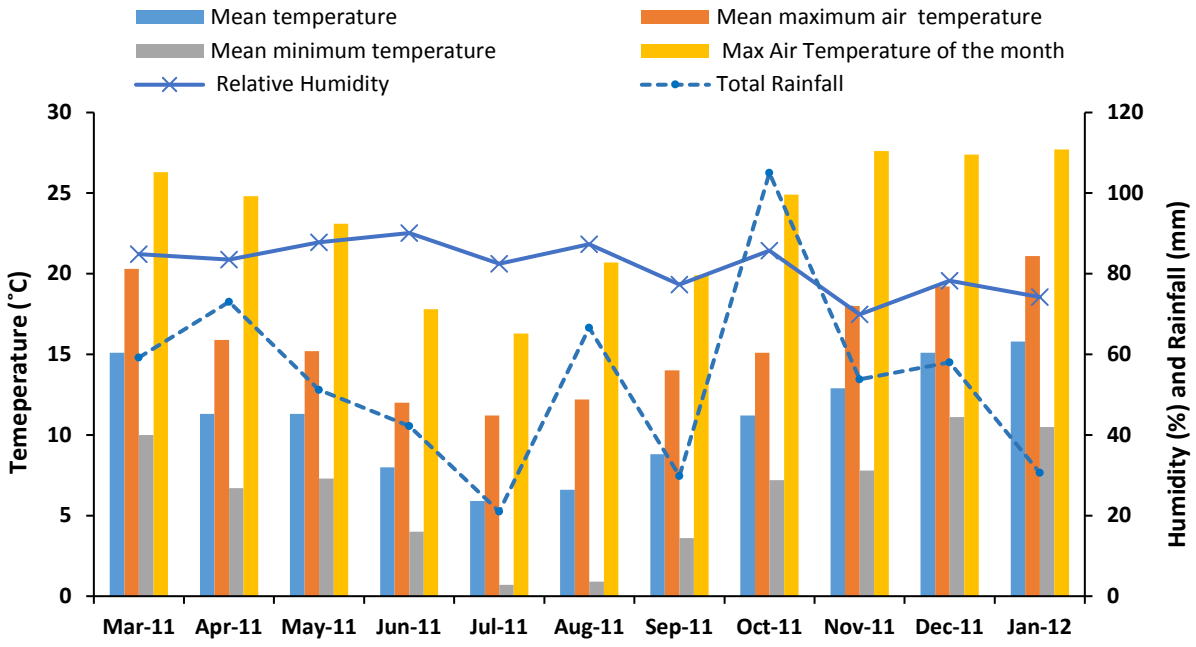


Figure 2.8 Weather data during forage rape growth period (March 2011-January 2012). Relative humidity (%) and total rainfall (mm) are presented on the secondary axis.

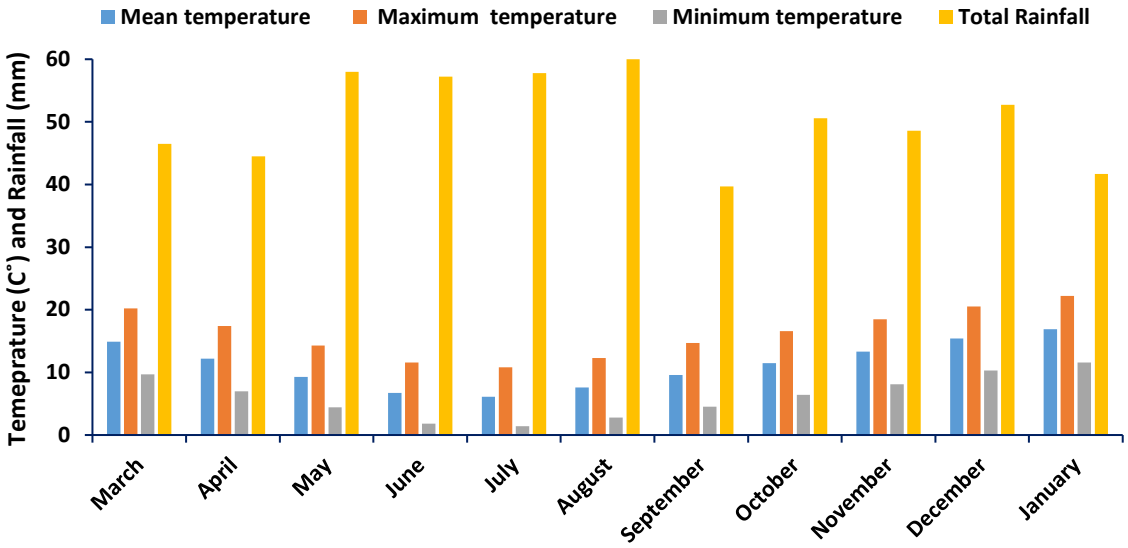


Figure 2.9 Monthly normal (average) temperature and rainfall for a 30 year period from March to January

2.4.2 Environmental conditions during reproductive growth

2.4.2.1 Environmental conditions during flowering and seed filling

The mean temperature during the time from flowering to the seed filling stage (80% SMC) was 12.3 and 13.2 ˚C for the March and April sowings respectively (Figure 2.10). The maximum temperature during this period was 17.0 and 17.6 ˚C and minimum temperature was 7.5 and 8.8 ˚C for the March and April sowings respectively (Figure 2.10). The March sowing received a total rainfall of 130.6 mm with an average rainfall of 2.3 mm during the period from flowering to seed filling. However, total rainfall received by the April sowing was 156.6 mm with average of 2.8 mm during the same period. The average R.H% was 77.1% and 77% during this period for the April and March sowings respectively (Figure 2.10). The March sown forage rape crop accumulated 420.6 GDD to reach the seed filling stage (≈80% SMC), while 493.2 GDD were accumulated for the April sown crop during this period (Table 2.4).

2.4.2.2 Environment during the time from seed filling to physiological maturity (≈ 80-50% SMC) stage of seed development.

The mean daily temperature during this period was around 14.2 ˚C for the March sowing but was slightly higher (15.1 ˚C) for the April sowing. The maximum and minimum temperatures during this time were around 17.7 ˚C and 10.8 ˚C for the March sowing and 19.3 ˚C and 10.9 ˚C respectively for the April sowing. The total rainfall during this period was 56.6 mm for the March but only 22.4 mm for the April sowing, while the average rainfall during the seed filling to PM stage was 3.0 and 1.5 mm for the March and April sowings respectively. The number of rainy days (> 1mm) was 5 and 4 for both sowing dates. The highest rainfall of 33 mm was received in one day for the March sowing. Seeds in

the March sowing took 19 days for SMC to fall from 80 to 50%, but this took only 16 days for the April sowing (Table 2.9-2.10) and minimum temperature during this time was around 17.7 ˚C and 10.8 ˚C for the March sowing and 19.3 ˚C and 10.9 ˚C respectively for the April sowing.

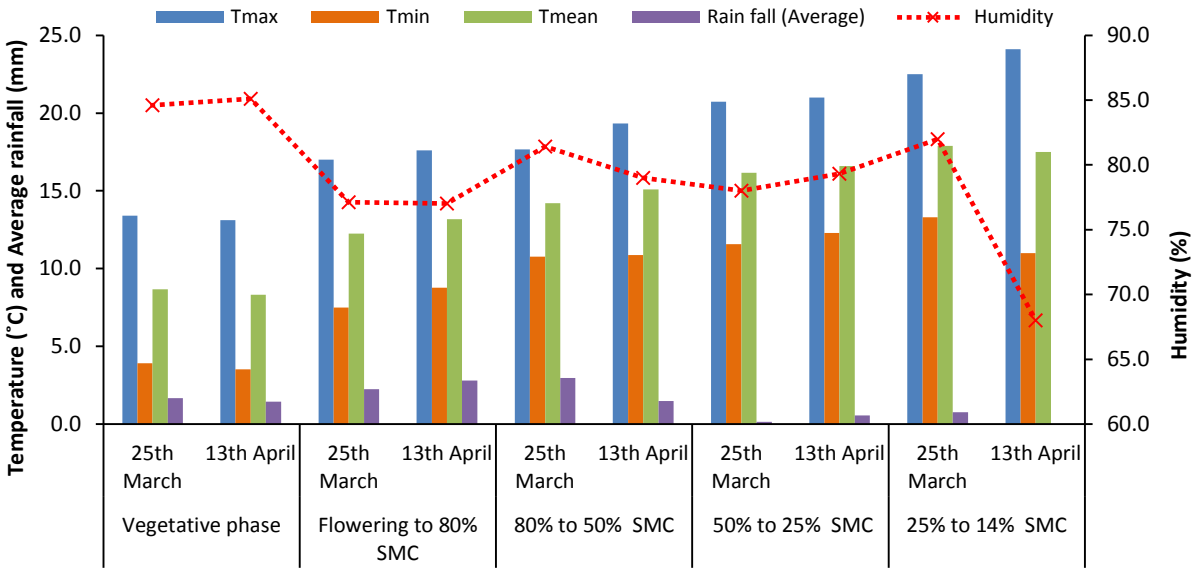


Figure 2.10 Environmental conditions during forage rape seed reproductive growth for the two sowing dates (25 March and 13 April).

The total rainfall during this period was 56.6 mm for the March sowing but only 22.4 mm for the April sowing, while the average rainfall during seed filling to PM was 3.0 and 1.5 mm for the March and April sowings respectively. The number of rainy days was 5 and 4 for both sowing dates. The highest rainfall of 33 mm was received in one day for the March sowing. RH (%) during this period was 81.4% for the March sowing and 79.0 % for the April sowing. Growing degree days (GDD) accumulated during this period were 175.1 and 161.1 for the March and April sown crops respectively (Table 2.5).

2.4.2.3 Environment between physiological maturity (45-50% SMC) and pre-desiccation final stage (≈25% SMC).

The mean temperatures for the March and April sowings during the time from PM to pre-desiccation final stage were 16.2 ˚C and 16.6 ˚C respectively (Figure 2.10). The maximum temperature during this time period was 20.7 ˚C for the March sowing and 21˚C for the April sowing. The minimum temperature for March sowing was 11.6 ˚C and 12.3 ˚C for the April sowing. The relative humidity (%) was 78% and 79.3% during the period from PM to desiccation stage for the March and April sowings respectively. Rainfall was higher for the April sowing (7.6 mm) than for the March sowing (2 mm) during this period. The number of rainy days (> 1mm) was higher for the April sowing (3 days) than for the March sowing

(1 day) (Figure 2.10 and Table 2.4). The growing degree days (GDD) accumulated during this period for the March sowing were 156.2 and for the April sowing were 161.1 (Table 2.5).

2.4.2.4 Environmental conditions from seed pre-desiccation final stage to harvest maturity (≈14% SMC).

The average mean daily temperature during the period for forage rape seeds to go from the desiccation stage to harvest maturity (≈ 14 % SMC) for the March sown crop was around 17.9 ˚C while it was 17.5 ˚C for the April sowing (Figure 2.10). The maximum mean daily temperature was higher for the April sowing (24.1 ˚C) during this period of seed maturation than for the March sown crop (22.5 ˚C).However, the minimum daily mean temperature during this period was 13.3 and 11.0 ˚C, and the average relative humidity was 82% and 68% for the March and April sowings respectively. No rainfall was recorded for the April sowing, however, 7mm total rainfall was received for the March sowing during the time from seed desiccation stage to harvest maturity (Figure 2.10). The GDD accumulated for this period were 116.2 and 122.6 for the March and April sowings respectively (Table 2.5).

Table 2.4 Effect of sowing date on forage rape growth stages, time and duration to complete the vegetative phase and to reach the seed physiological maturity, desiccation and harvest maturity stages

Sowing date	Vegetative phase			Flowering to seed filling (≈80% SMC) stage			Seed filling to PM* (≈45-50% SMC) stage			PM to final desiccation (≈25% SMC) stage			Final desiccation to harvest maturity (≈14% SMC) stage		
	Days to emergence	Period	Days	Period	Days	80% SMC stage reached	Period	Days	PM stage reached	Period	Days	25% SMC stage	Period	Days	HM stage reached
25 March	9	2 April-5 Oct	187	6 Oct-2 Dec	58	2 Dec	2 Dec-21 Dec	19	21 Dec	22 Dec-4 Jan	14	4 Jan	5 Jan-13 Jan	9	13 Jan
13 April	11	24 April-13 Oct	173	14 Oct-13 Dec	63	13 Dec	13 Dec-29 Dec	16	29 Dec	30 Dec-12 Jan	14	12 Jan	13 Jan-19 Jan	7	19 Jan

PM: Physiological maturity

Table 2.5 Growing degree days (GDD)*, average rainfall and the number of wet days during forage rape seed growth stages from vegetative to harvest maturity

Sowing date	Vegetative phase		Reproductive phase											
			Flowering to 80% SMC			80% to 50% SMC			50% to 25% SMC			25% to 14% SMC		
	Average Rainfall (mm/day)	wet days (> 1mm)	Average rainfall (mm/day)	wet days (> 1mm)	GDD†	Average rainfall (mm/day)	wet days (> 1mm)	GDD†	Average rainfall (mm/day)	No. of wet days (> 1mm)	GDD†	Average rainfall (mm/day)	wet days (>1mm)	GDD†
25 March	1.7	30	2.3	14	420.6	3.0	5	175.1	0.1	1	156.2	0.8	2	116.2
13 April	1.8	37	2.8	13	493.2	1.5	3	161.2	0.6	3	161.2	0.0	0	122.6

† Growing degree days accumulated in each seed development stage. Tb= 5

2.4.3 Forage brassica seed development and maturation pattern

The assessment of seed development stages started when seeds attained ≈ 80% SMC. The March sown forage rape crop reached at this at 1103.6 GDD, while the April sown crop reached this stage at 1068.4 GDD (Figure 2.11, (a) & (b)). Between 1103.6 GDD and 1278.7 GDD (March) and 1068.4 and 1230.0 GDD (April), a rapid and linear increase in both fresh and dry weight was recorded for both sowings. Fresh seed weight reached its maximum (PM) at 1278.7 and 1125.0 GDD for the March and April sowing respectively (Table 2.6) and thereafter, gradually declined until harvest maturity was reached at 1551 and 1479.3 GDD respectively (Figure 2.11 (a) & (b)). Dry weight increased rapidly from 1103.6 GDD and reached its maximum at 1278.7 GDD for the March sowing, while the April sown seeds achieved maximum dry weight at about 1230 GDD (Table 2.6). After attaining maximum seed mass maturity, changes in seed dry weight became negligible and further accumulation of dry matter ceased. Seed moisture content (SMC) changed markedly during seed development. At very early stages, SMC was very high but had fallen to around 50% at PM (Figure 2.11 (a) & (b)), SMC then dropped rapidly until it reached equilibrium with the RH of the surrounding air at around 14% SMC.

Table 2.6 Days after flowering and GDD required to reach four development stages in forage rape seed.

		Flowering to seed filling[1]	Seed filling to PM[2]	PM to pre-desiccation[3]	Pre- desiccation to H.M[4]
March	Date of sampling	02 Dec, 2011	21 Dec, -2011	04 Jan, 2012	13 Jan, 2012
	Days after flowing	58	77	91	100
	Growing degree days (GDD)	1103.6	1278.7	1434.8	1551.0
April	Date of sampling	13 Dec, 2011	16 Dec, 2011	12 Jan, 2012	19 Jan, 2012
	Days after flowing	63	79	93	100
	Growing degree days (GDD)	1068	1230.0	1391.7	1479.3

1. Period until SMC reached 80%
2. Period when SMC was between 80-50%
3. Period when SMC was between 50 to 25%
4. Period when SMC was between 25-14 %

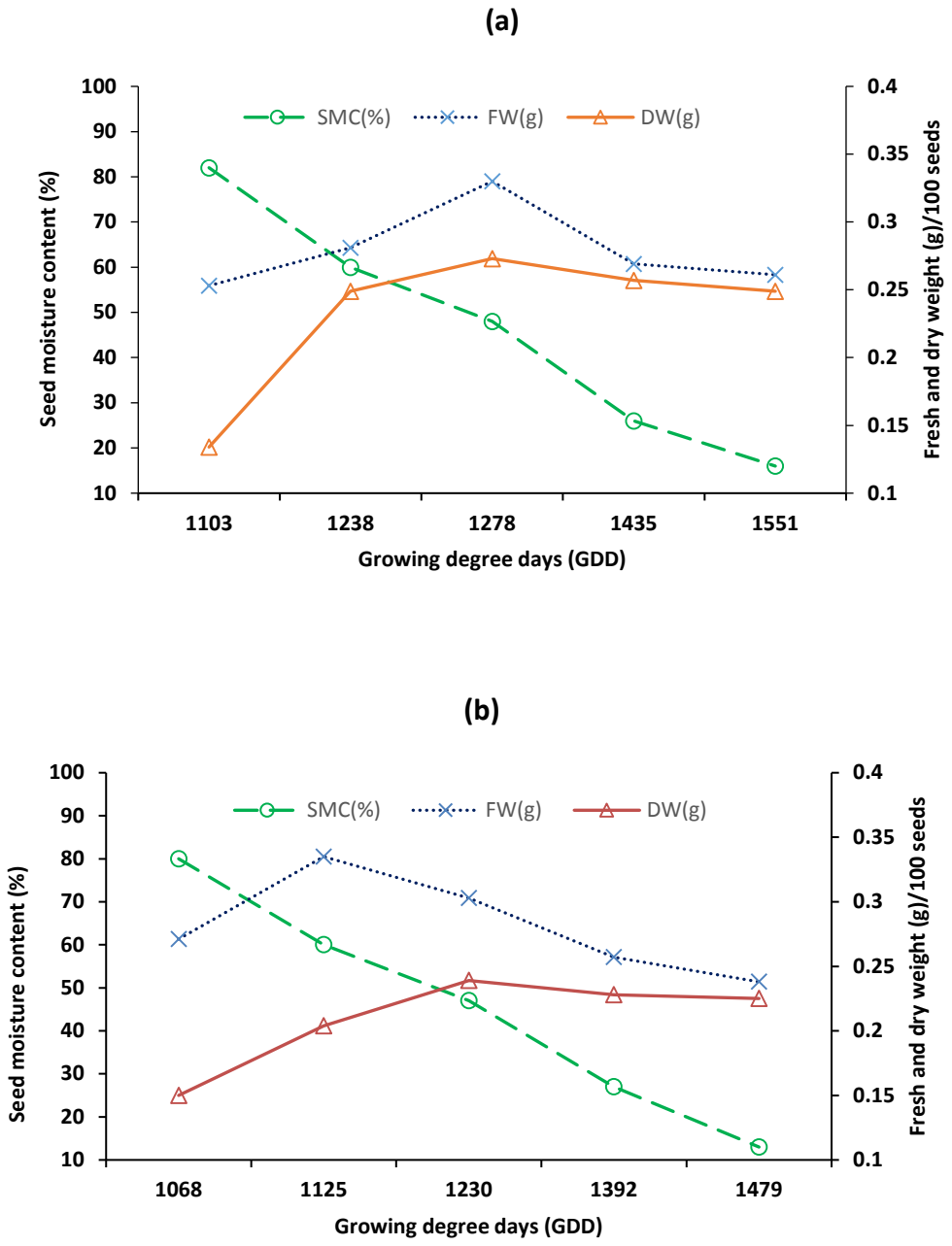


Figure 2.11 Growth pattern of developing forage brassica seed. Figure indicate changes in fresh weight (FW), dry weight (DW) and seed moisture content (SMC) during seed development. Each data point is the mean of three independent measurements (a) March and (b) April.

2.4.4 Seed quality determination

2.4.4.1 At physiological maturity

At physiological maturity (50% SMC), germination did not differ between the two sowing dates (Table 2.7). There was also no significant difference in vigour (as assessed by the AA and conductivity tests) between

the two sowing dates. However, TSW of seeds from the April sowing was significantly higher (*P<0.05*) than that from the March sowing.

Table 2.7 Effect of sowing date on forage rape seed quality hand harvested[a] at 50% SMC.

Sowing Date	Germination (%)	AA germ[n] (%)	Conductivity (µS cm^{-1} g^{-1})	TSW (g)
25th March	95	92	39.81	2.90
13th April	96	93	37.19	3.20
LSD (5%)	**3.7**	**2.6**	**5.3**	**0.12**
Significance of difference	*ns*	*ns*	*ns*	**
% CV	1.7	1.2	6.1	1.7
S.E.M.	0.8	0.6	1.2	0.02

ns= Non significant, *= Significant at *P< 0.05*, ** = Significant at *P< 0.01* ***= Significant at *P< 0.001*.
[a]= Harvested on 21/12/2011 (March sown) and 29/12/2011 (April sown).

2.4.4.2 At pre-desiccation stage (25% SMC)

Sowing date did not affect germination or vigour as assessed the by standard germination and AA test (Table 2.8). However, conductivity was significantly (*P<0.05*) higher for the March sowing (48.9 µS cm^{-1} g^{-1}) than the April sowing (42.48 µS cm^{-1} g^{-1}) (Table 2.8), while thousand seed weight (TSW) was lower for the March sowing (*P< 0.01*) than the April sowing (Table 2.8).

Table 2.8 Effect of sowing date on forage rape seed quality hand harvested at 25% SMC.

Sowing Date	Germination (%)	AA germ[n] (%)	Conductivity (µS cm^{-1} g^{-1})	TSW (g)
25th March	93	89	48.91	2.74
13th April	94	91	42.48	3.10
LSD (5%)	**3.0**	**4.3**	**4.6**	**0.13**
Significance of difference	*ns*	*ns*	*	**
% CV	1.5	2.1	4.5	1.9
S.E.M	0.7	0.9	1.0	0.03

ns= Non significant, *= Significant at *P< 0.05*, ** = Significant at *P< 0.01,* ***= Significant at *P<0.001*.
a= Harvested on 4/01/2012 (March sown) and 12/01/2012 (April sown)

2.4.4.3 At harvest maturity (14% SMC)

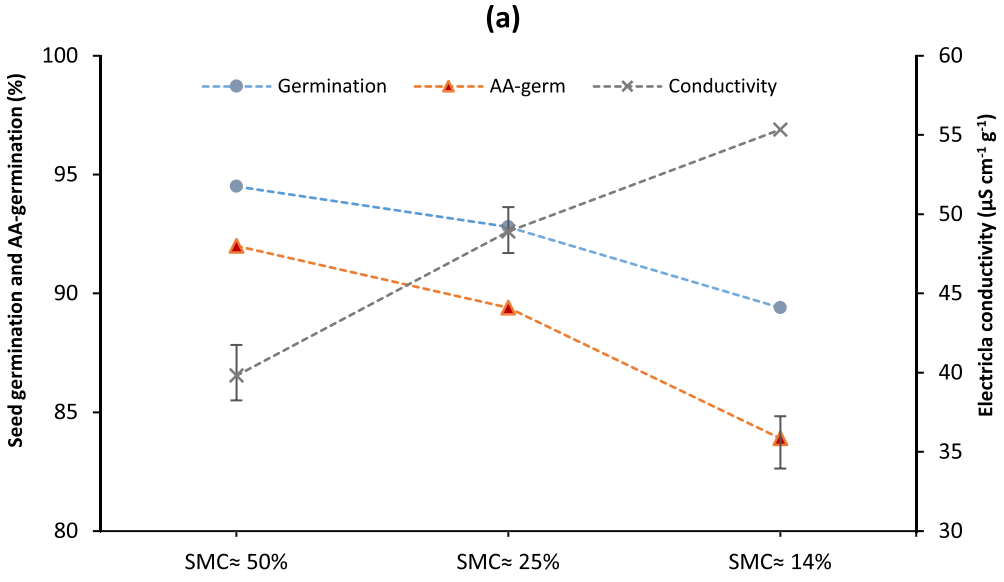
Germination and AA vigour did not differ significantly with sowing date for seeds harvested at harvest maturity stage (≈14 % SMC), but conductivity was significantly higher (*P <0.05*) for the March sowing than the April sowing (Table 2.9). Thousand seed weight of seeds harvested from March sown plots was significantly lower (*P<0.05*) than for the April sowings (Table 2.9).

Table 2.9 Effect of sowing date on forage rape seed quality hand harvested at 14% SMC.

Sowing Date	**Germination (%)**	**AA germ^{n} (%)**	**Conductivity (µS cm^{-1} g^{-1})**	**TSW (g)**
25th March	89	84	55.32	2.59
13th April	91	86	44.13	2.92
LSD (5%)	**2.7**	**4.4**	**10.7**	**0.22**
Significance of difference	*ns*	*ns*	*	*
% CV	1.3	2.3	9.6	3.6
S.E.M.	0.6	1.0	2.4	0.05

ns= Non significant, *= Significant at *P < 0.05*, ** = Significant at *P < 0.01*, ***= Significant at *P <0.001*.
a= Harvested on 9/01/2012 (March sown) and 19/01/2012 (April sown)

Seed quality showed a decreasing trend after PM for both sowings (Figure 2.12 (a) and (b)). The highest seed germination and vigour was found at PM, and thereafter a progressive decline in seed quality was observed (Figure 2.12).



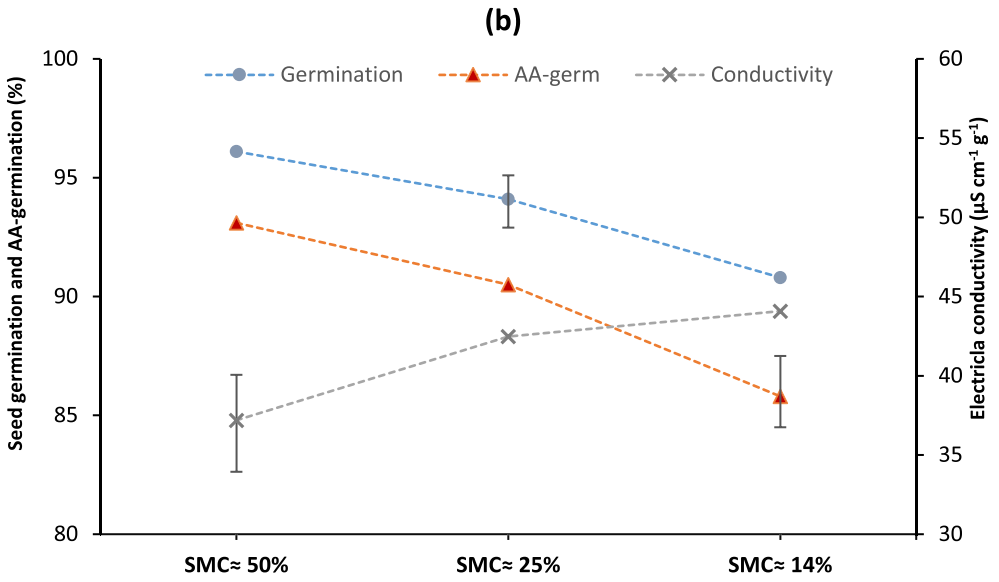


Figure 2.12 Seed quality trend from physiological maturity to harvest maturity level for March (a) and (b) April sowings. Vertical bars show LSD for each quality factor.

2.5 Discussion

2.5.1 Forage brassica seed development and maturation

Knowledge of seed development stages assists in organizing the best time for the harvest of the crop to get the highest yield and quality. Early harvesting may result in low yield and seed quality due to the presence of immature and undeveloped seeds and physical damage during the harvesting process, while harvesting too late may reduce yield because of pod shatter and bird damage, and seed quality losses due to weathering damage (Ekpong & Sukprakarn, 2008; Elias & Copeland, 2001; Oplinger et al., 1989; Wang et al., 2008).

The seed's ability to germinate depends upon its stage of development and degree and rate of maturation(Corbineau et al., 2000; Gosling et al., 1981). During the course of seed development, seeds undergo changes in both fresh and dry weight and hence the stage of seed development progresses or changes with the marked decrease in seed moisture content (SMC). Days after flowering (DAF) is considered to be an inaccurate indicator of seed development stage as the environment during the time in which seed development is occurring affects the rate of seed development and maturation (King, 1976; Prasad et al., 2006a; Prasad et al., 2002). Therefore, seed moisture content (SMC) is considered as a better index of monitoring seed development stages, as it can be better correlated with physiological stage (Fraser et al., 1982; King, 1976; Rondanini et al., 2007; Symmons et al., 1983).

The results of this study are in agreement with the results for seed development studies of other brassica species as reported for *Brassica napus* (Ghasemi-Golezani et al., 2011; Murphy & Cummins, 1989), *Brassica*

napus and *Brassica oleracea* (Still, 1999; Still & Bradford, 1998), *Brassica rapa* (Ren & Bewley, 1998) and *Arabidopsis thaliana* (Baud et al., 2002). There is, therefore, a constant pattern of physiological seed development among brassica species, and among *Brassica napus* cultivars (Still, 1999) although GDD required for seed development and reserve deposition may differ among different environments (Murphy & Cummins, 1989; Still & Bradford, 1998).

In most studies, the acquisition of maximum seed dry mass in Brassica spp. was reached at around 50% SMC. However, Elias and Copeland (2001) reported that in spring and winter cultivars of canola (*B. napus*), the acquisition of maximum seed dry weight was achieved at 20-36% SMC, which contradicts with the results of other studies. A difference in environmental conditions and/ or a genetic difference may have contributed to this difference in the SMC at which physiological maturity was reached, but this remains to be determined.

Variation in environmental conditions modifies the seed development process by affecting pod formation and the time to reach the PM stage (Elias & Copeland, 2001), and environments with high temperature prevail in major cropping zones of the world i.e. India, Africa and south east Asia (Stone, 2001). High temperature negatively affect crop growth, if it coincides with the sensitive reproductive growth stages, particularly seed filling (Farooq et al., 2011; Young et al., 2004). Elias and Copeland (2001) reported differences in brassica seed development between years and explained this by the variable environmental conditions (temperature, RH and rainfall) recorded during seed maturation between years. In this study mean temperature during forage rape seed development from November to December in Canterbury did not reach 20 ˚C (range 13-16 ˚C) in the 2011-12 season (Figure 2.8). As earlier stated, these mean temperatures were lower than those for the 30 year average. However, in Canterbury, mean temperature can exceed 20 ˚C during November to January, and daily maximum temperature often exceeds 30 ˚C (Hampton, 2004). In regions where temperature during seed development exceeds 30 ˚C, a negative effect on seed yield and quality has been reported (Morrison & Stewart, 2002). Even a moderate temperature of 28/23 ˚C was reported to decrease *Brassica napus* seed quality by altering the fatty acid profile of seeds (Aksouh-Harradj et al., 2006) . Similarly, when a temperature exceeding 30 ˚C (35/18 ˚C) was imposed during reproductive development, a 15-70 % reduction in seed yield per plant was found (Gan et al., 2004). During seed development, when temperature is high, the duration of seed filling declines (Prasad et al., 2008a) and/or the seed filling rate decreases (Dias & Lidon, 2009) which subsequently affects seed development. High temperature (33-35 ˚C) disrupts normal pod setting due to floral sterility and subsequently impairs seed filling (Young et al., 2004). Therefore, Morrison and Stewart (2002) proposed a heat stress index of 29.5 ˚C for all brassica species and suggested that temperatures greater than this would have a negative effect on reproductive development in three different brassica species (*Brassica napus*, *B*. rapa and *B*. *juncea*).

2.5.2 Differences in seed quality at different maturity levels

As already noted, changes occur in seed quality during the process of seed development and maturation. Seed maturation is a physiological process which contributes to seed quality and is a prerequisite for successful subsequent germination and emergence (Angelovici et al., 2010; Ghaderi-Far et al., 2011; Mehta et al., 1993). Stage of seed maturity at harvest is one of the important factors which can influence seed quality (Demir et al., 2011; Elias & Copeland, 2001), while physiological maturity (PM) is generally considered to be the time at which seeds attain maximum quality (Berti et al., 2007).However, there is still debate among researchers, and variations in attaining maximum seed quality have been reported among crops and growing locations (Coolbear, 1995; Dornbos, 1995; Hampton, 2000).

In the present study and for both sowing dates, seed germination and vigour (assessed by AA and electrical conductivity test) was greatest at physiological maturity (PM).Thereafter, seed germination and vigour decreased during the desiccation phase when seed SMC dropped from 50% to 14-16% (Figure 2.12). This supports the hypothesis that seeds attain maximum seed quality at physiological maturity (Harrington, 1972) and that quality may decrease from this point on, with the extent depending on environmental factors (Ellis & Hong, 1994). TeKrony et al. (1979) noted that following physiological maturity, seeds become vulnerable to environmental stress as the connection between the seed and maternal plant terminates due to breakage of the vascular connection. Seeds may begin to deteriorate, and in conditions of severe stress, seed germination and vigour can decline rapidly.

The environmental data for this study demonstrate a similar relationship. As the accumulated growing degree days (GDD) increased after PM (≈50% SMC) for both sowing dates (Table 2.5), a decreasing trend in seed quality was found (Figure 2.13). For the March sowing, the accumulated degree days over this period were 272.2 and for the April sowing were 249.0.

The correlation between GDD and seed germination was significant ($P< 0.012$) showing that with increasing GDD, a decreasing trend in seed germination was recorded. However, there was no difference between the two sowing dates ($P=0.66$) (Figure 2.13 (a)). Similarly, the correlation between GDD and AA-germination, conductivity; and TSW were also significant ($P<0.009$, $P<0.014$ and $P<0.05$ respectively) (Figure 2.13 (b), (c) and (d)). This analysis of covariance revealed that AA-germination and TSW decreased and conductivity increased with increasing GDD from PM to HM. There was no significant difference between the two sowings for AA-germination ($P=0.984$) and conductivity ($P=0.1$) but there was for TSW ($P<0.04$) (Figure 2.13 (b), (c) and (d)).

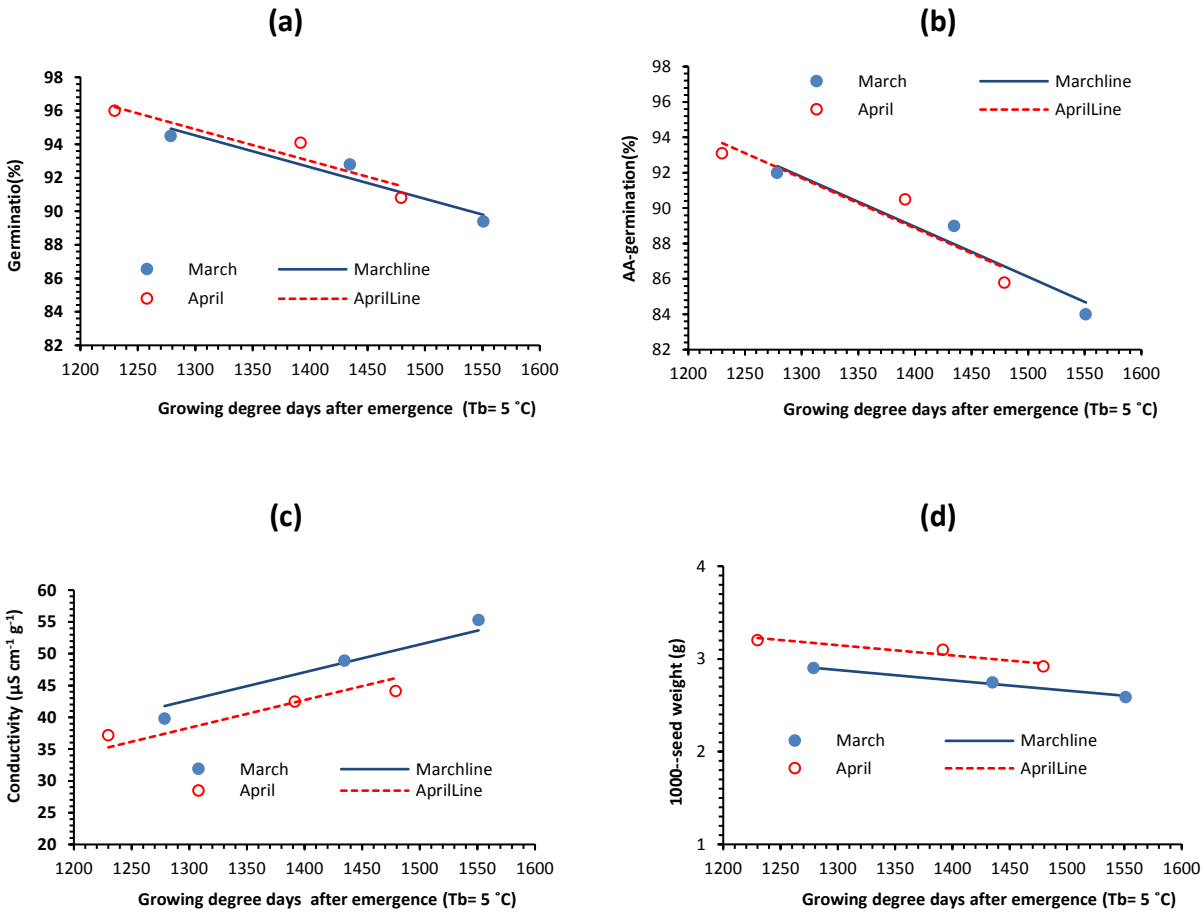


Figure 2.13 Relationship between GDD and seed quality.
An analysis of covariance (Ancova) was used to establish a common relationship between GDD accumulated at seed development stages (PM, pre-desiccation and H.M stage) and seed quality. (a) germination (b) AA-germination (c) Conductivity and (d) 1000-seed weight for the March and April sowings. The Ancova fitted parallel lines to the March and April sown plots were drawn. The statistical significance of the common slope was used as the test for relationship between GDD and seed quality.

2.5.3 Effect of sowing date on seed quality

Seed quality is a genetically inherited trait and variation in seed quality may occur because of the environmental conditions prevailing during the time between seed development and maturation. The variation in temperature and relative humidity resulting from different sowing dates may cause reductions in germination and vigour (TeKrony et al., 1980). If the crop experiences unfavourable environmental conditions during seed development, seed vigour is decreased (Castillo et al., 1994; Hampton et al., 2013). Therefore, optimum sowing time may not be similar for both seed yield and seed quality.

Siddique and Wright (2004) during a study on peas and flax with eight different sowing dates, found that delay in sowing had little effect on seed germination but had a significant effect on seed vigour. However, they did not find any difference in seed germination and vigour between sowing dates only 3-4 weeks apart (from 15 March to 12 April).The present field results are in line with this report. In this study, sowing time had no significant effect on germination or vigour as assessed by the AA test. However, conductivity was higher for the March than the April sowings, at 25% and 14% SMC, suggesting perhaps that in Brassicas the conductivity test is more sensitive to assess seed deterioration than the AA-germination test (Hampton, 2016, per comm.) (Table 2.7-2.9). The March sown crop took 114.8 GDD more to move from PM to harvest maturity and encountered a higher R.H and rainfall than the April sowing (Figure 2.10). This field weathering is likely to have damaged seed membrane integrity which promoted the increase in electrical conductivity and likely impaired seed performance (Pádua et al., 2009; Powell, 1986; Wang et al., 2012).

April sown harvested seed had a higher seed mass than the March sown crops at all three seed maturity stages, i.e. PM, desiccation stage and harvest maturity stage. This may have been because the temperature during the time from 80% SMC to PM was warmer for the April sown crop than the March sown crop, and this coupled with a good supply of moisture from the rain which fell during this time may have allowed a greater seed filling. This reasoning is supported by results from Trethewey (2012), who reported a higher forage rape seed yield in a late autumn sown crop than an early autumn sown crop. This was because the former had a superior crop canopy closure and significantly better light interception, providing a greater photosynthetic capacity and therefore better seed filling. Mandal and Sinha (2004) also reported that seed weight increases were related to a higher interception of photosynthetic active radiation (PAR) allowing a greater net assimilation rate in the crop. In the present trial the April sown crop had a more uniform distribution of plants than the March sowing which would have allowed a more uniform canopy and greater light interception. However, light interception was not recorded in this trial.

2.6 Conclusions

- In this season, sowing time had little effect on forage rape seed development. For both sowings, maximum seed quality was attained when seed reached 47-52% SMC.

- Sowing date had no effect on seed germination, because the prevailing environmental conditions between the sowings did not differ markedly and seeds matured under almost similar environmental conditions at this site in this season. However, the season was cooler than average; higher temperatures may have produced a different result.

- Seed vigour did not differ between sowing dates when assessed at the PM stage, but was lower at the pre-desiccation (25% SMC) and HM (14% SMC) stages, when determined by the electrical conductivity test. During seed maturation, seed vigour was higher for the April sowing than the March sowing.

- Seed mass was significantly greater for the April sowing, possibly because of better light interception and improved photosynthetic activity, although these were not recorded in this trial.

Chapter 3
Effect of high temperature stress at selected reproductive growth stages on forage brassica seed quality; Biotron study

3.1 Introduction

Worldwide, crop production is likely to face a serious threat from predicted global warming, with increased temperature during plant development having a deleterious effect on plant growth (Bita & Gerats, 2013; Hall, 2000). Heat stress has a considerable influence on plant growth and development by reducing crop duration, increasing respiration rate, inhibiting sucrose assimilation to seeds, hastening nutrient mineralization, decreasing fertilizer and water use efficiency and increasing evapotranspiration (Stone, 2001). Strong observational evidence indicates significant global changes in average temperature over the past half century. Surface temperatures have increased by approximately 0.7 ˚C since 1900 and 0.16 ˚C per decade since 1970. Carbon dioxide concentration is anticipated to rise from the current value of around 370 ppm to 550 ppm by 2050 and could approach the level of between 730 ppm to 1010 ppm by 2100 (Blunden et al., 2011; Solomon, 2007). With this rise in atmospheric CO_2 concentration and in other greenhouse gases (methane, chlorofluorocarbons and nitrous oxide), the trend in mean global temperature rise is predicted to be between 1.4 ˚C to 5.8 ˚C (Figure 3.1)(Houghton et al., 2001) and more recent studies have projected a rise of 1.6-6.9 °C by the end of the twenty-first century (Betts et al., 2011; Solomon, 2007).

Elevated CO_2 concentration is likely to allow an increase in the yield of most C_3 crop species by an average of 13% due to the increased rate of photosynthesis and increased vegetative growth under optimal light, temperature and growth conditions, but few changes are expected in C_4 plants (Jaggard et al., 2010; Kimball et al., 2002; Long et al., 2005). However, the deleterious impact of high temperature would not be balanced by the beneficial effects of elevated CO_2 on photosynthesis and plant growth, particularly, if these changes (i.e. 5 ˚C above ambient, upper end of climate change prediction) impact reproductive traits (pollen viability, seed set, seed size, and harvest index) and associated processes (Allen et al., 2000; Prasad et al., 2003; Thomas et al., 2009; Wheeler et al., 2000). The predicted increasing frequency of extreme weather events will affect all the dimensions of crop production (Gornall et al., 2010; Skoet & Stamoulis, 2006). The shortened growing season will force large regions of marginal agriculture out of production. In some countries, simulation studies anticipate that reductions in yield could be as much as 50% by 2020 and net revenues from crops could fall by 90%, with the most affected being small scale-farmers (Benhin, 2008; Kurukulasuriya et al., 2006; Radhouane, 2013). Our future climate will include extreme variability in temperature and periods of intense rainfall and drought, which are likely to affect all aspects of crop production.

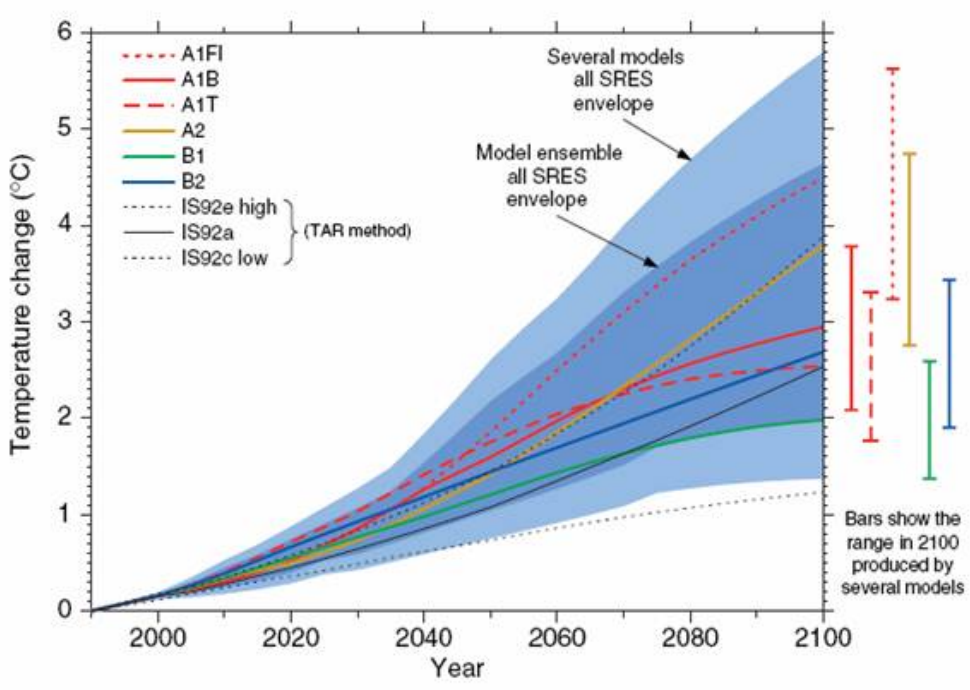


Figure 3.1. Predicted change in Temperature (˚C) over the period 1990-2100. The global mean Temperature is projected to rise by 1.4 to 5.8 over the period.
Source:- IPCC Third Assessment Report "Climate Change 2001". IPCC Summary for Policymakers (http://www.refresh.ucl.ac.uk/Work_Programme_3)

With an increase in temperature by 3-4 ˚C, crop yield is likely to fall by 15-30% in Africa and West Asia and 25-35% in the Middle East (FAO, 2008). A significant decline in crop yield of maize (*Zea mays* L.) was recorded in Germany as a result of heat a wave in Europe in 2003, which increased temperature by up to 6 ˚C above the long term means and reduced precipitation by 50% below the average (Ciais et al., 2005). The International Rice Research Institute (IRRI) has reported that rice yields will fall by 10% with every 1 ˚C increase in night time temperature (Nelson et al., 2009). Similarly, a decrease of 5.4% in mean global wheat yield and 8.3% in maize yield was predicted for every 1 ˚C increase in temperature (Lobell & Field, 2007). Most of the available literature has focused on the effects of climate change on seed yield and production and the effects on seed quality have been little addressed. The main challenge ahead for the seed industry is to cope with temporal variation in climatic variables that put seed quality under threat due to global warming.

Quality seed is a fundamental and crucial input for successful crop production in any environment. In the seed industry, the term seed quality includes physical and genetic purity, seed size, germination, viability, vigour, moisture content and seed health (Hampton, 2002). Each of these seed quality aspects can be influenced by climatic variables prevailing during reproductive growth (Hampton et al., 2013; Maity & Pramanik, 2013; Singh et al., 2013). Among the climatic variables, high temperature and moisture stress are most likely to affect seed quality. High temperatures stress is liable to shorten the growing cycle of

many crop species and particularly the reproductive phase. Most crops are only able to tolerate narrow temperature changes, which if exceeded, can reduce seed set and thus yield (Alqudah et al., 2011; Porter, 2005).The effects of increase in temperature on seed mass, germination and vigour have recently been reviewed (Hampton et al., 2013). Seed mass may be reduced by increased temperature during seed fill (Spears et al., 1997) as it hastens maturity and enhances the rate of seed growth (dry matter accumulation), thereby reducing the seed filling period (Wiegand & Cuellar, 1981; Young et al., 2004). However, seed mass of any seed lot may not always necessarily be related to germination and vigour (Castro et al., 2006; Powell, 1988). Seed germination can be negatively influenced by even small temperature variation, particularly during seed filling, which interrupts the normal seed development process, subsequently resulting in shrivelled and abnormal seeds (Gutterman, 2000; Spears et al., 1997).

High temperature during seed development may also reduce seed vigour both before and after physiological maturity (PM) (Gibson & Mullen, 1996; Hampton et al., 2013; Prasad et al., 2008b). Before PM, seed vigour may be reduced possibly due to negative effects on assimilate transport to the sink (seed) (Shinohara et al., 2006a; Spears et al., 1997). High temperature stress treatment after PM results in deterioration of cell membranes, allowing an increase in loss of cell contents (as measured by the conductivity test;.Castillo et al. (1993)), which is known to be the first indication of the start of seed deterioration (Powell, 1985).

Experiments concerning the effect of high temperature on reproductive growth have mostly been conducted under controlled conditions in a growth chamber, where the temperature stress has been applied for the entire growth period or for the reproductive phase of the crop (Canvin, 1965). Little information is available on the effect of short periods of high temperature stress during specific reproductive growth stages on seed quality in plants including *Brassica napus* (Angadi et al., 2000; Gan et al., 2004).

3.2 Objectives

To determine the effect of high temperature stress during seed development on forage rape seed quality. The hypothesis tested during this experiment was that high temperature stress for short periods both before and at PM would negatively affect seed germination, vigour and seed mass.

3.3 Materials and methods

Plants of March sown forage rape (*Brassica napus*) cv. 'Greenland', from a seed production trial in the 2011-12 season at AgResearch Farm, Lincoln, Canterbury (43° 38' S, 172° 28' E) (as described in Chapter 2, Section 2.3) were used for this experiment.

At the onset of reproductive growth (September, 2011), sixteen (16) plants from each of the four replicates of the plots were transplanted into 12.5 litre pots filled with field soil and left in the field (Figure 3.3). The

plants in the pots were arranged in four blocks (Randomized Complete Block Design) with 16 experimental units. Each experimental unit was comprised of 4 pots. After the start of seed development (pod filling), seed moisture content (SMC) was monitored on a weekly basis using the oven method as described in Chapter 2 (Section 2.3.3.1). SMC was measured for seeds produced at the three raceme positions i.e. top (T), middle (M) and base (B) on the plant (Figure 3.2) and SMC of seeds obtained from the middle of the raceme used as a reference for assessing the particular seed development stages. The experiment was conducted in a 3 x 4 m growth room (Biotron) equipped with cool white fluorescent lamps (model 840, Phillips) mounted above a clear glass barrier, and an upward flow distribution system using sufficient outdoor makeup air to provide ambient CO_2 levels inside the room. Growth room air temperature was 30/25 ˚C (SD ± 2/1 ˚C) during the light/dark period. The relative humidity was maintained at 70% in the room. The photosynthetically active radiation (PAR) at the top of the canopy was 400 µmol m^{-2} s^{-1} (SD ±10 µmol m^{-2} s^{-1}) while a 12-hour photoperiod cycle was maintained. The first set of four experimental units (pots) selected randomly from within each block (total 16 experimental units) was transported to the Biotron (Figure 3.4) at approximately ≈ 80% SMC, the second set at approximately at ≈50 % SMC and the third set were transported to the Biotron twice, at ≈80% and 50% SMC. The pots were watered daily and covered with plastic bags to save the plants from moisture loss (Figure 3.4). In the Biotron, the plants were exposed to 30 ˚C day/ 25 ˚C night for four days (240 ˚C hour) and then returned to the field until seed harvest. The fourth set of experimental units was left in the field as the control. The four treatments made up a 2x2 factorial, with factors (A) nil or some heat-stress at 80% SMC and (B) nil or some heat-stress at 50% SMC. Once pods had started to ripen, the pots were covered with netting to protect against seed loss from birds. At harvest maturity (14% SMC), racemes from each plant per experimental unit were cut into three parts to collect the pods from the top, middle and base positions of the raceme (Figure 3.2). Pods were hand shelled and the seeds from the same positions on the raceme were combined to make a working sample-seed lot to conduct TSW, germination and vigour (conductivity and AA) tests. Methods for the quality tests have been given in Chapter 2 (Section 2.3.3).

The experiment was repeated in the 2012-13 season, but this time forage rape seeds were sown directly in 12.5 litre pots (3 seeds/pot) filled with field soil, to raise the forage brassica plants for the heat stress treatments. At 30 days after sowing, plants were thinned to one healthy plant per pot. The plants were watered daily with an automatic sprinkler system. The pots were organized in the same manner as described above for the first trial for the high temperature treatments in a growth room.

Forage rape plants were exposed to the temperature stress (240 ˚C hours) at the two seed development stages, seed filling (≈ 80% SMC), physiological maturity (≈50% SMC), neither and at both stages in each of two seasons (2011-12 and 2012-13).

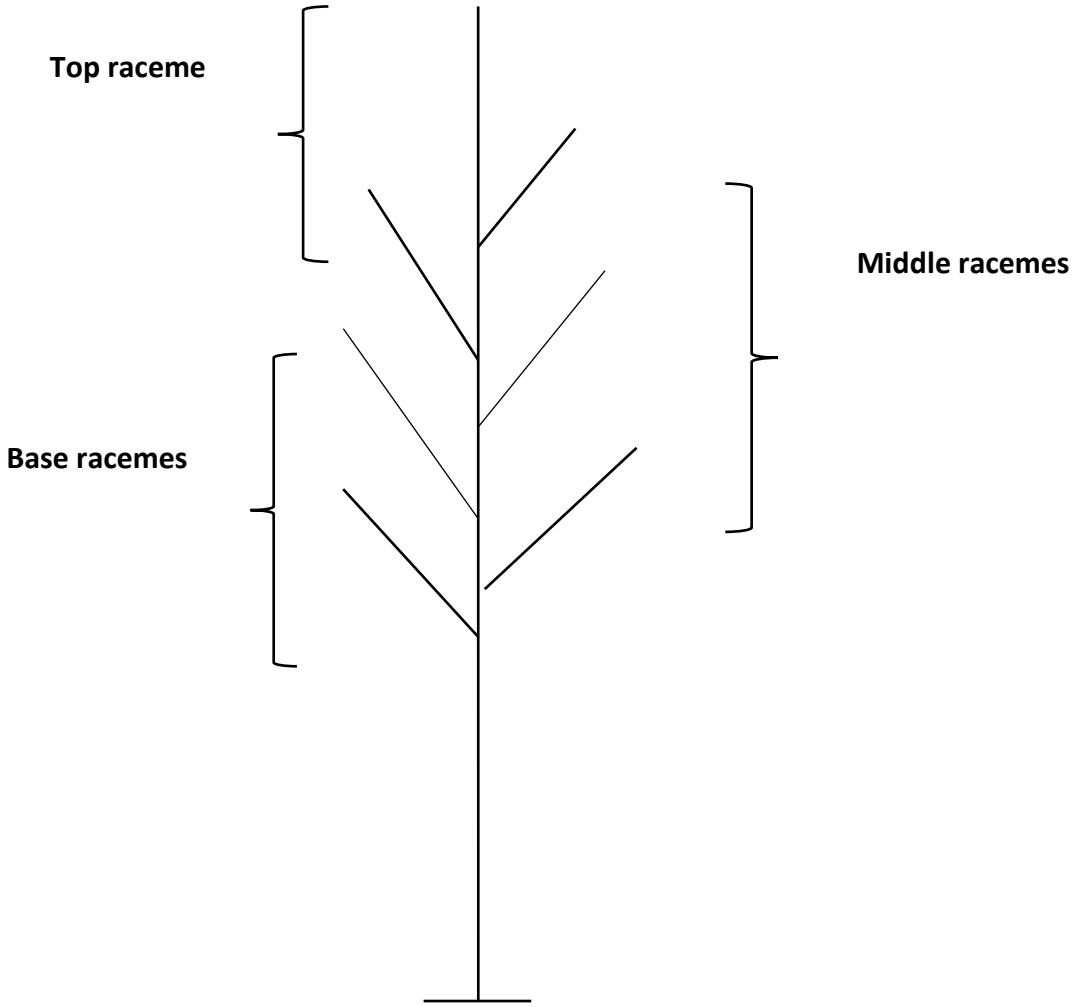


Figure 3.2 Schematic diagram of forage rape inflorescence divided into top, middle and base racemes to obtain seed from the three raceme positions for seed quality tests

Figure 3.3 A view of forage rape plants growing in 12.5 litre pots in the field.

Figure 3.4 A view of forage rape plants exposed to high temperature stress in the Biotron.

3.3.1 Statistical analysis

The results of the quality tests were compared using analysis of variance for the four blocks of 2x2 factorial treatments. A randomized complete block design with four replications was used for the germination test, vigour (AA test and conductivity) test and seed mass. Means for each of the quality test were compared

using the LSD test at *P>0.05*, using Genstat Software (16th Edition, VSN International Ltd, Hemel Hempstead, UK). Seeds harvested from the three raceme positions of the plants from within the 4 blocks were also tested for quality, i.e. germination, vigour (AA test and conductivity test) and seed mass. Statistical analyses of these data were also performed using Genstat software (16th Edition). Analysis of variance (ANOVA) was used to compare the means of seed quality tests from each of the heat stress treatments at a particular seed development stage. The impact of heat stress at two seed development stages (≈80% and ≈50% SMC) on seed quality was analysed in a 2x2 factorial design with heat stress at ≈80% SMC (+, -) and at ≈50% SMC (+, -) as treatment factors. Least significant difference (LSD 5%) was used to compare means of treatments between factors. The interaction between the two treatments was calculated, and least significant interaction (LS interaction (5%) = LSD X√2) was used to test for interaction significance. Least significant effect (LSE 5%) was used to compare the treatment effects on the seeds between the racemes of any three sections of the plant i.e. top, middle and base. A combined analysis of two years data was conducted to estimate the magnitude of treatment effects over the two seasons (2011-12 and 2012-13).

Simple linear correlation analyses were calculated using the mean results of electrical conductivity and AA tests (AA-germination) to evaluate the relationship between the two vigour test methods.

3.4 Results

3.4.1 Seed moisture content at different raceme positions

The seed moisture content of seeds harvested from the three raceme positions was measured. The target SMC was set as a reference point for heat stress application at the particular seed development stages (Table 3.1). The top raceme seeds always had a lower SMC and the base raceme seeds had a higher moisture content than the target SMC (Table 3.1).

Table 3.1 Seed moisture content (%) of forage rape seeds at different raceme position

Target SMC	**Seed moisture content (SMC) at the time of heat stress application**		
	Top raceme seeds	Middle raceme seeds	Base raceme seeds
2011-12 season			
≈80% SMC	65	80	85
≈50% SMC	42	51	55
2012-13 season			
≈80% SMC	73	82	84
≈50% SMC	46	52	59

3.4.2 Seed germination

For control plants, the germination of harvested seeds was > 90% in both the 2011-12 and 2012-13 seasons, with a mean for the two seasons of 93% (Table 3.2). However, when the heat stress was applied, a significant effect of heat stress on seed germination was observed (Table 3.2). Heat stress at ≈ 80% SMC significantly reduced germination in both the 2011-12 season (*P<0.001*) and in the 2012-13 season (*P<0.05*) (Table 3.2). Heat stress at physiological maturity (50% SMC) had a larger effect than at ≈80% SMC, significantly reducing germination in the 2011-12 (*P < 0.001*) and 2012-13 (*P<0.01*) seasons. There was a significant interaction for heat stress application time (80% SMC + 50% SMC) in terms of reduction in germination in the 2011-12 (*P<0.05*) season but not in the 2012-13 season (*P= 0.082*). In general, much larger effects were observed in 2011-12 than in 2012-13 (e.g., the 80%+50% SMC treatment differed from the control by 15% in 2011-12 but only 3% in 2012-13) (Table 3.2). Where germination was reduced following heat stress, it was mostly because of the production of abnormal seedlings, as no dead seeds were recorded. However, some 1-2 % of fresh un-germinated seeds were recorded. Most of the abnormal seedlings were missing their primary root, were stunted or were trapped in the seed coat (Figure 3.5 (i) & (ii)).

A combined analysis of the two years' germination data (2011-12 and 2012-13 seasons) showed that the main effects of heat stress treatments, both at ≈80% SMC and ≈50% SMC and the interaction effect were statistically non-significant, probably because of the marked differences in response between the two seasons (*P = 0.273*, *P=0.119* and *P=0.642* respectively) (Table 3.2).

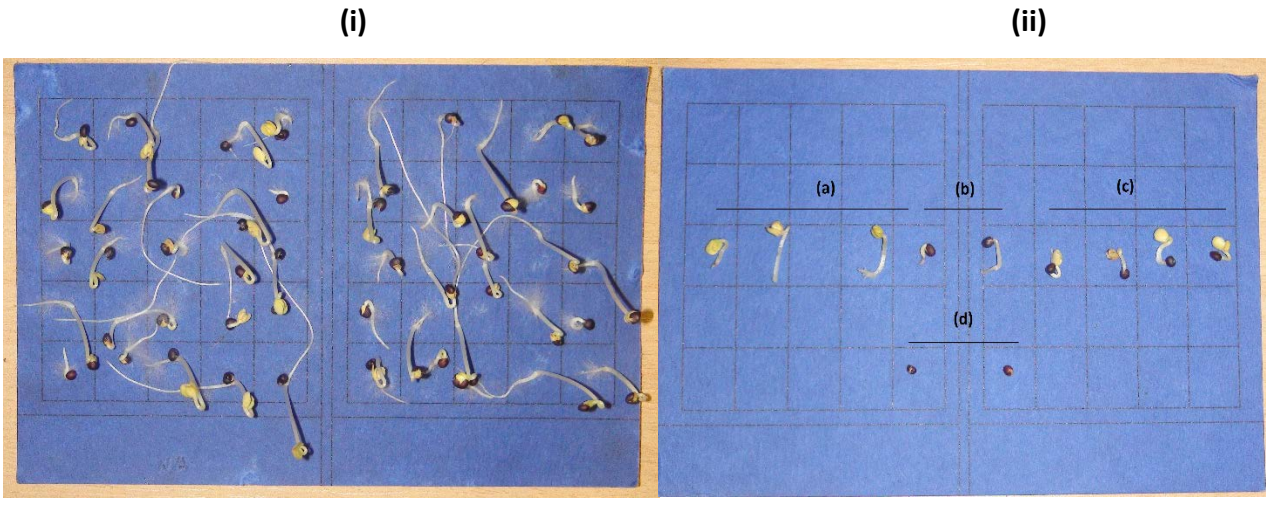


Figure 3.5 (i) Germination test ready to evaluate after seven days (ii) Abnormal seedlings and non-germinated seeds after seven days, (a) missing primary roots (b) cotyledon trapped in seed coat (c) roots trapped in seed coat (d) fresh un-germinated seed.

Table 3.2 Effect of high temperature (240̊Ch) during seed development on forage rape germination (%) in the 2011-12 and 2012-13 seasons.

Treatments	Germination (%)		
	2011-12	**2012-13**	**Mean**
Main effect of heat stress at 80% SMC			
Nil (no stress)	88.8	93.3	91.0
Heat stress at 80% SMC	83.0	92.3	87.8
LSD (5%)	**1.5**	**0.9**	**7.7**
Significance of difference	***	*	*ns*
Main effect of heat stress at 50% SMC			
Nil (no stress)	90.4	93.5	92.0
Heat stress at 50% SMC	81.5	92.1	86.8
LSD (5%)	**1.5**	**0.9**	**7.7**
Significance of difference	***	**	*ns*
Treatment means			
Control [1]	92.3	93.7	93.0
Heat stress at 80% SMC	88.5	93.3	91.0
Heat stress at 50% SMC	85.4	93.0	89.0
Heat stress at (80% SMC+ 50% SMC)	77.5	91.2	84.5
LSD (5%)	**2.1**	**1.2**	**10.9**
SEM	0.7	0.4	2.4
CV%	1.5	0.8	3.8
Interaction effect (80% SMC X 50% SMC)	-4.2	-1.5	-2.5
L.S. interaction (5%)	**2.9**	**1.7**	**15.4**
Significance of interaction	*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*,
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)] /2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)] /2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)] /2
Heat stress at 50% SMC=[(SMC 50%) + (SMC 80+50%)] /2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Germination of seeds from control plants harvested from all three raceme positions (top (T), middle (M) and base (B)) were > 90% in both years (Table 3.3). When the heat stress was applied at ≈ 80% SMC,

germination was significantly reduced in seeds from all three raceme positions in the 2011-12 season (*P < 0.001*) but there were no significant differences in the 2012-13 season (Table 3.3). Heat stress at physiological maturity (PM) significantly reduced germination in seed harvested from all three raceme positions in both years with the exception of the middle raceme position in the 2012-13. Heat stress at both 80% SMC and PM also significantly reduced germination of seeds at all raceme positions in both seasons. However, there was a larger negative effect on seed germination in the 2011-12 season from all raceme positions than in the 2012-13 season. The interaction between applying heat stress at the two seed development stages was significant for the middle and base raceme positions, but not the top position in the first season (*P<0.01* and *P<0.05* respectively) (Table 3.3), but there were no significant interactions in the second season.

Heat stress applied at ≈80% SMC significantly increased the difference in seed germination between the middle and top sections of the raceme (*P<0.05*) and between the base and top sections of the raceme (*P<0.05*), in the 2011-12 season only (Table 3.4). Heat stress at physiological maturity had no significant effect on differences in germination between raceme positions in either season, nor were any significant interactions detected.

Calculation of least significant effects (LSE) allowed an evaluation of the effect of raceme position on seed germination for each of the four treatments individually (Table 3.4). Germination of the seeds from the middle section of the raceme (M) was higher than that of the seeds from the top section of the raceme (T) when heat stress was applied either at ≈80% SMC (*P<0.05*) and at physiological maturity (≈50% SMC) or at both seed development stages in the 2011-12 season. However, there was no significant interaction between these treatments in either season (Table 3.4). Mean seed germination for the middle section of the raceme (M) did not differ from the average germination for the basal raceme (B) seeds in any treatment or season except for the heat stress at the physiological maturity (≈50% SMC) only treatment in the 2012-13 season. Basal raceme (B) seeds had a significantly higher mean germination (*P<0.05*) than seeds from the top section of the raceme (T) in the presence of heat stress, either at ≈80% SMC only or at both development stages in the first season, but not in the second season. The interaction effect was non-significant in both seasons (Table 3.4).

Table 3.3 Effect of high temperature (240°Ch) during seed development on seed germination (%) of forage rape from different raceme positions in the 2011-12 and 2012-13 seasons.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	87.5	92.5	90.1	94.5	88.9	93.0
Heat stress at 80% SMC	80.0	91.2	84.9	93.3	84.1	92.4
LSD (5%)	**2.5**	**1.5**	**1.3**	**1.3**	**2.1**	**1.0**
Significance of difference	***	*ns*	***	*ns*	***	*ns*
Main effect of heat stress at 50% SMC						
Nil (no stress)	88.6	92.8	91.5	94.3	91.0	93.5
Heat stress at 50% SMC	78.9	90.8	83.5	93.5	82.0	91.9
LSD (5%)	**2.5**	**1.5**	**1.3**	**1.3**	**2.1**	**1.0**
Significance of difference	***	*	***	*ns*	***	**
Treatment means						
Control [1]	91.5	93.0	93.0	94.5	92.3	93.5
Heat stress at 80% SMC	85.8	92.5	90.0	94.0	89.8	93.5
Heat stress at 50% SMC	83.5	92.0	87.3	94.5	85.5	92.5
Heat stress at (80 SMC+50% SMC)	74.3	89.8	79.8	92.5	78.5	91.3
LSD (5%)	**3.5**	**2.1**	**1.8**	**1.9**	**2.9**	**1.4**
SEM	1.1	0.7	0.6	0.6	0.9	0.4
CV%	2.6	1.5	1.3	1.3	2.1	1.0
Interaction effect (80% SMC X 50% SMC)	-3.5	-1.7	-4.6	-1.5	-4.5	-1.3
L.S. interaction (5%)	**4.9**	**3.0**	**2.5**	**2.7**	**4.1**	**2.0**
Significance of interaction	*ns*	*ns*	**	*ns*	*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant *at P< 0.001*.
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)] /2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)] /2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + (SMC 80%)] /2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)] /2
Interaction Effect = [(SMC 80+50%)-(SMC 50%) - (SMC 80%) - (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. interaction** = LSD (Treatment means) x √2

Table 3.4 Comparison of germination (%) of forage rape seeds from different raceme positions in the 2011-12 and 2012-13 seasons. An * indicates the difference (e.g. M-T) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	M-T		B-T		M-B	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	2.6*	2.0*	1.4	0.5	1.3	1.5*
Heat stress at 80% SMC	4.9*	2.1*	4.1*	1.3	0.8	0.9
LSD (5%)	**1.8**	**1.7**	**2.6**	**1.8**	**2.3**	**1.6**
Significance of difference	*	*ns*	*	*ns*	*ns*	*ns*
LSE (5%)	**1.3**	**1.2**	**1.9**	**1.3**	**1.7**	**1.1**
Main effect of heat stress at 50% SMC						
Nil (no stress)	2.9*	1.5*	2.4	0.8	0.5	0.8
Heat stress at 50% SMC	4.6*	2.6*	3.1*	1.0	1.5	1.6*
LSD (5%)	**1.8**	**1.7**	**2.6**	**1.8**	**2.3**	**1.6**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**1.3**	**1.2**	**1.9**	**1.3**	**1.7**	**1.1**
Treatment means						
Control [1]	1.5	1.5	0.8	0.5	0.8	1.0
Heat stress at 80% SMC	4.3*	1.5	4.0*	1.0	0.3	0.5
Heat stress at 50% SMC	3.8*	2.5*	2.0	0.5	1.8	2.0*
Heat stress at (80% SMC+ 50% SMC)	5.5*	2.8*	4.3*	1.5	1.3	1.3
S.E.M	0.8	0.8	1.2	0.8	1.0	0.7
LSD (5%)	**2.6**	**2.3**	**3.7**	**2.5**	**3.3**	**2.2**
LSE (5%)	**1.8**	**1.7**	**2.6**	**1.8**	**2.3**	**1.6**
Interaction effect (80% SMC X 50% SMC)	-1.0	0.3	-1.0	0.5	0.0	-0.3
L.S. interaction (5%)	**3.6**	**3.4**	**5.2**	**3.5**	**4.7**	**3.1**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*;
[1] Plants in the pots left in the field.
An * to the right side of means indicates a significant difference between two raceme positions.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)] /2
Heat stress at 80% SMC= [(80%) + (80+50% SMC)] /2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + (80% SMC)] /2
Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)] /2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - (80% SMC) – (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, LSE **(5%) (Main Effect)** = LSD (Main effect)/√2

3.4.3 Seed vigour- Accelerated Ageing

Seed vigour of forage rape seeds as assessed by the accelerated ageing (AA) test, was highest from the control plants. Heat stress significantly (*P<0.001*) reduced seed vigour, when applied either during the seed filling stage (≈80% SMC) or at PM stage (≈50% SMC) in both seasons (Table 3.5).There was a significant (*P<0.001*) decrease in seed vigour for the heat stress at ≈80% SMC and the reduction in seed vigour was very similar between the seasons (12-13%) (Table 3.5). The largest seed vigour reduction was recorded for heat stress at PM (≈50% SMC) which reduced seed vigour significantly (*P<0.001*) in both season; the difference between the pre- and post-AA germination was 23% in the 2011-12 season and 18 % in the 2012-13 season (Table 3.5). There was a significant interaction for heat stress between seed development stages (80% SMC + 50% SMC), which was larger in the 2011-12 (*P<0.01*) than in the 2012-13 (*P<0.05*) season (Table 3.5). Generally, a larger seed vigour reduction was observed in the 2011-12 season than in the 2012-13 season, particularly for heat stress treatment at PM (Table 3.5). Heat stress application at both ≈80% SMC and ≈50% SMC reduced seed vigour with the difference between the pre- and post-AA germination being 34% in the first season and 29% in the second season (Table 3.5). The analysis of mean data for the two years indicated that the main effect of heat stress treatment both at ≈80% SMC and ≈50% SMC, and the interaction between the treatments were significant, indicating an almost similar magnitude of vigour loss in both years and less variability between the years (Table 3.5).

Seed vigour from control plants was highest in both seasons from all three raceme positions (Table 3.6). Heat stress during seed development either at ≈80% SMC or at PM (≈ 50% SMC), significantly (*P<0.001*) decreased seed vigour in seeds harvested from all three raceme positions in both seasons (Table 3.6). The interaction of the two heat stress treatment was significant with respect to seed vigour for the top and middle raceme positions (*P<0.01 and P<0.05*, respectively) in both seasons but was non-significant for the seeds from the basal raceme position both in the 2011-12 and 2012-13 seasons (*P=0.11 and P=0.22* respectively) (Table 3.6) Application of heat stress at the two seed development stages, first at seed filling (≈80% SMC) and later at seed physiological maturity (≈50% SMC) , significantly reduced seed vigour from all three raceme positions in both years but the reduction was larger in the 2011-12 season and on seeds harvested from the top raceme position in both seasons (36% and 33% respectively) (Table 3.6).

Table 3.5 Effect of high temperature (240̊Ch) during seed development on forage rape AA-germination (%) in the 2011-12 and 2012-13 seasons.

Treatments	AA-germination (%)		
	2011-12	**2012-13**	**Mean**
Main effect of heat stress at 80% SMC			
Nil (no stress)	80.1	83.9	82.0
Heat stress at 80% SMC	69.3	73.5	71.5
LSD (5%)	**1.9**	**2.3**	**2.9**
Significance of difference	***	***	**
Main effect of heat stress at 50% SMC			
Nil (no stress)	84.4	86.8	85.8
Heat stress at 50% SMC	65.0	70.6	67.8
LSD (5%)	**1.9**	**2.3**	**2.9**
Significance of difference	***	***	***
Treatment means			
Control [1]	88.3	90.8	89.5
Heat stress at 80% SMC	80.5	82.8	82.0
Heat stress at 50% SMC	71.8	77.1	74.5
Heat stress at (80%SMC+50% SMC)	58.2	64.1	61.0
LSD (5%)	**2.7**	**3.2**	**4.1**
SEM	0.8	1.0	0.9
CV%	2.2	2.6	1.7
Interaction effect (80% SMC X 50% SMC)	-7.0	-5.0	-6.0
L.S. interaction (5%)	**3.8**	**4.5**	**5.8**
Significance of interaction	**	*	*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*,
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)] /2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)] /2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)] /2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)] /2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 3.6 Effect of high temperature (240̊Ch) during seed development on AA-germination(%) of forage rape from different raceme positions in the 2011-12 and 2012-13 seasons.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	78.5	81.9	81.4	86.3	80.4	83.8
Heat stress at 80% SMC	66.8	69.9	71.9	75.5	69.3	75.0
LSD (5%)	**2.2**	**2.6**	**1.9**	**2.5**	**2.8**	**3.9**
Significance of difference	***	***	***	***	***	***
Main effect of heat stress at 50% SMC						
Nil (no stress)	82.6	84.6	86.1	88.6	84.5	87.3
Heat stress at 50% SMC	62.8	67.1	67.1	73.1	65.1	71.5
LSD (5%)	**2.2**	**2.6**	**1.9**	**2.5**	**2.8**	**3.9**
Significance of difference	***	***	***	***	***	***
Treatment means						
Control [1]	86.5	89.3	89.5	92.8	89.0	90.5
Heat stress at 80% SMC	78.8	80.0	82.8	84.5	80.0	84.0
Heat stress at 50% SMC	70.5	74.5	73.3	79.8	71.8	77.0
Heat stress at (80 SMC 50% SMC)	55.0	59.8	61.0	66.5	58.5	66.0
LSD (5%)	**3.1**	**3.7**	**2.8**	**3.5**	**3.9**	**5.5**
SEM	0.9	1.2	0.9	1.1	1.2	1.7
CV%	2.7	3.0	2.3	2.7	3.2	4.3
Interaction effect (80% SMC X 50% SMC)	-7.5	-5.4	-5.6	-5.0	-4.3	-4.5
L.S. interaction (5%)	**4.4**	**5.2**	**3.9**	**4.9**	**5.5**	**7.8**
Significance of interaction	**	*	*	*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*.

[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2; **LS. Interaction** = LSD (Treatment means) x√2

When heat stress was applied at ≈ 80% SMC, seeds harvested from the basal raceme position had higher seed vigour than seeds from the top raceme position (*P<0.05*), but only in the 2012-13 season (Table 3.7). Seed vigour did not differ between the seeds of any other raceme positions in either season, nor within any of the heat stress treatments. There was no significant interaction between the two heat stress treatments with respect to differences in seed vigour between any raceme positions in either season apart from in the 2011-12 season, where once again seeds from the basal section of the raceme had a higher seed vigour than those from the top raceme (Table 3.7).

Seed vigour from the middle section of the raceme was higher than that of the seeds from the top section of the raceme in both seasons for all the heat stress treatment. However, there was no significant interaction between these treatments in either season (Table 3.7). No differences in seed vigour between the middle and basal raceme seeds occurred for either season or treatment, except for the heat stress at ≈80% SMC in the 2011-12 season, but no significant interaction was recorded in either season (Table 3.7). Basal seeds had a higher seed vigour than those from the top raceme position for the double stress treatment (mean effect) in both seasons, and there was a significant interaction for these treatments in the 2011-12 season (Table 3.7).

3.4.4 Conductivity test

Seed vigour in the conductivity test is estimated by electrolyte leakage from the seed into water. Conductivity was lowest and therefore vigour was highest in forage rape seeds harvested from the control plants. Heat stress during seed development increased electrolyte leakage and therefore reduced seed vigour (Table 3.8). Heat stress during seed filling (≈80% SMC) significantly increased conductivity but the effect was larger in the 2011-12 (*P<0.001*) than in the 2012-13 (*P<0.01*) season. Heat stress at PM significantly (*P<0.001*) increased conductivity in both seasons (Table 3.8). No significant interaction effect of heat stress between the two seed development stages (80% SMC+ 50% SMC) occurred in the 2011-12 and 2012-13 seasons (*P=0.609 and P= 0.722*, respectively) (Table 3.8).

Heat stress at both seed development stages (80 +50% SMC) also increased conductivity in both seasons, but the increase was greater in the 2011-12 than in the 2012-13 season (77.8 cf. 65.3 µS cm^{-1} g^{-1}) (Table 3.8).

The mean seed conductivity for two years' (2011-12 and 2012-13 seasons) indicates that heat stress (main effects) increased conductivity at 80% SMC (*P<0.05*) and at ≈50% SMC (*P<0.01*) but the interaction was statistically non-significant (*P= 0.698*) which shows the consistency of heat stress treatment response over the two seasons (Table 3.8).

Table 3.7 Comparison of AA-germination (%) of forage rape seeds between different raceme positions in the 2011- 12 and 2012-13 seasons.
An * indicates the difference (e.g. M-T) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	M-T		B-T		M-B	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	2.9*	4.4*	1.9*	1.9	1.0	2.5
Heat stress at 80% SMC	5.0*	5.6*	2.4*	5.1*	2.6*	0.5
LSD (5%)	**2.6**	**3.1**	**1.6**	**3.1**	**2.7**	**4.2**
Significance of difference	*ns*	*ns*	*ns*	*	*ns*	*ns*
LSE (5%)	**1.9**	**2.2**	**1.1**	**2.2**	**1.9**	**3.0**
Main effect of heat stress at 50% SMC						
Nil (no stress)	3.5*	4.0*	1.9*	2.6*	1.6	1.4
Heat stress at 50% SMC	4.4*	6.0*	2.4*	4.4*	2.0*	1.6
LSD (5%)	**2.6**	**3.1**	**1.6**	**3.1**	**2.7**	**4.2**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**1.9**	2.2	1.1	2.2	1.9	3.0
Treatment means						
Control [1]	3.0*	3.5*	2.5*	1.3	0.5	2.3
Heat stress at 80% SMC	4.0*	4.5*	1.3	4.0*	2.8*	0.5
Heat stress at 50% SMC	2.8*	5.3*	1.3	2.5	1.5	2.8
Heat stress at (80% SMC+ 50% SMC)	6.0*	6.8*	3.5*	6.3*	2.5	0.5
S.E.M	0.8	1.4	0.7	1.4	1.2	1.9
LSD (5%)	**3.7**	**4.4**	**2.3**	**4.4**	**3.9**	**5.9**
LSE (5%)	**2.6**	**3.2**	**1.6**	**3.1**	**2.7**	**4.2**
Interaction effect (80% SMC X 50% SMC)	2.3	0.5	3.5	1.1	-1.3	-0.5
L.S. interaction (5%)	**5.2**	**6.2**	**3.2**	**6.2**	**5.4**	**8.4**
Significance of interaction	*ns*	*ns*	*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001*

[1] Plants in the pots left in the field.

An * to the right side of means indicates a significant difference between two raceme position.

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(50% SMC)]/2

Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(80% SMC)]/2

Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2

Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].

LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD(Treatment means) x√2,

LSE (5%) (Treatment means)=LSD (Treatment means)/√2, LSE**(5%)** (Main Effect**)** =LSD (Main effect)/√2

Forage rape seeds harvested from all three raceme positions (top (T), middle (M) and base (B)) of the control plants had the lowest conductivity in both seasons. Heat stress at ≈80% SMC and at 50% SMC significantly increased conductivity of seeds from all three raceme positions, but the increase was greater in the 2011-12 season than in the 2012-13 season (Table 3.9). Heat stress at both 80% SMC and 50% SMC further increased conductivity in seeds from all raceme positions, but the interaction of heat stress treatments between these two seed development stages was non-significant for seed from all three raceme positions (Table 3.9).

Heat stress, either at ≈80% SMC or at ≈50% SMC had no significant effect on differences in seed conductivity between any raceme positions in either season except between the top and middle raceme positions in the 2012-13 season where conductivity was lower *(P<0.05)* in the seeds from the middle section of the raceme. There was no significant interaction for differences in seed conductivity between any raceme positions for either season (Table 3.10).

For treatment means, differences in conductivity were recorded between the seeds from the top and middle raceme position for control plants and for all heat stress treatments in both seasons. Seeds from the top raceme position had a higher conductivity than the seeds from the middle section of the raceme in both years. However, the conductivity of the seeds from the top of the raceme position did not differ with the basal raceme for any individual heat stress treatment in 2012-13, but they had a higher conductivity for the double stress. There was no difference in mean conductivity of seeds between basal and middle position for any heat stress treatment in either season (Table 3.10).

Table 3.8 Effect of high temperature (24ͦ Ch) during seed development on forage rape seed conductivity (µS cm^{-1} g^{-1}) over two seasons 2011-12 and 2012-13.

Treatments	Conductivity (µS cm^{-1} g^{-1})		
	2011-12	**2012-13**	**Mean**
Main effect of heat stress at 80% SMC			
Nil (no stress)	55.9	49.5	52.7
Heat stress at 80% SMC	68.9	59.1	63.9
LSD (5%)	**3.8**	**5.1**	**6.3**
Significance of difference	***	**	*
Main effect of heat stress at 50% SMC			
Nil (no stress)	52.9	47.9	50.5
Heat stress at 50% SMC	71.8	60.7	66.2
LSD (5%)	**3.8**	**5.1**	**6.3**
Significance of difference	***	***	**
Treatment means			
Control [1]	46.1	42.7	44.4
Heat stress at 80% SMC	59.9	53.1	56.5
Heat stress at 50% SMC	65.7	56.3	61.0
Heat stress at (80 SMC + 50% SMC)	77.8	65.0	71.4
LSD (5%)	**5.3**	**7.2**	**8.9**
SEM	1.7	2.3	1.9
CV%	5.3	8.3	4.8
Interaction effect[2] (80% SMC X 50% SMC)	-1.7	-1.7	-1.7
L.S. interaction (5%)	**7.5**	**10.2**	**12.6**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*.
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)]/2
Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(80% SMC)]/2
Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].
LSD (Main Effect) = LSD/√2., **LS. Interaction** = LSD x√2

Table 3.9 Effect of high temperature (240°Ch) during seed development on seed conductivity (µS cm^{-1} g^{1}) of forage rape from different raceme positions in the 2011-12 and 2012-13 seasons.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	59.4	52.0	53.3	47.2	55.0	49.4
Heat stress at 80% SMC	74.0	62.6	66.5	55.0	66.1	59.6
LSD (5%)	**5.3**	**5.2**	**4.5**	**6.1**	**6.0**	**6.1**
Significance of difference	***	**	***	*	**	**
Main effect of heat stress at 50% SMC						
Nil (no stress)	56.4	51.0	50.7	45.1	51.9	47.6
Heat stress at 50% SMC	77.0	63.6	69.0	57.0	69.3	61.4
LSD (5%)	**5.3**	**5.2**	**4.5**	**6.1**	**6.0**	**6.1**
Significance of difference	***	***	***	**	***	***
Treatment means						
Control [1]	48.7	44.8	43.8	41.0	45.6	42.4
Heat stress at 80% SMC	64.1	57.2	57.5	49.3	58.1	52.8
Heat stress at 50% SMC	70.1	59.2	62.7	53.4	64.4	56.5
Heat stress at (80%SMC+ 50% SMC)	83.9	68.1	75.4	60.6	74.2	66.4
LSD (5%)	**7.4**	**7.3**	**6.3**	**8.6**	**8.5**	**8.6**
SEM	2.3	2.3	2.0	2.7	2.7	2.8
CV%	6.9	7.9	6.5	10.6	8.8	9.8
Interaction effect (80% SMC X 50% SMC)	-1.60	-3.5	-1.0	-1.1	-2.7	-0.5
L.S. interaction (5%)	**10.4**	**10.3**	**8.9**	**12.1**	**12.0**	**12.1**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non-significant; *= significant at *P< 0.05*; ** =Significant at *P< 0.01*; **= Significant at *P< 0.001*.
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC=[(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC=[(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 3.10 Comparison of Conductivity (µS cm^{-1} g^{-1}) in forage rape seeds from different raceme positions in the 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	T-M		T-B		B-M	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	6.1*	4.8*	4.4	2.5	1.7	2.3
Heat stress at 80% SMC	7.6*	7.7*	7.9*	3.1	-0.3	4.6*
LSD (5%)	4.5	2.8	8.4	4.4	5.6	6.2
Significance of difference	*ns*	*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**3.2**	**2.0**	**6.0**	**3.1**	**3.9**	**4.4**
Main effect of heat stress at 50% SMC						
Nil (no stress)	5.8*	5.9*	4.6	3.4*	1.2	2.5
Heat stress at 50% SMC	7.9*	6.6*	7.7*	2.2	0.2	4.4
LSD (5%)	**4.5**	**2.8**	**8.4**	**4.4**	**5.6**	**6.2**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**3.2**	**2.0**	**6.0**	**3.1**	**3.9**	**4.4**
Treatment means						
Control [1]	4.8*	3.8*	3.1	2.4	1.8	1.5
Heat stress at 80% SMC	6.6*	7.9*	6.1	4.4	0.5	3.5
Heat stress at 50% SMC	7.4*	5.8*	5.7	2.7	1.6	3.1
Heat stress at (80% SMC + 50% SMC)	8.6*	7.5*	9.8*	1.7	-1.2	5.8
S.E.M	1.9	1.3	3.7	1.9	2.8	2.7
LSD (5%)	**6.3**	**3.9**	**11.9**	**6.2**	**7.9**	**8.8**
LSE (5%)	**4.5**	**2.7**	**8.4**	**4.4**	**5.6**	**6.2**
Interaction effect (80% SMC X 50% SMC)	-0.6	-2.4	1.1	-3.0	-1.5	0.7
L.S. interaction (5%)	**8.9**	**5.5**	**16.8**	**8.7**	**11.1**	**12.4**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*;
[1] Plants in the pots left in the field.
An * to the right side of means indicates a significant difference between two raceme positions.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)]/2
Heat stress at 80% SMC=[(80%) + (80+50% SMC)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(80% SMC)]/2
Heat stress at 50% SMC=[(50% SMC) + (80+50% SMC)]/2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, LS**. Interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, LSE **(5%)** (Main Effect) =LSD (Main effect)/√2

3.4.5 Relationship between the two vigour tests (AA and electrical conductivity) of forage rape seed

Electrical conductivity of forage rape seed lots subjected to heat stress at particular seed development stages was plotted against AA-germination. There was a significant negative linear relationship between electrical conductivity and AA-germination in both individual seasons (2011-12 and 2012-13 Figure 3.6) and when the two season's data were combined (r = -0.981 and *P<0.05*). The variability of electrical conductivity accounted for by AA-germination was about 96% in both seasons. The greater the heat stress, the lower the AA-germination and the higher the electrical conductivity (Figure 3.6 a & b).

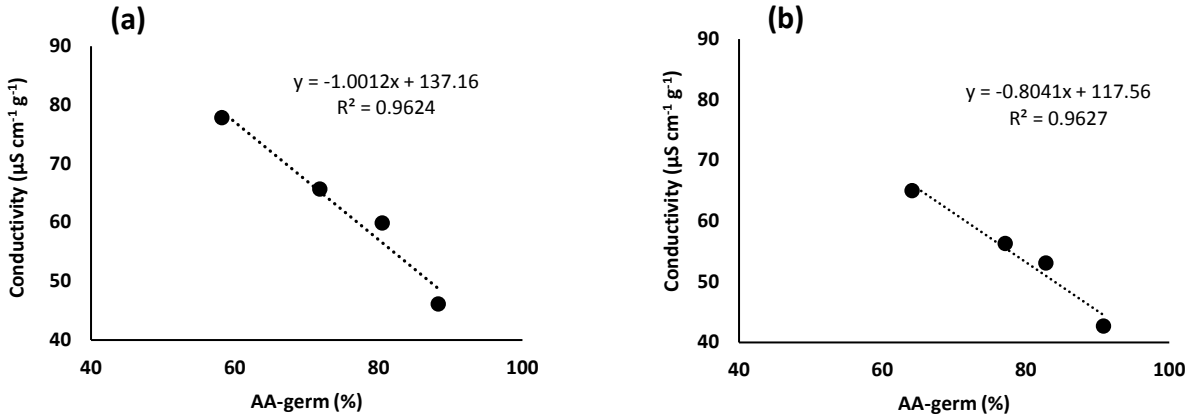


Figure 3.6 Relationship between the two vigour tests (electrical conductivity and AA) of forage rape seed lots subjected to heat stress at particular seed development stages during the (a) 2011-12 and (b) 2012-13 seasons.

3.4.6 Thousand seed weight (TSW)

Seed mass of forage rape as measured by thousand seed weight (TSW) was highest for the control plants in both seasons (Table 3.11). TSW was significantly (*P<0.001*) reduced with the heat stress at ≈80% SMC in both seasons but not at physiological maturity (≈50% SMC) (Table 3.11). There was no significant interaction for heat stress between the two seed development stages (80% SMC x 50% SMC) in either season (Table 3.11). A combined analysis of two years' data indicated that reduction in TSW was significant for heat stress at the seed filling stage (≈80% SMC) (*P <0.001*) but not significant for the PM stage (Table 3.11), and the interaction was non-significant demonstrating the consistency of the heat stress response on seed mass between the two seasons (Table 3.11).

Seed mass was highest from all raceme positions for control plants in both seasons. There was a larger negative impact of heat stress at the seed filling stage (≈80% SMC) than at PM (≈50% SMC). Heat stress

at the seed filling stage (≈80% SMC) significantly reduced TSW in both seasons in seed harvested from all three raceme positions (Table 3.12). The reduction in seed mass after heat stress at this stage of seed development was significant at *P< 0.05* and *P< 0.01* for seeds from the top and basal positions of the raceme respectively in both seasons (Table 3.12). Heat stress at PM did not produce a significant reduction in the TSW for seeds at all three raceme positions in either season. A significant reduction in TSW was caused by the double heat stress at seed filling and at PM stage relative to the non-stressed seeds in both seasons. The interaction between the double heat stresses (80% SMC x 50% SMC) was non-significant for seeds at all three positions in both seasons (Table 3.12).

Heat stress did not produce significant differences in TSW between the seeds from any raceme position (Table 3.13). There was no significant interaction between heat stress applications at the two seed development stages (80% SMC x 50% SMC) between any of the raceme positions. Middle raceme seeds had a higher TSW than that of seeds from the top section racemes in non-stressed seeds only for both seed development stages in both seasons (Table 3.13). TSW did not differ between other raceme positions (e.g. B-T and M-B) for heat stress at any seed development stage in either season (Table 3.13).

Table 3.11 Effect of high temperature (240°Ch) during seed development on forage rape thousand seed weight (TSW) over two seasons 2011-12 and 2012-13.

Treatments	TSW(g)		
	2011-12	2012-13	Mean
Main effect of heat stress at 80% SMC			
Nil (no stress)	2.91	2.83	2.87
Heat stress at 80% SMC	2.69	2.67	2.68
LSD (5%)	**0.07**	**0.04**	**0.05**
Significance of difference	***	***	***
Main effect of heat stress at 50% SMC			
Nil (no stress)	2.83	2.77	2.80
Heat stress at 50% SMC	2.78	2.73	2.75
LSD (5%)	**0.07**	**0.04**	**0.05**
Significance of difference	*ns*	*ns*	*ns*
Treatment means			
Control [1]	2.94	2.86	2.90
Heat stress at 80% SMC	2.71	2.69	2.70
Heat stress at 50% SMC	2.88	2.81	2.84
Heat stress at (80% SMC + 50% SMC)	2.67	2.65	2.66
LSD (5%)	**0.1**	**0.07**	**0.07**
SEM	0.03	0.02	0.02
CV%	2.30	1.70	0.80
Interaction effect (80% SMC X 50% SMC)	0.02	0.01	0.02
L.S. interaction (5%)	**0.14**	**0.10**	**0.10**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*.
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)]/2
Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(80% SMC)]/2
Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].
LSD (Main Effect) = LSD/√2., **LS. Interaction** = LSD x√2

Table 3.12 Effect of high temperature (240C h) during seed development on TSW of forage rape from different raceme positions in the 2011-12 and 2012-13 seasons.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	2.82	2.75	3.00	2.94	2.91	2.82
Heat stress at 80% SMC	2.64	2.66	2.75	2.71	2.68	2.63
LSD (5%)	**0.16**	**0.09**	**0.11**	**0.12**	**0.12**	**0.12**
Significance of difference	*	*	***	**	**	**
Main effect of heat stress at 50% SMC						
Nil (no stress)	2.75	2.73	2.91	2.85	2.82	2.74
Heat stress at 50% SMC	2.71	2.68	2.84	2.80	2.77	2.71
LSD (5%)	**0.16**	**0.09**	**0.11**	**0.12**	**0.12**	**0.12**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Treatment means						
Control [1]	2.82	2.74	3.05	2.97	2.95	2.87
Heat stress at 80% SMC	2.67	2.71	2.77	2.74	2.68	2.61
Heat stress at 50% SMC	2.82	2.75	2.96	2.91	2.86	2.76
Heat stress at (80 SMC + 50% SMC)	2.62	2.60	2.72	2.68	2.67	2.65
LSD (5%)	**0.22**	**0.13**	**0.16**	**0.17**	**0.17**	**0.17**
SEM	0.07	0.04	0.05	0.05	0.05	0.05
CV%	5.00	3.10	3.50	3.80	3.80	4.00
Interaction effect (80% SMC X 50% SMC)	-0.05	-0.12	0.04	-0.01	0.08	0.15
L.S. interaction (5%)	**0.31**	**0.18**	**0.23**	**0.24**	**0.24**	**0.24**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non-significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*.
[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC=[(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) -(Control)].
LSD (Main Effect) = LSD (Treatment means)/√2; **LS. Interaction** = LSD (Treatment means) x√2

Table 3.13 Comparison of TSW (g) in forage rape seeds of different raceme positions in the 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. M-T) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*)

Treatments	M-T		B-T		M-B	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of heat stress at 80% SMC						
Nil (no stress)	0.18*	0.19*	0.09	0.07	0.98	0.12
Heat stress at 80% SMC	0.10	0.06	0.04	-0.03	0.07	0.08
LSD (5%)	0.21	0.17	0.15	0.17	0.19	0.18
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**0.15**	**0.12**	**0.11**	**0.12**	**0.14**	**0.13**
Main effect of heat stress at 50% SMC						
Nil (no stress)	0.16*	0.13*	0.07	0.01	0.09	0.11
Heat stress at 50% SMC	0.13	0.12	0.05	0.03	0.07	0.09
LSD (5%)	**0.21**	**0.17**	**0.15**	**0.17**	**0.19**	**0.18**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**0.15**	**0.12**	**0.11**	**0.12**	**0.14**	**0.13**
Treatment means						
Control [1]	0.22*	0.22*	0.13	0.12	0.09	0.10
Heat stress at 80% SMC	0.09	0.03	0.01	-0.10	0.09	0.13
Heat stress at 50% SMC	0.14	0.16	0.04	0.01	0.10	0.15
Heat stress at (80% SMC+ 50% SMC)	0.11	0.08	0.06	0.05	0.05	0.32
S.E.M	0.09	0.08	0.06	0.08	0.08	0.08
LSD (5%)	**0.30**	**0.25**	**0.21**	**0.24**	**0.27**	**0.26**
LSE (5%)	**0.21**	**0.17**	**0.15**	**0.17**	**0.19**	**0.18**
Interaction effect (80% SMC X 50% SMC)	0.10	0.11	0.14	0.26	-0.05	0.14
L.S. interaction (5%)	**0.42**	**0.34**	**0.30**	**0.34**	**0.38**	**0.37**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*;
[1] Plants in the pots left in the field.
An * to the right side of means indicate the least significant difference between two raceme position.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)]/2
Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(80% SMC)]/2
Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, LS**. Interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, LSE **(5%)** (Main Effect) = LSD (Main effect)/√2

3.5 Discussion

3.5.1 Seed germination

The germination test of a seed lot (ISTA, 2016), involves the evaluation of normal and abnormal seedlings and the un-germinated seeds. The normal seedling count is reported as the percent germination of the seed lot (ISTA, 2016).Theoretically, if dormancy is not a factor, a germination percentage below 100% is a signal of seed deterioration (Dornbos, 1995). However, Hampton and Coolbear (1990) did not agree with this and suggested that for temperate species, any test result below a commercially acceptable germination percent (e.g. 90%), indicates that the performance of the seed lot has been impaired due to seed deterioration.

There are many factors which may contribute to the reduction in germination. Environmental stress (e.g. high temperature, humidity) has been reported to negatively affect seed germination (Egli et al., 2005). In our study high temperature (heat stress) and humidity did have a small negative effect on subsequent seed germination. Heat stress both at the seed filling stage (≈80% SMC) and at PM (≈50% SMC) reduced seed germination more so in the first than the second season. The reduction was marginally greater for the heat stress at physiologically maturity (PM). This stress did not kill seeds. Germination was reduced because more of the seeds which germinated in a physiological sense (protrusion of the radical) produced abnormal seedlings. Most of the abnormal seedlings produced had either missing roots or had a stunted primary root and/or shorter hypocotyl, or the emerging roots were trapped in the seed coat. Abnormal seedlings are a result of either mechanical injury to the embryo, plant pathogen attack, or deficiencies in the physiological makeup of the seed and embryo (Egli et al., 2005; Gillen et al., 2012; Wang & Hampton, 1989). As seeds were hand podded and sorted, mechanical injury was not the cause of the abnormalities found, and no pathogens were detected during the germination tests. This means that physiological deterioration of the heat stressed seeds had begun (Dornbos & McDonald, 1986; Toledo et al., 2011). Many physiological processes are involved in root production (radicle protrusion) from seed under normal conditions, including the controlled production of ROS (especially H_2O_2) and a sufficient supply of energy (Bewley & Black, 2012; McDonald, 1999) which comes from storage reserve mobilization and the synthesis of new compounds (Bailly et al., 2002a; Kupidłowska et al., 2006).Heat stress probably disturbed the balance between ROS production and ROS scavenging enzymes and partly reduced the metabolic activity necessary to continue and drive normal root growth. Uncontrolled accumulation of H_2O_2 and reduced energy supply through damage to the mitochondria can prevent radical protrusion and/or result in the production of abnormal seedlings (Bailly et al., 2008; Kupidłowska et al., 2006). This is further discussed in Chapter 5 and 6. Other authors have reported a reduction in germination in response to heat stress (Dornbos, 1995; Egli et al., 2005; Khalil et al., 2001; Ren et al., 2009; Shinohara et al., 2006a).

Brassicas have an indeterminate growth pattern and as flowering occurs over an extended period of time, the harvested seed lot may have a mixed maturity (Still, 1999), because pod setting time differs for seeds produced at different raceme positions (Vallejos et al., 2011). The position of the seeds or time of pod setting at different positions of the plant may contribute to the variation within the plant in physical (seed weight and shape) and physiological (e.g. seed viability and vigour) (Hampton et al., 1996; Illipronti et al., 2000) seed attributes.

The results obtained in this study revealed that in the first season only, seed harvested from the top raceme position had a slightly lower germination than those from other raceme positions. Seeds from the middle raceme had the highest germination, followed by those from the base and top of the raceme positions and this difference became slightly more pronounced under heat stress. The probable reason for this very small variation for raceme positions might be due to small differences in seed moisture content (SMC) (Castillo et al., 1992) before heat stress treatment. In this study, SMC was measured for seed from the middle raceme position and this was used as a reference point for deciding the seed development stage for heat stress. This is because 70-85% of the seeds are produced from the middle racemes of forage rape (Trethewey, 2009). Although, for the heat stress treatment in this study at the seed filling stage the SMC was ≈80% for seeds from the middle raceme position, the actual seed moisture content for seeds from the top and basal raceme was between 60-65% and > 85% respectively (see Table 3.1). Similarly, for heat stress at PM, SMC for the seeds from the top, middle and base was ≈35-40%, 50% and 55% respectively. The lower SMC for the seeds from top raceme position was because of the earlier flowering and pod setting (Tayo & Morgan, 1975). They therefore received a greater time per day exposure to higher temperature (Castillo et al., 1993) before and after the heat stress treatment (see Figure 2.8, Chapter 2, for weather data), suggesting that these seeds had already started to deteriorate (Dornbos, 1995; Illipronti et al., 2000; TeKrony et al., 1980).The SMC of seeds from the basal raceme was almost similar to that of middle raceme, and differences in germination were small. These findings are consistent with those of Khalil et al. (2010), who reported that heat stress during seed filling and maturation reduced seed germination, and that seed harvested from the middle position of the raceme had the highest seed germination, followed by seeds from the basal and top positions. Illipronti et al. (2000) noted that seeds produced earlier often had a poorer germination than seeds produced later and suggested that time of seed set may be more important than seed position because of time to start seed set differ for seed produced at different positions on the plant.

Although the 2012-13 season was warmer than 2011-12, the effect of heat stress on germination of seeds harvested in 2011-12 was greater than on those in 2012-13. It is possibly due to some transplanting shock experienced by the forage rape plants in the 2011-12 season. Forage rape plants at the early reproductive growth stage were transplanted into the pots in the 2011-12 season for transporting to the growth room for heat stress treatment while in the 2012-13 season, forage rape

plants were grown in pots and did not experience any unexpected stress during the reproductive stage. The negative effect of transplanting on seed germination may therefore have occurred due to transplanting shock, as previously been reported by Bell et al. (2004) and Hu et al. (1996). Although this transplanting shock and its subsequent effect on seed germination was not found in non-stressed seeds, the heat stress seeds produced a larger effect on seed germination. Whether this was the reason is not known.

3.5.2 Seed vigour

Environmental factors, particularly high temperature during seed development have been reported to have considerable influence on seed vigour (Dornbos, 1995; Wilson et al., 2004). Much of the seed quality variation reported occurs because of exposure of the mother plant to adverse environmental conditions during seed set, development and maturation (Dornbos, 1995; Kaushal et al., 2016).However, this depends upon the stage of seed development. PM is generally accepted as the stage where the seed attains its maximum seed quality and dry weight, and the supply of assimilates ceases from the parent plant; from this point the seed maturation phase starts (Bewley & Black, 1994). Seed vigour loss has been reported by many researchers, both before PM (Egli et al., 2005; Shinohara et al., 2006a; Spears et al., 1997) and post- PM (Hampton, 2000; TeKrony et al., 1980). But most of these studies were conducted on either soybean or field peas. In a New Zealand study, variation in seed vigour was significantly correlated with high temperature during seed development i.e. the higher the temperature, the lower the seed vigour (Hampton et al., 2013).

This study has shown that a short period of heat stress (30 ˚C/25 ˚C day and night, 240 ˚Ch) both before and at PM significantly reduced seed vigour in both seasons. For the AA test, heat stressed seeds still germinated physiologically, but an increased percentage of the seedlings so produced were abnormal (20-24%).This vigour loss was confirmed by the electrical conductivity results, whereby conductivity increased with heat stress during seed filling and at PM relative to the control plants in both seasons. The relationship between the two vigour tests (Figure 3.6) was strong and negative, confirming that the higher the seed conductivity, the lower the seed vigour.

High temperature during seed filling has been reported to reduce seed vigour in soybean (Egli et al., 2005). Heat stress can differentially affect the various processes involved in seed filling i.e. nutrient uptake, assimilate supply, partitioning and remobilization of nutrients and subsequently seed composition; and or all the biochemical events (Kaushal et al., 2016; Prasad et al., 2008b; Spears et al., 1997; Thuzar et al., 2010).During seed filling, assimilates are either translocated from current assimilation or from the pre-anthesis stored reserves. Heat stress may substantially alter the relative contribution of assimilate supply from current assimilation or pre-anthesis stored reserves (Blum, 1998). This reduced accumulation of assimilates may also cause weaker membrane integrity (Flinn & Pate, 1968; Powell, 1988), as recorded by increased electrical conductivity. Alternatively, heat stress

may disrupt normal structures and function of cell organelles, including cellular membranes or mitochondrial activity, thereby reducing respiratory activity (Grass & Burris, 1995b) and/or biochemical, metabolic and physiological alterations (McDonald, 1999; Priestley, 1986) e.g. ROS accumulation and reduced activity in the antioxidant enzyme system, which is also likely to affect membrane function (Fath et al., 2002) resulting in lower seed vigour. Fully functioning essential structures and physiological process are necessary for vigorous cellular activity which is acquired by seed gradually during seed development (Dornbos, 1995; Sairam et al., 2000).

Heat stress has been reported to modify the structure and functions of membranes by compositional changes of fatty acid in phospholipids resulting in fluidity of membrane lipids. These modifications in phospholipids affect many cellular functions and properties of certain membrane bound enzymes which leads to the increased leakage of ions and loss of membrane functions (Ren et al., 2009; Spector & Yorek, 1985; Taiz & Zeiger, 1998). Grass and Burris (1995a) demonstrated that heat stress applied at PM caused damage to seed so that conductivity was increased, and suggested that heat stress possibly altered seed metabolism and caused physiological changes. Increase in conductivity is well associated with heat stress in peas (Castillo et al., 1993) and in soybean (Spears et al., 1997). Dornbos et al. (1989) also reported that the impact of heat stress on phospholipids consequently decreased the ability to maintain optimum metabolic activity during seed development and germination. The results of the current study are consistent with these reports. Grass and Burris (1995b) reported that the effect of heat stress at PM was to reduce metabolic activity by reducing the level of ATP and mitochondrial activity, resulting in reduced vigour. This is further discussed in Chapter 5 and 6.

Seed location in the canopy or raceme has been reported as a parameter which can be influenced by an unfavourable environment, particularly temperature stress during seed development and maturation. High day and night temperature is significantly associated with quality of seed produced at different positions on the soybean plant (Khalil et al., 2010). Seed development occurs at different times at the different locations on the plant with respect to the distance from the main stem and photosynthates supply and is greatly influenced by temperature (Bewley & Black, 1994). Gupta et al. (2009) related seed location to the source sink relationship/competition for the availability of assimilate supply. The indeterminate growth habit of the forage rape plant results in seeds with varying developmental stages at the different raceme positions (Vallejos et al., 2011), and thus the development of seeds at these different location on the plant takes place under varying environmental conditions with regard to temperature and photoperiod (Munshi et al., 2003).

Gbikpi and Crookston (1981) reported higher quality seed from the pods on upper canopy section than that from pods on the lower canopy position in indeterminate soybean and, similarly Adam et al. (1989) observed that seeds from the top position of the plant produced higher seed quality in terms

of viability and seed weight than those from the bottom position, whilst Keigley and Mullen (1986) found higher seed quality from the middle stem section of the indeterminate soybean compared with the other sections of the plant. The results of this study demonstrated that the heat stress significantly reduced seed vigour both before and after physiological maturity in both years, and that seeds from the top raceme position had lower seed vigour then seeds from the middle raceme and basal raceme positions. These results are consistent with the results of Keigley and Mullen (1986) , Still (1999) and Illipronti et al. (2000). As stated in the previous section, this difference in seed vigour is likely to be due to differences in time of pod set and maturity levels of seeds located on the different raceme positions. Seeds produced earlier experienced higher temperature for a longer time during seed development which may have created differences in seed vigour compared with the seeds produced on the middle raceme position.

3.5.3 Seed mass

Seed mass may have an influence on the performance of a seed lot in establishing and maintaining the optimum plant population. It can play an important role in the population dynamics of plants and affects many processes e.g. seed germination (Aiken & Springer, 1995; Larsen & Andreasen, 2004), emergence of seedlings and growth rate (Zhang & Maun, 1990) and establishment (Schaal, 1980; Wang et al., 2014).

The seed industry assesses seed mass by means of seed weight and usually expresses it as thousand seed weight to help to estimate the value of a seed lot (Castro et al., 2006; Hampton et al., 2013). Seed mass is considered to be least variable component of yield due to uniformity in seed mass through breeding and grading by removing small seeds (Almekinders & Louwaars, 1999).

Variability in seed mass is influenced by ecological niche (Murray et al., 2004; Murray et al., 2003) nutrient and water availability (Castro et al., 2006), biotic and abiotic factors (Kesavan et al., 2013) and also by genetic factors (Wang et al., 2014).During the seed development phase, high temperature (heat stress) may decrease seed mass (Siddique et al., 1999; Spears et al., 1997) because of an increased seed filling rate and decrease in the length of the seed filling duration (Prasad et al., 2008a; Young et al., 2004). In the present study, heat stress during seed filling significantly reduced seed mass (TSW) during seed filling in both seasons (2011-12 and 2012-13) but heat stress at PM did not. This suggests that the heat stress disrupted normal seed development processes by reducing seed filling duration and disturbed the source-sink relationship which subsequently decreased seed weight (Prasad et al., 2015).

Heat stress has previously been reported to reduce seed mass in brassica species (Gan et al., 2004). High temperature stress during seed filling may alter seed composition and subsequently decrease seed mass (Yu et al., 2014). Gan et al. (2004) reported that a moderate high temperature regime (28/ 18 ˚C) increased seed mass, and they attributed this to a decreased seed set and subsequently seeds

per pod due to a sink limitation which allowed an increased seed mass (Mendham & Salisbury, 1995). In contrast to moderate heat stress, heat stress of 35/ 18 ˚C significantly reduced seed mass. Morrison and Stewart (2002) suggested a threshold temperature of 29.5 ˚C for brassica species (*B. napus*, *B. rapa* and *B. juncea*) during flowering and reported that temperatures above this resulted in yield loss exclusively due to a reduction in seed mass. Similar results were obtained by Aksouh-Harradj et al. (2006) who reported that a short period of high temperature (28/23 ˚C) for five days during seed filling significantly reduced seed weight of *B. napus*. The results of the present study are similar to these reports. It is likely that assimilate supply to seeds as a sink was limited due to the shortening of the seed development process due to high temperature. Therefore, fewer assimilates were translocated to seeds and seed filling became limited, which negatively affected thousand seed weight (seed mass) (Dreccer et al., 2000).

3.6 Conclusions

- In the present study, a heat stress of 30/25 ˚C for 3 days (240 ˚C h) reduced seed quality when the stress was applied both during seed filling and at the PM stage. When the data from the two seasons were combined, germination was not affected by heat stress, but seed vigour was significantly reduced by heat stress at both seed development stages in both seasons. Seed mass in terms of thousand seed weight was only affected by heat stress at the seed filling stage.
- When seeds were harvested from the three different raceme position, seeds from the top raceme position were more susceptible to heat stress for all seed quality attributes i.e. seed germination, vigour and seed mass, than those from the middle and basal racemes.

Chapter 4
Effect of high temperature stress at selected reproductive growth stages on forage brassica seed quality; field study

4.1 Introduction

One of the important environmental factors affecting the growth, yield and quality of crops is high temperature stress (Van der Merwe et al., 2015). Plants grown in the field environment often experience fluctuating high day and night temperatures that can have a profound effect on plant metabolism and plant reproductive processes (Lavania et al., 2015; Teixeira et al., 2013). Plants are more sensitive during reproductive growth than vegetative growth (Farooq et al., 2011; Nguyen et al., 2013; Prasad et al., 2006b). Adverse environmental conditions, particularly high temperature stress (HTS) during the plant reproductive phase not only have a significant negative effect on crop yield worldwide, but also on the geographical distribution of plant species (Young et al., 2004). The general reproductive processes that are negatively influenced by heat stress are pollination and fertilization, embryo growth, seed number formation, duration and rate of seed filling and seed reserve accumulation (protein, carbohydrates and fats etc.) and their mobilization (Chimenti & Hall, 2001; Prasad et al., 2008b). Many of these effects as a result of heat stress are detrimental and irreversible once they have occurred, and if these stresses are too great, can lead to the plant death. The present world trend for increasing mean temperatures could lead to intense and more frequent heat waves (Hansen et al., 2012; Meehl & Tebaldi, 2004). This frequency and intensity of heat waves during seed development will have a noticeable effect on seed yield and quality (Thomas et al., 2009). During the seed filling period for every 1 °C rise in temperature > 25 °C, final seed mass could be reduced (Chimenti & Hall, 2001).

Long durations of high temperature occurs in many crop production areas, including New Zealand, where long days with high temperature and photoperiod during seed development and maturation may negatively affect seed vigour (Hampton et al., 2013). Increases in global temperature are likely to accompanied by change in the pattern of rainfall and extreme weather events (Seimon, 2011), which may also negatively affect seed quality.

Studies on high temperature during seed development and maturation and its effects on seed quality are limited. Most previous work has been done either by applying a short episode of heat stress at particular seed development stages, or growing plants in a controlled environment with specific day/night temperature during the entire life cycle of the plants.

4.2 Objectives

The current study was initiated to determine the effect of elevating the ambient day and night temperature on seed quality in a field environment. The hypothesis was that seed quality, in terms of seed germination, vigour and seed mass, would decrease as a result of elevated field temperatures during seed development.

4.3 Materials and methods

This experiment was designed to increase the ambient field temperature by covering a section of the March sown forage rape plots (see Section 2.3) with clear plastic sheets during seed development and maturation to assess the effects of elevated temperature on seed quality attributes i.e. seed germination, vigour and seed mass. The details of the 2011-12 March sown plots were described in Section 2.3. The experiment was repeated in the 2012-13 season.

Beginning at 10-15 days after flowering, when pod development had started, seed moisture content was determined by hand sampling all pods from three randomly selected plants from the middle of each of the control plots in a block. The pods were hand harvested from the middle of the racemes, placed into plastic bags and then transported directly to the seed laboratory where the seeds were hand removed from the pods and seed moisture content (SMC) determined using the low constant temperature oven method (see Section 2.3.3.1). SMC of control plots was then monitored weekly to estimate the onset of each seed development stage. SMC of control plots was also measured for seeds developing at each of the three raceme positions (see Section 2.3). However, only SMC of seeds from middle of the raceme position was used as the reference point to assess each seed development stage. Ambient field temperature was increased over three seed development stages as follows:

1. Phase-I from seed filling (≈ 80% SMC) to PM (45-50% SMC)
2. Phase-II from PM (45-50% SMC) to HM (≈ 14% SMC)
3. Phase-III from seed filling to HM (phase-I + phase-II)

When the seeds had reached the particular seed development stage i.e. seed filling (≈80% SMC) and PM (≈50% SMC), a section of each plot (2.7 x 2 m) was covered with plastic sheet supported on iron posts around the plots (Figure 4.1), to increase the ambient field temperature. Some open spaces were left in the clear plastic sheets to allow free air movement inside the cages.

There were therefore four treatments as follows:

1. Treatment (T_0) (Control). No covering. Plants in these plots were exposed to ambient field temperature during the entire seed development and maturation period.

2. Treatment (T_1). Covered with plastic sheets during phase-I of seed development

3. Treatment (T_2). Covered with plastic sheets during phase-II of seed development

4. Treatment ($T_3 = T_1 + T_2$). Covered with plastic sheets during the entire period of seed development (phase-I + phase-II).

Hourly mean temperature and relative humidity (RH) were measured in both the control and covered plots using HOBO series-8 data loggers. Inside the plastic sheet covered plots, the data loggers were placed on a wooden pole above the plant canopy. Ambient field temperature was recorded by installing the Hobo data loggers 1m above the canopy of the control plots. The environmental data before and after elevated temperature treatments and during particular seed development phases were also recorded.

Hourly temperature was used rather than daily mean temperature as daily mean temperature rarely exceeded 25 ˚C. Hourly mean temperature data were recorded for each of the treatments and converted to the number of degree hours exceeding the base temperature (Tb) of 25 ˚C (using a minimum of 1˚C temperature increase above 25 ˚C, not less than 1 ˚C). The hourly thermal time was also measured as an integral of hourly mean temperature above the base temperature (Tb=25 ˚C) during these phases by using the formula ($\Sigma [(T_{min} + T_{max})/2 - Tb]$), where Tb, T_{min} and T_{max} are the base, minimum and maximum temperatures respectively.

Figure 4.1 A section (2.7 x 2 m) in each replicate covered with plastic sheeting to elevate the temperature.

4.3.1 Collection of data

At harvest maturity (14% SMC) all plants from within each plot (but excluding border rows) were cut at the base of the raceme and the raceme divided into top (T), middle (M) and base (B) sections (see Figure 3.2, Section 3.3) and placed into bags separately. All pods from each raceme position were hand removed. Seeds were then removed by hand from the pods and tested for quality i.e. germination, vigour (conductivity and AA tests) and seed mass (thousand seed-weight).The details of all these methods are provided in Chapter 2 (Section 2.3.3).

4.3.2 Statistical analysis

The four treatments were arranged in a randomized complete block design with four replications of each treatment. The effect of heat stress (T_1 and T_2) during two seed development phases on seed quality was analysed as treatment factors. Each treatment factor had two levels T_1 (+,-) and T_2 (+, -), which made up a 2 x 2 factorial design.

Results of quality tests were analysed using analysis of variance for 4 blocks with 2 x 2 factorial treatments. Means of quality tests i.e. seed germination, vigour (Conductivity and AA-test) and thousand seed weight (TSW) were compared using the least significant difference (LSD) at $P < 0.05$ using Genstat Software (16th Edition, VSN International ltd, Hemel Hempstead, UK).

To determine seed quality variations among the three raceme positions i.e. top, middle and base, the statistical measure “least significant effect” (LSE) was calculated to determine if the difference between one raceme position and other (e.g. middle (M) and top (T) was significant, i.e. different from zero. If the difference in seed quality attributes between two raceme positions was greater than its LSE, the difference was significant.

4.4 Results

4.4.1 Environmental conditions during seed development

In the 2011-12 season, daily maximum and minimum field temperature during phase-I of seed development was 27.2 ˚C and 6.9 ˚C (Figure 4.2), while in the next season (2012-13) the daily mean maximum and minimum temperature during this phase was 29.3 ˚C and 3.4 ˚C (Figure 4.3). During phase-II of seed development (after PM), the maximum and minimum temperature in the 2011-12 season was 28.4 ˚C and 3.4 ˚C (Figure 4.2), but in the second season these temperatures were 31.6 ˚C and 2.5 ˚C (Figure 4.3).

The period between when developing seeds were at approximately ≈ 80% SMC and when they reached 14% SMC was 39 days in 2011-12 and 44 days in 2012-13 in the control plots (Table 4.1). Ambient temperature was warmer in the second season than in the first season, with 30 h above 25 ˚C being

reached over this time in 2012-13 compared with only 9 h in 2011-12. Hourly thermal time above the Tb of 25 ˚C was 15.6 ˚Ch in the first season and 30.0 ˚C h in the second season (Table 4.1). The difference was mostly explained by the warmer temperature both before and after PM in the 2012-13 season.

Covering the plots prior to PM (phase-I) increased the time to reach PM by 3-4 days. This delay was mostly explained by slow seed moisture loss due to humid conditions experienced by the plants inside the covered plots (Table 4.1). During this time there were 40 hours when the temperature exceeded 25 C˚ in the first season and 84 h in the second season, resulting in an hourly thermal time of around 101.3 ˚C h and 255.5 ˚C h respectively. Heat stress prior to PM (phase-I) increased HTT by 111 ˚C hours and 283˚C hours respectively, while the number of hours >25 ˚C after the covers were removed, were 47 and 102 hours in the 2011-12 and 2012-13 respectively. Likewise, when section of plots were covered after PM to raise ambient temperature (phase-II), this increased hours above 25 ˚C by 118 h in 2011-12 and 159 h in 2012-13, which resulted in an HTT of 334.4 ˚C h and 577.6 ˚C h in the 2011-12 and 2012-13 seasons respectively (Table 4.1). Therefore, the total time above 25 ˚C and HTT experienced by the plants between periods from 80% SMC to reach 14 % SMC for treatment T_2 was 121 h and 340.1 ˚C h in the 2011-12 season and 173 h and 605.7 ˚C h in the 2012-13 season (Table 4.1).

When the sections of the plots were covered with plastic sheets for the entire seed development period (phase-I + phase-II), the hourly temperature above 25 ˚C increased to 149 and hourly thermal time (HTT) to 425.2 ˚C h in the 2011-12 season. During the 2012-13 season, the hourly increase in the temperature above 25 ˚C was 228 which resultantly increased HTT to 783.2 ˚C h (Table 4.1).

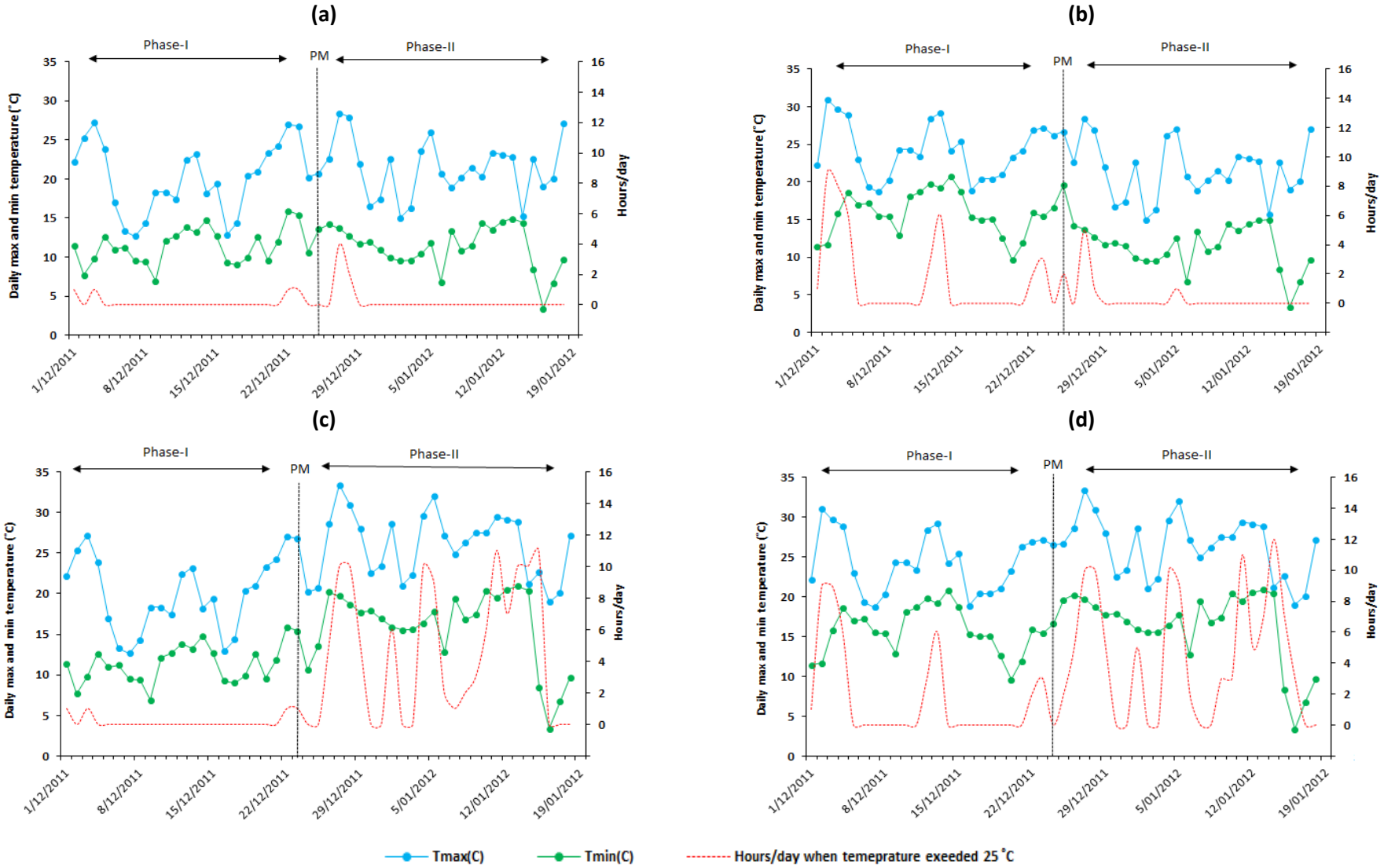


Figure 4.2 Daily maximum and minimum temperature and number of hours per day when temperature exceeded 25 ˚C in the 2011-12 season. (a) Control plants, section of plots exposed to ambient field temperature (b) section of plots covered with plastic sheets during phase-I (c) section of plots covered with plastic sheets during phase-II (d) section of plots covered with plastic sheets during phase-I + phase-II

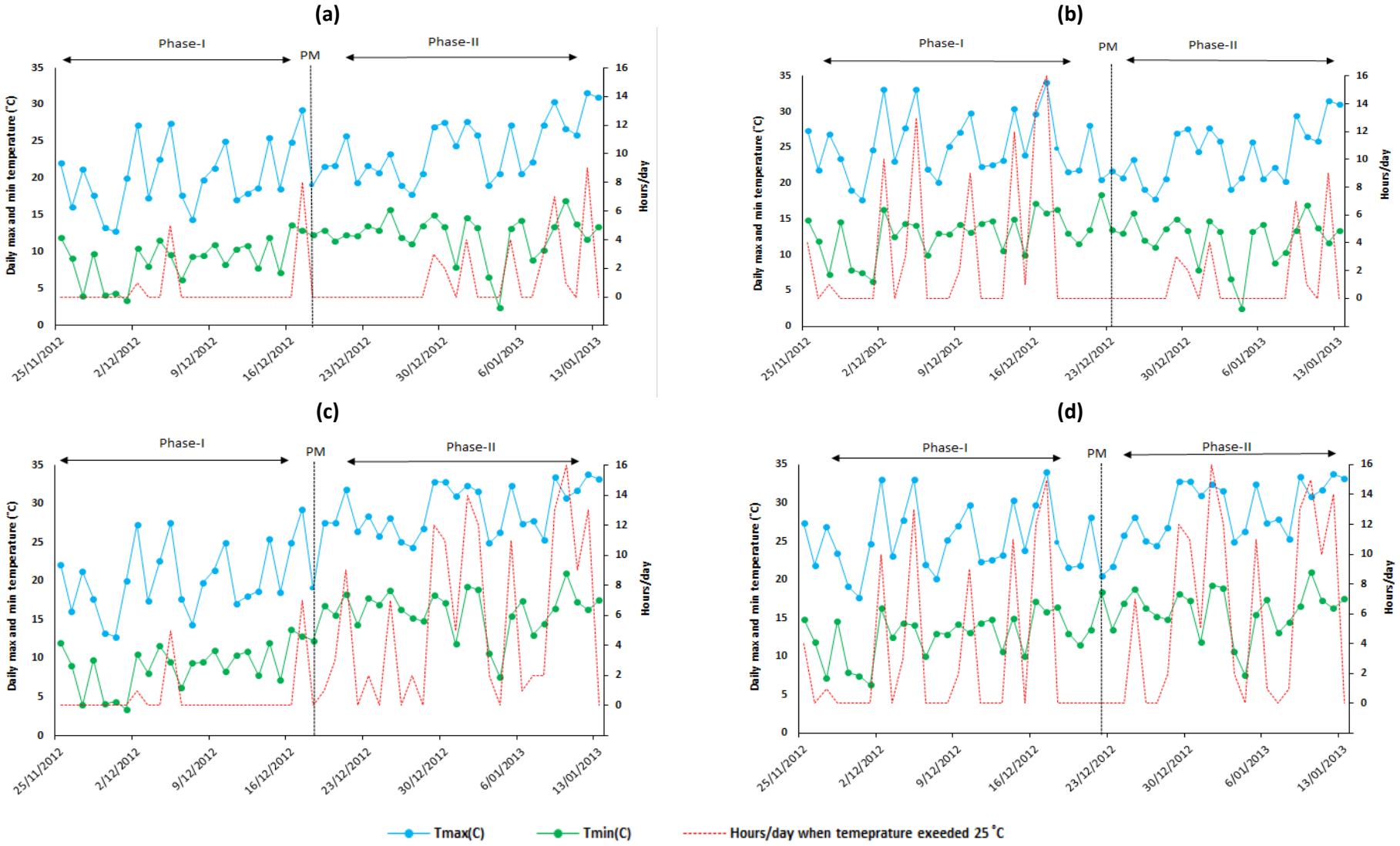


Figure 4.3 Daily maximum and minimum temperature and number of hours per day when temperature exceeded 25 ˚C in the 2012-13 season.
(a) Control plants, section of plots exposed to ambient field temperature (b) section of plots covered with plastic sheets during phase-I
(c) section of plots covered with plastic sheets during phase-II (d) section of plots covered with plastic sheets during phase-I + phase-II

Table 4.1 Increase in air temperature at different reproductive growth stages of forage plants during seed development in (2 x 2.7 m) plots covered with plastic sheets to raise the temperature (above ambient) during different seed development stages, hourly thermal time (HTT; Tb = 25 ˚C) and number of hours of exposure to elevated temperature exceeding 25 ˚C during the period of seed development when plants were covered with plastic sheets at ≈80% - 50% SMC (PM), PM -14% SMC (HM) and from 80% SMC to HM.

Treatment	Growing season	Phase-I (before PM) SMC (≈ 80-50%)				Phase-II (after PM) SMC (≈50-14%)				Total (Phase-I + Phase-II) SMC (≈80-14%)		
		Period (Days)	No. of hours (>25 ˚C)	HTT (˚C h)	R.H (%)	Period (Days)	No. of hours >25 ˚C	HTT (˚C h)	R.H %	No. of hours (>25 ˚C)	HTT (˚C h)	R.H (%)
T_0 (Control)	2011-12	2 Dec-23 Dec	3	6.7	81	24 Dec-10 Jan	6	8.9	78	9	15.6	79
	2012-13	25 Nov-18 Dec	14	28.1	75	19 Dec-8 Jan	16	23.4	72	30	51.5	74
T_1	2011-12	2 Dec-26 Dec	40	101.3	86	27 Dec-13 Jan	7	9.7	79	47	111.0	82
	2012-13	25 Nov-22 Dec	84	255.5	78	23 Dec-11 Jan	18	27.5	69	102	283.0	74
T_2	2011-12	2 Dec- 23 Dec	3	6.7	81	24 Dec-15 Jan	118	333.4	82	121	340.1	81
	2012-13	25 Nov-18 Dec	14	28.1	75	19 Dec-13 Jan	159	577.6	71	173	605.7	73
$T_3 = (T_1+T_2)$	2011-12	2 Dec-25 Dec	38	96.9	87	26 Dec-16 Jan	111	328.3	81	149.0	425.2	84.1
	2012-13	25 Nov-22 Dec	81	247.5	80	23 Dec-14 Jan	147	535.7	70	228.0	783.2	76

T_0 = Control plants, section of plots not covered with plastic sheets. Temperature (HTT and hours > 25 ˚C) per day of plots were monitored throughout the reproductive growth during seed development and maturation.

T_1 = Plants covered with plastic sheets at ≈80% SMC and removed at P.M (≈50% SMC).

T_2 = Plants covered with plastic sheets at ≈50% SMC and removed at H.M (≈14% SMC).

T_3 = **T_1+T_2** Plants covered with plastic sheets at ≈80% SMC and removed at H.M (≈14% SMC)

4.4.2 Seed moisture content (SMC) at different raceme positions

The seed moisture contents were obtained from the three raceme positions i.e. top, middle and base. The target SMC was set as a reference point for heat stress treatment at the particular seed development stages (Table 4.2). The top raceme seeds always had a lower SMC and the base raceme seeds had a higher moisture content than the target SMC (Table 4.2).

Table 4.2 Seed moisture content (%) of seeds at different raceme position.

Target SMC	Seed moisture content (SMC) at the time of heat stress application		
	Top raceme seeds	Middle raceme seeds	Base raceme seeds
2011-12 season			
≈80% SMC	73	82	86
≈50% SMC	42	51	55
2012-13 season			
≈80% SMC	70	80	88
≈50% SMC	44	49	60

4.4.3 Seed quality

4.4.3.1 Seed germination

Seed obtained from control plants had a germination of 92% in both seasons (Table 4.3). A negative influence of elevated temperature on seed germination was observed in both seasons 2011-12 (Table 4.3). Heat stress treatment both before PM (phase-I) and after PM (phase-II) reduced germination (Table 4.3). Heat stress during phase-I (T_1) reduced seed germination in both years; however, the germination reduction due to heat stress at this time was greater in the 2012-13 season (*P<0.001*) compared to the 2011-12 season (*P<0.05*) (Table 4.3). Also, heat stress during phase-II had a greater effect than during phase-I, reducing seed germination in both seasons (*P<0.001*) (Table 4.3). The interaction of the two treatment factors (T_1 x T_2) for seed germination was non-significant in both seasons (*P = 0.382* and *P = 0.331* respectively).

As an individual treatment, heat stress (T_1) during phase-I of seed development caused a larger reduction in seed germination in the 2012-13 (-7%) than in the 2011-12 season (-2%) (Table 4.3). Heat stress during phase-II of seed development (T_2) significantly reduced seed germination by 12 % in the 2012-13 season but only by 4% in the 2011-12 season. The largest reduction in germination occurred in T_3, when plants were exposed to the elevated temperature stress during the entire period of seed

development and maturation (Phase-I+II). Again, the reduction was greater in 2012-13 (-16%) than in 2011-12 (-7%).

The mean data over the two seasons indicate that the main effect of treatment (T_1) did not differ from the control (Table 4.3). However, the main effect of treatment (T_2) was significant in the combined analysis, although there was no interaction between T_1 and T_2 (Table 4.3).

For treatment means, the control germination was >90% for seeds from all three positions on the raceme in both seasons (Table 4.4). Heat stress before PM (T_1) did not reduce germination at any position in 2011-12, but significantly reduced germination at all three positions on the raceme in 2012-13. Heat stress after PM (T_2) significantly reduced germination at all three raceme positions in both seasons, as did heat stress both before and after PM (T_3). There were no significant interactions between T_1 and T_2 at any raceme position in either season.

For the main effect of T_1 in 2011-12, germination was reduced only at the basal (B) position, but in the next season germination was reduced significantly at all three positions (*P<0.001*) (Table 4.4). For the main effect of T_2, germination was significantly reduced at all three positions in both seasons.

Where germination loss occurred, it was always greater in 2012-13 (*P< 0.001*) than in 2011-12 (*P<0.01*) e.g. for T_3 at the top of the raceme. The germination loss was nearly always explained by a corresponding increase in the percentage of abnormal seedlings (stunted roots, deformed and stunted cotyledons, or roots which were trapped in the seed coat) rather than dead seeds.

A statistical comparison of germination of seeds from the three raceme positions was achieved using the “least significant effect” (LSE) (Table 4.5). There was no significant difference in seed germination between seeds of any raceme position in control plants i.e. (M-T), (B-T) and (M-B) in either the 2011-12 or 2012-13 seasons (Table 4.5). The germination of seeds from the middle section of the raceme was greater than that of seeds from pods of the top section of the raceme (M-T) in all three heat stress treatments in the 2012-13 season and in treatment (T_1) in 2011-12. No significant variation (B-T) was found in germination between seeds harvested from the top and base sections of the raceme in any treatment in both years. Likewise, middle section seeds had significantly higher germination than that of seeds from the base section (M-B) for treatments (T_1) and (T_2) in 2012-13, and for treatments (T_1) and (T_1+T_2) in 2011-12 (Table 4.5).

Table 4.3 Effect of elevated temperature during seed development on seed germination (%) of forage rape in the 2011-12 and 2012-13 seasons.

Treatments	Germination (%)		
	2011-12	**2012-13**	**Mean**
Main effect of T_1			
Nil (no stress)	90.7	87.0	88.9
T_1 (HS at ≈80% →50% SMC)	88.9	80.8	84.9
LSD (5%)	**1.3**	**1.3**	**6.6**
Significance of difference	*	***	*ns*
Main effect of T_2			
Nil (no stress)	92.1	89.0	90.6
T_2 (HS at ≈50% →14% SMC)	87.5	78.8	83.2
LSD (5%)	**1.3**	**1.3**	**6.6**
Significance of difference	***	***	*
Treatment means			
T_0 (Control)	92.8	92.4	92.6
T_1 (HS at ≈80% →50% SMC)	91.5	85.7	88.6
T_2 (HS at ≈50% →14% SMC)	88.7	81.6	85.2
T_3 (HS at 80%→14% SMC)	86.3	76.0	81.2
LSD (5%)	**1.9**	**1.8**	**9.3**
SEM	0.6	0.6	2.1
CV%	1.3	1.4	3.4
Interaction effect (T_1x T_2)	-1.1	1.2	0.0
L.S. interaction (5%)	**2.7**	**2.5**	**13.1**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*

Main effect of T_1

Nil (no stress) = $[(T_0) + [(T_2)] /2$

$T_1 = [(T_1) + (T_3)]/2$

Main effect of T_2

Nil (no stress) = $[(T_0) + (T_1)] /2$

$T_2 = [(T_2) + (T_3)]/2$

Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 – T_0]$

LSD (Main Effect) = LSD (Treatment means) /√2; LS. Interaction = LSD (Treatment means) x√2

Table 4.4 Effect of elevated temperature during seed development on seed germination (%) of forage rape from different raceme positions during 2011-12 and 2012-13.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	89.1	86.0	91.8	89.0	91.3	86.0
T_1 (HS at ≈80% →50% SMC)	88.0	78.9	90.9	83.3	87.8	80.4
LSD (5%)	**2.3**	**1.8**	**1.5**	**1.3**	**2.6**	**2.3**
Significance of difference	*ns*	***	*ns*	***	*	***
Main effect of T_2						
Nil (no stress)	91.0	88.0	94.1	90.9	91.6	88.3
T_2 (HS at ≈50% →14% SMC)	87.0	76.9	88.5	81.4	87.4	78.1
LSD (5%)	**2.3**	**1.8**	**1.5**	**1.3**	**2.6**	**2.3**
Significance of difference	**	***	***	***	**	***
Treatment means						
T_0 (Control)	91.0	92.0	94.3	93.8	93.0	91.5
T_1 (HS at ≈80% →50% SMC)	90.3	84.0	94.0	88.0	90.3	85.0
T_2 (HS at ≈50% →14% SMC)	87.3	80.0	89.3	84.3	89.5	80.5
T_3 (HS at 80%→14% SMC)	86.0	73.8	87.8	78.5	85.3	75.8
LSD (5%)	**3.3**	**2.5**	**2.1**	**1.8**	**3.7**	**3.3**
SEM	1.0	0.8	0.7	0.8	1.2	1.0
CV%	2.3	1.9	1.5	1.3	2.6	2.5
Interaction effect (T_1x T_2)	-0.5	1.8	-1.3	0.0	-1.5	1.8
L.S. interaction (5%)	**4.6**	**3.6**	**3.0**	**2.5**	**5.2**	**4.6**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P < 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P <0.001*

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)] /2$
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)] /2$
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 – T_0]$
LSD (Main Effect) = LSD (Treatment means) /√2; **LS. Interaction** = LSD (Treatment means) x√2

Table 4.5 Comparison of standard germination (%) in forage rape seeds from different raceme positions during 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. M-T) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*), as judged using the LSE=least significant effect.

Treatments	M-T		B-T		M-B	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	2.6*	3.0*	2.1	0.0	0.5	3.0*
T_1 (HS at ≈80% →50% SMC)	2.8*	4.4*	-0.4	1.5	3.1*	2.9*
LSD (5%)	**3.3**	**2.2**	**3.3**	**2.4**	**2.4**	**2.3**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*	*ns*
LSE (5%)	**2.3**	**1.5**	**2.4**	**1.7**	**1.7**	**1.6**
Main effect of T_2						
Nil (no stress)	3.5*	2.9*	1.0	0.3	2.5*	2.6*
T_2 (HS at ≈50% →14% SMC)	2.8*	4.5*	0.8	1.3	1.1	3.3*
LSD (5%)	**3.3**	**2.2**	**3.3**	**2.4**	**2.4**	**2.3**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**2.3**	**1.5**	**2.4**	**1.7**	**1.7**	**1.6**
Treatment means						
T_0 (Control)	3.3	1.8	2.0	-0.5	1.3	2.3
T_1 (HS at ≈80% →50% SMC)	3.8*	4.0*	0.0	1.0	3.8*	3.0*
T_2 (HS at ≈50% →14% SMC)	2.0	4.3*	2.3	0.5	-0.3	3.8*
T_3 (HS at 80%→14% SMC)	1.8	4.8*	-0.8	2.0	2.5*	2.8*
S.E.M	1.4	1.0	1.5	1.1	1.0	1.0
LSD (5%)	**4.6**	**3.1**	**4.7**	**3.4**	**3.4**	**3.2**
LSE (5%)	**3.3**	**2.2**	**3.3**	**2.4**	**2.4**	**2.3**
Interaction effect (T_1x T_2)	-0.8	-1.8	-1.0	0.0	0.3	-1.8
L.S. interaction (5%)	**6.5**	**4.3**	**6.6**	**4.8**	**4.7**	**4.5**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*, ***= Significant at *P<0.001*

Main effect of T_1
Nil (no stress) = [(T_0) + [(T_2)] /2
T_1= [(T_1) + (T_3)]/2
Main effect of T_2
Nil (no stress) = [(T_0) + (T_1)] /2
T_2 = [(T_2) + (T_3)]/2
Interaction Effect **(** T_1x T_2**)** = [(T_3)-(T_1)]-[T_2 - T_0]
LSD (Main Effect) = LSD (Treatment means) /√2, LS**. interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, **LSE (5%) (Main Effect)** = LSD (Main effect)/√2

4.4.3.2 Seed vigour- Accelerated ageing

Germination of seeds harvested from control plants after accelerated ageing (AA) was ≈ 90% in both seasons (Table 4.6).Treatments T_1, T_2 and T_3 all significantly reduced seed vigour in both seasons (Table 4.5).The main effect of heat stress before PM (T_1) significantly reduced seed vigour in both seasons *(P <0.001)* (Table 4.6). Similarly, significant seed vigour loss was also found for heat stress treatment (T_2) after PM *(P< 0.001)* in both seasons (Table 4.6). However, the negative effect of heat stress was larger for plants exposed to this stress during the entire period of seed development and maturation (phase-I + phase-II) in 2012-13 than in 2011-12 (Table 4.6). The interaction of the two treatment factors (T_1 X T_2) was non-significant in 2011-12 *(P= 0.791)* but was significant in the 2012-13 season *(P <0.05).*

During the post-PM (phase-II), heat stress produced a larger decline in seed vigour than that of heat stress before PM (phase-I) in both seasons. For the treatment means, heat stress (T_1) had a similar effect, reducing AA-germination by 6-7% in both seasons. For the heat stress (T_2), the reduction in AA-germination was larger in 2012-13 (13%) than in 2011-12 (9%) (Table 4.5). The largest reduction in AA-germination was found for heat stress (T_3) (phase-I+II) in both seasons. The reduction was larger in 2012-13 (32%) then in 2011-12 (15%) (Table 4.6).

The response of elevated temperature during the phase-I (T_1) and the interaction of treatments during the entire seed development period did not result in significant seed vigour loss as expressed by the combined data analysis over two seasons (2011-12 and 2012-13). However, the effect of treatment (T_2) reducing seed vigour was consistent over the two seasons (Table 4.6).

Table 4.6 Effect of elevated temperature during seed development on AA- germination (%) of forage rape in the 2011-12 and 2012-13 seasons.

Treatments	AA-germination (%)		
	2011-12	**2012-13**	**Mean**
Main effect of T_1			
Nil (no stress)	85.4	82.4	83.9
T_1 (HS at ≈80% →50% SMC)	79.6	69.0	74.3
LSD (5%)	**2.1**	**5.6**	**12.1**
Significance of difference	***	***	*ns*
Main effect of T_2			
Nil (no stress)	87.2	85.2	86.2
T_2 (HS at ≈50% →14% SMC)	77.8	66.2	72.0
LSD (5%)	**2.1**	**5.6**	**12.1**
Significance of difference	***	***	*
Treatment means			
T_0 (Control)	90.2	88.8	89.5
T_1 (HS at ≈80% →50% SMC)	84.2	81.6	82.9
T_2 (HS at ≈50% →14% SMC)	80.6	75.9	78.2
T_3 (HS at 80%→14% SMC)	75.1	56.5	65.8
LSD (5%)	**2.9**	**7.9**	**17.0**
SEM	0.9	2.5	3.8
CV%	2.2	6.5	7.1
Interaction effect (T_1x T_2)	0.5	-12.2	-4.8
L.S. interaction (5%)	**4.1**	**11.2**	**24.0**
Significance of interaction	*ns*	*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)] /2$
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)] /2$
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 – T_0]$
SD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Heat stress (T_1) before PM did not reduce vigour of seeds harvested from the top (T) of the raceme position in 2011-12 and seeds from the middle and basal racemes in 2012-13, however, T_1 reduced vigour of seeds from the top (T) of the raceme in 2012-13 and from the middle and basal raceme in 2011-12 (Table 4.7). Heat stress after PM (T_2) significantly reduced seed vigour at all three raceme positions in both seasons. The largest negative effect on seed vigour was found for heat stress both before and after PM (T_3) in seeds harvested from all three raceme positions (Table 4.7). There was a significant interaction between heat stress treatments (T_1 x T_2) with respect to the vigour of seeds harvested from the top (T) and middle (M) of the raceme in the 2012-13 season *(P<0.05)* but not in the 2011-12 season (Table 4.7). There was no significant interaction for seeds from the base (B) of the raceme in either season (Table 4.7).

For the main effect of heat stress (T_1) in 2011-12, seed vigour was significantly reduced at the top (T), middle (M) and base (B) raceme positions in both seasons (Table 4.7). Heat stress (T_2) also significantly reduced seed vigour at all three raceme positions in both seasons *(P<0.001)* (Table 4.7).

Heat stress, either applied before or after PM, produced differences in seed vigour between seeds harvested from the middle (M) and top (T) raceme positions in both seasons for both stressed and non-stressed seeds, and this difference in seed vigour was also significant in the 2012-13 season for both heat stress treatments (Table 4.8). Seeds from the middle position of the raceme position had higher vigour than seeds from the top raceme seeds (M-T) for both non-heat stressed (Control) and stressed seeds in both seasons (Table 4.8), however, the difference in vigour (M-T) was more pronounced for heat stress for both the main effect of heat stress treatments T_1 and T_2 (*P<0.05* and *P<0.01* respectively) in 2012-13 season (Table 4.8). Seeds from the basal position of the raceme had higher vigour, than those from the top of the raceme *(P<0.01)* for T_1 in the 2012-13 season but not in 2011-12, and there were no significant difference for T_2 in either season. There was no significant interaction between T_1 and T_2 heat stress treatments with regard to differences in seed vigour between any of the raceme positions (Table 4.8).

For treatment means, seeds from the middle section of the raceme had higher vigour than those from the top of the raceme section for all three treatments in 2012-13 but only for T_2 and T_3 in the 2011-12 season (Table 4.8). Similarly basal raceme seeds had higher vigour than from the top of the raceme position seeds for T_2 and T_3 in 2011-12 but only for T_3 in 2012-13 (Table 4.8).There was no difference in seed vigour between the middle and basal raceme positions for T_3 in either season but was higher vigour for T_1 in 2011-12 and for T_2 in 2012-13 (Table 4.8).

Table 4.7 Effect of elevated temperature seed development on AA- germination (%) of forage rape from different raceme position during 2011-12 and 2012-13.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	82.1	81.6	87.1	85.0	86.9	80.5
T_1 (HS at ≈80% →50% SMC)	76.5	66.2	82.8	71.8	79.6	69.3
LSD (5%)	**4.1**	**6.0**	**3.6**	**5.6**	**2.1**	**5.9**
Significance of difference	*	***	*	***	***	**
Main effect of T_2						
Nil (no stress)	85.5	83.9	89.6	87.0	86.4	84.8
T_2 (HS at ≈50% →14% SMC)	73.1	63.9	80.3	69.8	80.1	65.0
LSD (5%)	**4.1**	**6.0**	**3.6**	**5.6**	**2.1**	**5.9**
Significance of difference	***	***	***	***	***	***
Treatment means						
T_0 (Control)	88.0	88.2	92.3	90.2	90.3	88.0
T_1 (HS at ≈80% →50% SMC)	83.0	79.5	87.0	83.8	82.5	81.5
T_2 (HS at ≈50% →14% SMC)	76.3	75.0	82.0	79.8	83.5	73.0
T_3 (HS at 80%→14% SMC)	70.1	52.8	78.5	59.8	76.8	57.0
LSD (5%)	**5.8**	**8.5**	**5.1**	**7.9**	**2.9**	**8.3**
SEM	1.8	2.6	1.6	2.5	0.9	2.6
CV%	4.6	7.2	3.7	6.3	2.2	6.9
Interaction effect (T_1x T_2)	-1.2	-13.5	1.8	-13.6	1.0	-9.5
L.S. interaction (5%)	**8.2**	**11.9**	**7.2**	**11.1**	**4.1**	**11.7**
Significance of interaction	*ns*	*	*ns*	*	*ns*	*ns*

ns= Non significant; *= Significant at P< *0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001*.

Main effect of T_1
Nil (no stress) = [(T_0) + [(T_2)] /2
T_1= [(T_1) + (T_3)]/2
Main effect of T_2
Nil (no stress) = [(T_0) + (T_1)] /2
T_2 = [(T_2) + (T_3)]/2
Interaction Effect (T_1x T_2) = [(T_3)-(T_1)]-[T_2 – T_0]
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 4.8 Comparison of AA- germination (%) in forage rape seeds from different raceme positions in the 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	M-T		B-T		M-B	
	2011-12	**2012-13**	**2011-12**	**2012-13**	**2011-12**	**2012-13**
Main effect of T_1						
Nil (no stress)	5.0*	3.4*	4.7*	-1.1	0.3	4.5
T_1 (HS at ≈80% →50% SMC)	6.3*	5.7*	3.2	3.1*	3.1*	2.5*
LSD (5%)	**5.1**	**1.7**	**4.8**	**2.6**	**3.8**	**3.3**
Significance of difference	*ns*	*	*ns*	**	*ns*	*ns*
LSE (5%)	**3.6**	**1.2**	**3.4**	**1.8**	**2.7**	**2.3**
Main effect of T_2						
Nil (no stress)	4.1*	3.2	0.9	0.9	3.3*	2.3
T_2 (HS at ≈50% →14% SMC)	7.2*	5.9	7.0*	1.1	0.1	4.8*
LSD (5%)	**5.1**	**1.7**	**4.8**	**2.6**	**3.8**	**3.3**
Significance of difference	*ns*	**	*	*ns*	*ns*	*ns*
LSE (5%)	**3.6**	**1.2**	**3.4**	**1.8**	**2.7**	**2.3**
Treatment means						
T_0 (Control)	4.2	2.0	2.2	-0.3	2.0	2.3
T_1 (HS at ≈80% →50% SMC)	4.0	4.3*	-0.5	2.0	4.5*	2.3
T_2 (HS at ≈50% →14% SMC)	5.8*	4.8*	7.2*	-2.0	-1.5	6.8*
T_3 (HS at 80%→14% SMC)	8.5*	7.0*	6.8*	4.2*	1.8	2.8
S.E.M	2.3	0.8	2.1	1.1	1.7	1.4
LSD (5%)	**7.2**	**2.4**	**6.7**	**3.6**	**5.4**	**4.6**
LSE (5%)	**5.1**	**1.7**	**4.8**	**2.6**	**3.8**	**3.3**
Interaction effect (T_1x T_2)	2.9	-0.1	2.3	4.0	0.8	-4.0
L.S. interaction (5%)	**10.2**	**3.4**	**9.4**	**5.1**	**7.6**	**6.5**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001*.

Main effect of T_1
Nil (no stress) = [(T_0) + [(T_2)] /2
T_1= [(T_1) + (T_3)]/2
Main effect of T_2
Nil (no stress) = [(T_0) + (T_1)] /2
T_2 = [(T_2) + (T_3)]/2
Interaction Effect **(** T_1x T_2**)** = [(T_3)-(T_1)]-[T_2 - T_0]
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, **LSE(5%) (Main Effect)** = LSD (Main effect)/√2

4.4.3.3 Conductivity test

For both seasons, the seeds harvested from control plants had the lowest conductivity. All three heat stress treatments significantly increased conductivity. Heat stress before PM (T_1) significantly increased conductivity in both seasons relative to the control, as did heat stress after PM (T_2) (Table 4.9). The largest increase in conductivity was found for heat stress (T_3) (phase-I + phase-II). There was no significant interaction between T_1 and T_2 in either season, (*P=0.185* and *P=0.451* respectively*)* (Table 4.9).

The main effect of heat stress before PM (T_1) was to significantly increase conductivity of seeds in both seasons (*P<0.001*). Similarly, an increase in conductivity was also found for the main effect of heat stress after PM (T_2) in both seasons (*P<0.001*) (Table 4.9).

The two years data indicate that conductivity of forage rape seed under different heat stress treatments ranged from 41.3 to 84.0 µS cm^{-1} g^{-1} in 2011-12 and 48.5 to 94.2 µS cm^{-1} g^{-1} in the 2012-13 season. The increase in seed conductivity as a response of main effects of heat stress before PM (T_1) and after PM (T_2) was almost similar in both seasons (*P<0.001*) as well as in the two years pooled data (Table 4.8). However, the interaction between two factor heat stresses (T_1 x T_2) was non-significant in both seasons and the two season's pooled data, which shows the consistency of heat stress effect over two seasons (Table 4.9).

In both seasons, the conductivity of non-heat stressed (control) seeds was lowest at all three raceme positions. Heat stress significantly increased conductivity of seeds harvested from all three raceme positions in both seasons. However, the seeds from the top raceme position had a higher conductivity than those from the other two positions on the raceme for all treatments in both seasons. The lowest increase in conductivity was observed in seeds from the middle section of the raceme in both years followed by the seeds from the basal raceme position (Table 4.10). The highest increase in conductivity was found for heat stress (T_3) for all three raceme positions (Table 4.10). However, the interaction for the two heat stress treatments (T_1 X T_2) was non-significant for seeds harvested from all three raceme positions in both seasons (Table 4.10).

For the main effects of individual heat stress treatments, heat stress before PM (T_1) and after PM (T_2) significantly increased conductivity of seeds harvested from all three raceme positions in both seasons (*P>0.001*). However, heat stress both before (T_1) and after PM (T_2) had a larger effect on increasing conductivity of seeds in 2012-13 than in 2011-12 (Table 4.10).

Table 4.9 Effect of elevated temperature during seed development on conductivity (µS cm^{-1} g^{-1}) of forage rape seeds in the 2011-12 and 2012-13 seasons.

Treatments	Conductivity (µS cm^{-1} g^{-1})		
	2011-12	**2012-13**	**Mean**
Main effect of T_1			
Nil (no stress)	54.1	62.5	58.3
T_1 (HS at ≈80% →50% SMC)	74.9	83.0	78.9
LSD (5%)	**5.9**	**4.5**	**3.4**
Significance of difference	***	***	***
Main effect of T_2			
Nil (no stress)	54.1	60.3	56.9
T_2 (HS at ≈50% →14% SMC)	75.9	85.2	80.3
LSD (5%)	**5.9**	**4.5**	**3.4**
Significance of difference	***	***	***
Treatment means			
T_0 (Control)	41.3	49.3	45.3
T_1 (HS at ≈80% →50% SMC)	65.9	71.4	68.6
T_2 (HS at ≈50% →14% SMC)	66.9	75.7	71.3
T_3 (HS at 80%→14% SMC)	84.0	94.6	89.3
LSD (5%)	**8.4**	**6.3**	**4.8**
SEM	2.6	1.9	1.1
CV%	8.1	5.4	2.2
Interaction effect (T_1x T_2)	-7.5	-3.12	-5.3
L.S. interaction (5%)	**11.8**	**8.9**	**6.8**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)] /2$
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)] /2$
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 – T_0]$
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Heat stress, either before PM (T_1) or after PM (T_2) had no significant effect on differences in seed conductivity between seeds of any raceme position in either season (Table 4.11). There was no significant interaction for differences in seed conductivity between any raceme position in either season, expect between seeds from the top (T) raceme and middle (M) raceme position in the 2011-2012 season (Table 4.11). All three heat stress treatments (T_1, T_2 and T_3) increased the differences in seed conductivity between the top and middle raceme positions. Seeds from the top of the raceme had a higher conductivity than the seeds from the middle of the raceme in both heat stressed and non-stressed seeds in both seasons except for non-stressed seeds in 2012-13. Similarly, in both seasons, conductivity of these seeds was higher than that of the basal raceme for T_1 and T_3 heat stress treatments but did not differ for the heat stress after PM (T_2). However, for the main effect of heat stress, a difference was found for both heat stress treatments, before (T_1) and after PM (T_2) in both seasons. There was no difference in mean conductivity of seeds from the basal and middle section of the raceme for either heat stress treatment in both seasons (Table 4.11).

Table 4.10 Effect of elevated temperature during seed development on conductivity (µS cm^{-1} g^{-1}) of forage rape seed from different raceme positions in 2011-12 and 2012-13.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	58.5	68.1	50.6	58.6	53.3	60.8
T_1 (HS at ≈80% →50% SMC)	81.7	90.0	70.9	78.9	72.2	80.2
LSD (5%)	**7.8**	**5.9**	**6.9**	**7.8**	**5.2**	**5.7**
Significance of difference	***	***	***	***	***	***
Main effect of T_2						
Nil (no stress)	59.4	67.0	49.4	55.9	51.9	58.1
T_2 (HS at ≈50% →14% SMC)	80.8	91.0	72.0	81.6	73.5	82.9
LSD (5%)	**7.8**	**5.9**	**6.9**	**7.8**	**5.2**	**5.7**
Significance of difference	***	***	***	***	***	***
Treatment means						
T_0 (Control)	44.4	54.8	38.9	45.8	40.5	47.2
T_1 (HS at ≈80% →50% SMC)	74.4	79.2	59.9	65.9	63.3	69.0
T_2 (HS at ≈50% →14% SMC)	72.6	81.3	62.2	71.4	66.0	74.3
T_3 (HS at 80%→14% SMC)	89.0	100.7	81.8	91.8	81.0	91.4
LSD (5%)	**11.0**	**8.3**	**9.7**	**11.0**	**7.3**	**8.1**
SEM	4.9	2.6	3.0	3.5	2.3	2.5
CV%	9.9	6.6	10.0	10.0	7.3	7.2
Interaction effect (T_1x T_2)	-13.6	-5.0	-1.4	0.3	-7.8	-4.7
L.S. interaction (5%)	**15.1**	**11.7**	**13.7**	**15.6**	**10.3**	**11.4**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P < 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P <0.001*

Main effect of T_1
Nil (no stress) = [(T_0) + [(T_2)] /2
T_1= [(T_1) + (T_3)]/2
Main effect of T_2
Nil (no stress) = [(T_0) + (T_1)] /2
T_2 = [(T_2) + (T_3)]/2
Interaction Effect (T_1x T_2) = [(T_3)-(T_1)]-[T_2 – T_0]
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 4.11 Comparison of conductivity (µS cm^{-1} g^{-1}) of forage rape seeds from different raceme positions in the 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	T-M		T-B		B-M	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	8.0*	9.5*	5.3*	7.3*	2.7	2.2
T_1 (HS at ≈80% →50% SMC)	10.9*	11.1*	9.5*	9.8*	1.3	1.4
LSD (5%)	**4.8**	**9.8**	**6.9**	**8.4**	**4.3**	**6.2**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**3.4**	**6.9**	**4.9**	**5.9**	**3.1**	**4.4**
Main effect of T_2						
Nil (no stress)	10.0*	11.2*	7.5*	8.9*	2.5	2.3
T_2 (HS at ≈50% →14% SMC)	8.8*	9.4*	7.3*	8.2*	1.5	1.3
LSD (5%)	4.8	9.8	6.9	8.4	4.3	6.2
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**3.4**	**6.9**	**4.9**	**5.9**	**3.1**	**4.4**
Treatment means						
T_0 (Control)	5.5*	9.0	3.9	7.6	1.6	1.4
T_1 (HS at ≈80% →50% SMC)	14.5*	13.3*	11.0*	10.3*	3.5	3.1
T_2 (HS at ≈50% →14% SMC)	10.4*	9.9*	6.6	7.0	3.8	2.9
T_3 (HS at 80%→14% SMC)	7.2*	8.9*	8.0*	9.3*	-0.8	-0.4
S.E.M	2.1	0.8	1.2	3.7	1.9	2.7
LSD (5%)	**6.8**	**13.8**	**9.7**	**11.8**	**6.1**	**8.8**
LSE (5%)	**4.8**	**9.8**	**6.9**	**8.4**	**4.3**	**6.2**
Interaction effect (T_1x T_2)	-12.2	-5.3	-5.7	-0.4	-6.5	-5.0
L.S. interaction (5%)	**9.6**	**19.5**	**13.7**	**16.6**	**8.6**	**12.4**
Significance of interaction	*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant *at P< 0.05*; ** = Significant at *P < 0.01*, ***= Significant at *P <0.001*

Main effect of T_1
Nil (no stress) = [(T_0) + [(T_2)] /2
T_1= [(T_1) + (T_3)]/2
Main effect of T_2
Nil (no stress) = [(T_0) + (T_1)] /2
T_2 = [(T_2) + (T_3)]/2
Interaction Effect **(** T_1x T_2**)** = [(T_3)-(T_1)]-[T_2 - T_0]
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2, LSE**(5%) (Main Effect)** = LSD (Main effect)/√2

4.4.3.4 Relationship between the two vigour tests (electrical conductivity and AA test) of forage rape seed over two seasons 2011-12 and 2012-13

Conductivity of forage rape seed lots from both seasons (2011-12 and 2012-13) after heat stress was plotted against the AA-germination test results. The relationship between the two vigour tests was linear and significant (*P<0.05*) in both seasons. The correlation coefficient was 0.95 in 2011-12 and 0.90 in the 2012-13 season. The relationship demonstrated that as heat stress increased, AA-germination was reduced and conductivity increased (Figure 4.4 (a) and (b)).

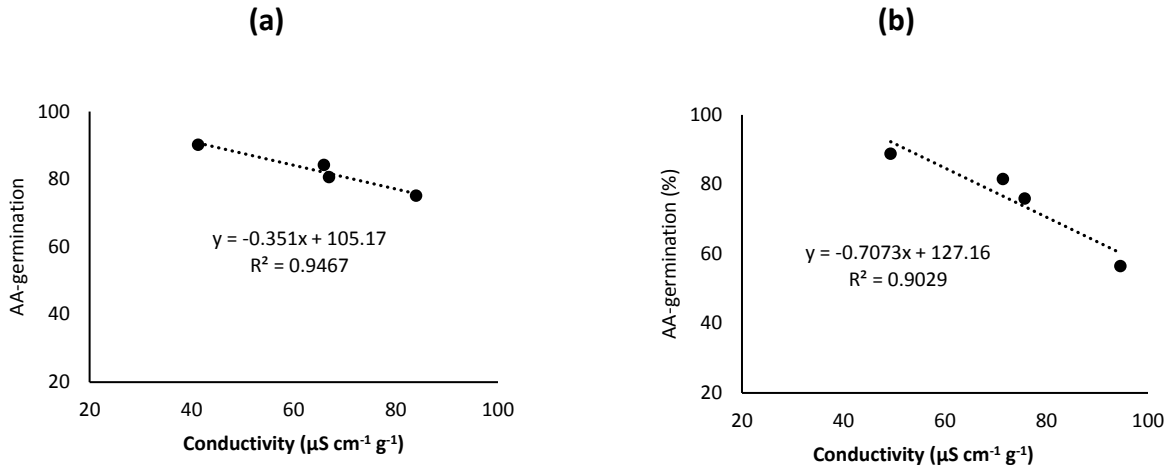


Figure 4.4 Relationship between the two vigour tests (conductivity and AA) of forage rape seed lots subjected to heat stress during two phases of seed devolopment (before and after PM) during the (a) 2011-12 and (b) 2012-13 seasons.

4.4.3.5 Seed mass

Mean seed mass was determined by thousand seed weight (TSW). Thousand seed weight was highest for the non-heat stressed seeds. TSW was significantly reduced following the heat stress before PM (T_1) but not after PM (T_2). The reduction in TSW was larger for heat stress before PM (T_1) in both seasons (Table 4.12). This maximum reduction in TSW was recorded for heat stress (T_3) in both seasons. However, there was no significant interaction between heat stress treatments before and after PM (T_1 X T_2) in both seasons (Table 4.12).

For the main effects of heat stress treatments, TSW was significantly reduced with the heat stress before PM (T_1) in both seasons (*P<0.001*). The reduction in TSW was not significant for heat stress after PM (T_2) (Table 4.12). The effect of heat stress in a combined analysis of two years data did not show significant difference in results, when TSW data from the first season 2011-12 were compared with the second season 2012-13 (Table 4.12). Two season's TSW pooled mean was significantly different for before (T_1) but not after PM (T_2) heat stress treatments (*P<0.01)* The interaction between heat stress treatments (T1 x T2) was not significant, which shows the consistency in effect of heat stress on TSW in both seasons (Table 4.12).

Non-heat stressed (control) seeds harvested from all three raceme positions were heavier than those from any heat stressed treatment. Heat stress before PM (T_1) reduced TSW but not after PM (T_2) in both seasons for seeds from all three raceme positions, except for after PM from the middle position of the raceme in 2012-13. However, there was a larger reduction in TSW of seeds from all three raceme positions with heat stress before PM (T_1) in both seasons (Table 4.13). The largest reduction in TSW in seeds harvested from all three raceme positions was recorded for heat stress treatment (T_3) in both seasons (Table 4.13). The interaction between two heat stress treatments (T_1 X T_2) was not significant for seeds from any of the raceme positions in both seasons (Table 4.13).

Heat stress before PM (T_1) significantly reduced TSW of seeds harvested from all three raceme positions i.e. top (T), middle (M) and basal (B) in the 2011-12 season (*P<0.01, P>0.05 and P>0.01* respectively). However, this heat stress caused a greater impact on seed mass with a significant reduction in TSW *(P>0.001)* for seeds from all three raceme positions in the 2012-13 season (Table 4.13). No significant reduction in TSW was recorded for seeds from any position of the raceme for heat stress (T_2) treatment in both seasons, except for the seeds from middle of the raceme position in 2012-13 (*P<0.05*) (Table 4.13).

Heat stress before PM (T_1) or after PM (T_2) did not produced any significant differences in TSW between the seeds harvested from any of the three raceme positions in either season. Similarly, there was no significant interaction between two heat stress treatments (T_1 X T_2) for differences in TSW between any raceme positions in both seasons (Table 4.14). Least significant effect (LSE) was used to evaluate the differences in TSW of the seeds of any two raceme position. Seeds from the middle of the raceme had a higher TSW than that of seeds from top (T) of the raceme position in (T_1) in 2011-12 but not in 2012-13. There was no difference in TSW between seeds from the basal and top raceme for all heat stress treatments in both seasons. However, the seeds from the middle of the raceme had higher TSW than seeds from the basal position of the raceme for the heat stress T_1 and T_3 treatments in 2011-12, but for only non- stressed (control) seeds in 2011-12 season (Table 4.14).

Table 4.12 Effect of elevated temperature during seed development on Thousand Seed weight (TSW) of forage rape seeds in the 2011-12 and 2012-13 seasons.

Treatments	Thousand Seed Weight (TSW) (g)		
	2011-12	**2012-13**	**Mean**
Main effect of T_1			
Nil (no stress)	2.88	2.97	2.92
T_1 (HS at ≈80% →50% SMC)	2.60	2.60	2.60
LSD (5%)	**0.13**	**0.07**	**0.10**
Significance of difference	***	***	**
Main effect of T_2			
Nil (no stress)	2.80	2.81	2.81
T_2 (HS at ≈50% →14% SMC)	2.68	2.76	2.71
LSD (5%)	**0.13**	**0.07**	**0.10**
Significance of difference	*ns*	*ns*	*ns*
Treatment means			
T_0 (Control)	2.96	3.00	2.98
T_1 (HS at ≈80% →50% SMC)	2.64	2.63	2.63
T_2 (HS at ≈50% →14% SMC)	2.80	2.93	2.86
T_3 (HS at 80%→14% SMC)	2.55	2.58	2.57
LSD (5%)	**0.18**	**0.10**	**0.14**
SEM	0.06	0.03	0.03
CV%	4.10	2.20	1.60
Interaction effect (T_1x T_2)	0.07	0.02	0.06
L.S. interaction (5%)	**0.25**	**0.14**	**0.20**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)] /2$
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)] /2$
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 - T_0]$
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 4.13 Effect of elevated temperature during seed development on thousand seed weight (g) of forage rape from different raceme positions in the 2011-12 and 2012-13 seasons.

Treatment	Top (T)		Middle (M)		Base (B)	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	2.80	2.93	3.00	3.10	2.84	2.91
T_1 (HS at ≈80% →50% SMC)	2.52	2.57	2.81	2.64	2.46	2.60
LSD (5%)	**0.16**	**0.11**	**0.16**	**0.11**	**0.20**	**0.10**
Significance of difference	**	***	*	***	**	***
Main effect of T_2						
Nil (no stress)	2.70	2.76	2.98	2.91	2.73	2.77
T_2 (HS at ≈50% →14% SMC)	2.63	2.73	2.83	2.79	2.57	2.73
LSD (5%)	**0.16**	**0.11**	**0.16**	**0.11**	**0.20**	**0.10**
Significance of difference	*ns*	*ns*	*ns*	*	*ns*	*ns*
Treatment means						
T_0 (Control)	2.89	2.98	3.01	3.12	2.93	2.91
T_1 (HS at ≈80% →50% SMC)	2.50	2.54	2.91	2.70	2.52	2.63
T_2 (HS at ≈50% →14% SMC)	2.71	2.87	2.95	3.01	2.75	2.91
T_3 (HS at 80%→14% SMC)	2.54	2.60	2.72	2.57	2.40	2.56
LSD (5%)	**0.22**	**0.16**	**0.23**	**0.15**	**0.28**	**0.14**
SEM	0.07	0.05	0.07	0.05	0.08	0.04
CV%	5.2	3.6	4.9	3.2	6.6	3.2
Interaction effect (T_1x T_2)	0.22	0.17	-0.13	-0.02	0.06	0.02
L.S. interaction (5%)	**0.31**	**0.23**	**0.32**	**0.21**	**0.39**	**0.20**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P < 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P <0.001*

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)] /2$
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)] /2$
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 - T_0]$
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 4.14 Comparison of thousand seed weight (g) in forage rape seeds from different raceme positions in the 2011-12 and 2012-13 seasons.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatments	M-T		B-T		M-B	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
Main effect of T_1						
Nil (no stress)	0.21	0.13*	0.04	-0.02	0.16	0.15*
T_1 (HS at ≈80% →50% SMC)	0.29*	0.07	-0.06	0.03	0.35*	0.04
LSD (5%)	**0.21**	**0.11**	**0.13**	**0.16**	**0.24**	**0.15**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**0.15**	**0.08**	**0.10**	**0.11**	**1.17**	**0.11**
Main effect of T_2						
Nil (no stress)	0.28*	0.16*	0.03	0.02	0.26*	0.14*
T_2 (HS at ≈50% →14% SMC)	0.20	0.05	-0.05	0.00	0.26*	0.05
LSD (5%)	**0.21**	**0.11**	**0.13**	**0.16**	**0.24**	**0.15**
Significance of difference	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
LSE (5%)	**0.15**	**0.08**	**0.10**	**0.11**	**0.17**	**0.11**
Treatment means						
T_0 (Control)	0.17	0.14*	0.04	-0.07	0.13	0.21*
T_1 (HS at ≈80% →50% SMC)	0.41*	0.17*	0.02	0.10	0.39*	0.07
T_2 (HS at ≈50% →14% SMC)	0.24*	0.13*	0.04	0.04	0.20	0.09
T_3 (HS at 80%→14% SMC)	0.17	-0.02	-0.15	-0.04	0.32*	0.02
S.E.M	0.09	0.05	0.06	0.07	0.11	0.07
LSD (5%)	**0.29**	**0.15**	**0.19**	**0.22**	**0.34**	**0.21**
LSE (5%)	**0.21**	**0.11**	**0.13**	**0.16**	**0.24**	**0.15**
Interaction effect (T_1x T_2)	-0.30	-0.18	-0.17	-0.25	-0.14	0.07
L.S. interaction (5%)	**0.41**	**0.21**	**0.27**	**0.31**	**0.48**	**0.30**
Significance of interaction	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*, ***= Significant at *P<0.001*.

Main effect of T_1
Nil (no stress) = $[(T_0) + [(T_2)]$ /2
$T_1 = [(T_1) + (T_3)]/2$
Main effect of T_2
Nil (no stress) = $[(T_0) + (T_1)]$ /2
$T_2 = [(T_2) + (T_3)]/2$
Interaction Effect (T_1x T_2) = $[(T_3)-(T_1)]-[T_2 - T_0]$
LSD (Main Effect) = LSD (Treatment means) /√2, **LS.Interaction**=LSD (Treatment means) x√2,
LSE (5%)(Treatment means)=LSD (Treatment means)/√2, **LSE(5%) (Main Effect)** = LSD (Main effect)/√2

4.4.3.6 Corelation between hourly thermal time (HTT) and seed quality attributes

Correlation between hourly thermal time (HTT) and seed quality attributes i.e. seed germination, AA-germination, electrical conductivity and thousand seed weight (TSW) was determined by using analysis of covariance (ANCOVA). The analysis of covariance revealed that the difference between the two seasons (2011-12 and 2012-13) for germination, AA-germination, seed conductivity and TWS was not significant *(P< 0.108, P=0.786, P=0.534* and *0.729* respectively*)* (Figure 4.5-4.8). However, the covariate (HTT) (independent variable) was significant for seed germination (*P<0.001*), AA-germination (*P< 0.01*), electrical conductivity (*P<0.01*) but not for TSW (*P=0.128)* (Figure 4.5-4.8).

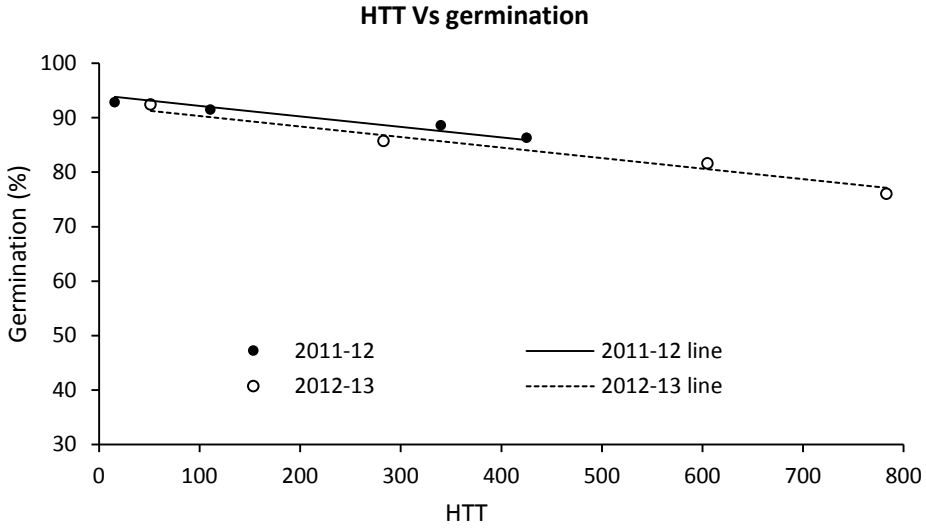


Figure 4.5 Correlation between HTT and seed germination of forage rape plants exposed to elevated temperature during seed development over two seasons (using analysis of covariance). Solid and dotted are for two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (HTT) and dependent variable over two seasons. The two lines revealed that that there was no difference in seed germination between the two seasons (*P= 0.11*). The covariate (HTT) is significant (*P< 0.001*), showing that increasing HTT, significantly reduced seed germination (the common slope of parallel lines was – 0.0193).

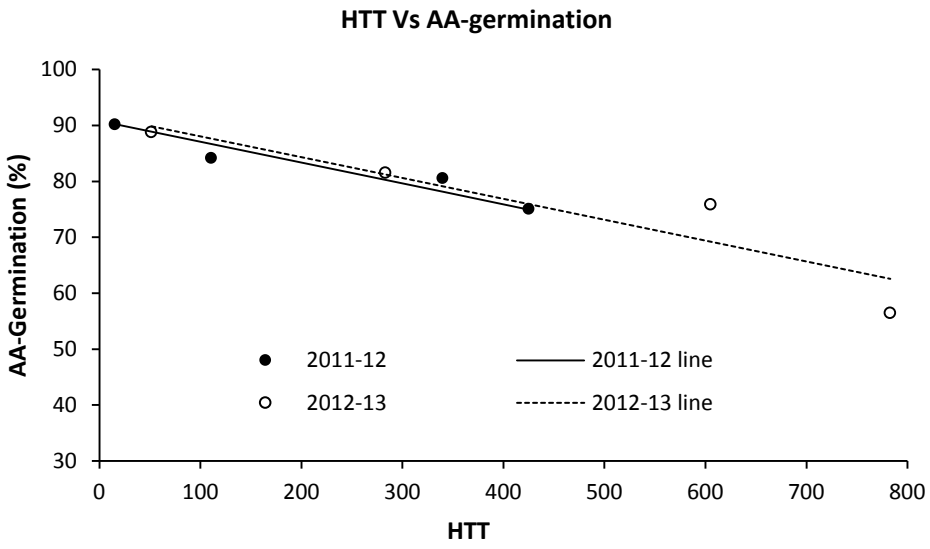


Figure 4.6 Correlation between HTT and AA-germination of forage rape plants exposed to elevated temperature during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles shows means of the independent variable (HTT) and dependent variable over two seasons. The two lines revealed that that there was no difference in AA-germination between the two seasons (*P=0.786*). The covariate (HTT) is significant (*P< 0.01*), showing that increasing HTT significantly reduced seed AA-germination (the common slope of parallel lines was -0.0373).

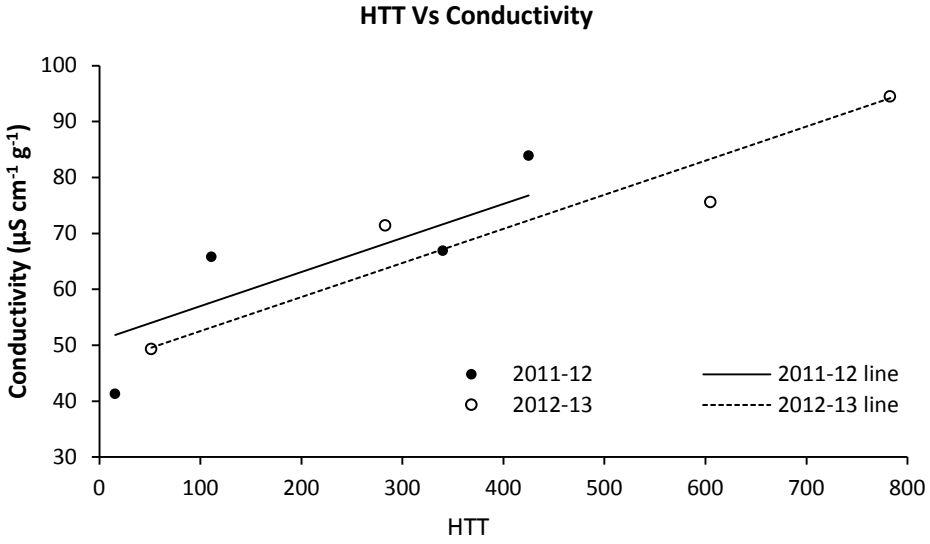


Figure 4.7 Correlation between HTT and electrical conductivity of forage rape plants exposed to elevated temperature during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (HTT) and dependent variable over two seasons. The two lines revealed that there was no difference in electrical conductivity between the two seasons (*P=0.54*). The covariate (HTT) is significant (*P< 0.01*), showing that increasing HTT, significantly increased electrical conductivity (the common slope of parallel lines was 0.061).

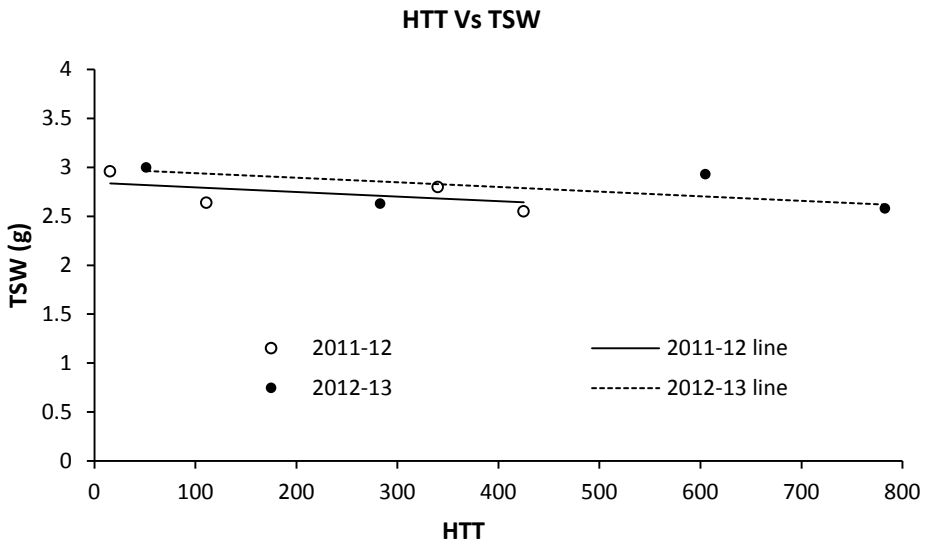


Figure 4.8 Correlation between HTT and TSW of forage rape plants exposed to elevated temperature during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (HTT) and dependent variable over two seasons. The two lines revealed that there was no difference in TSW between the two seasons (*P=0.73*). The covariate (HTT) is also non-significant (*P=0.06*), showing that increasing HTT did not have a significant effect on TSW (the common slope of parallel lines was -0.00047).

4.4.3.7 Corelation between the number of hours when temperature > 25 ˚C and seed quality attributes

The number of hours > 25 ˚C (Tb= 25 C) was plotted against seed germination, AA-germination, electrical conductivity and TSW to establish correlation between the two seasons (2011-12 and 2012-13). There was no significant difference between the two seasons for germination (*P= 0.10*), AA-germination (*P=0.993*), electrical conductivity (*P=0.576*) or TSW (*P=0.77*) (Figure 4.9- 4.12). However, the relationship between the number of hours >25 ˚C (independent variable) and seed germination (*P< 0.001*), AA-germination (*P< 0.01*), conductivity (*P< 0.001*), but not TSW (*P= 0.114)* was significant. As the number of hours > 25 ˚C increased, seed germination and seed vigour decreased (Figure 4.9-4.12)

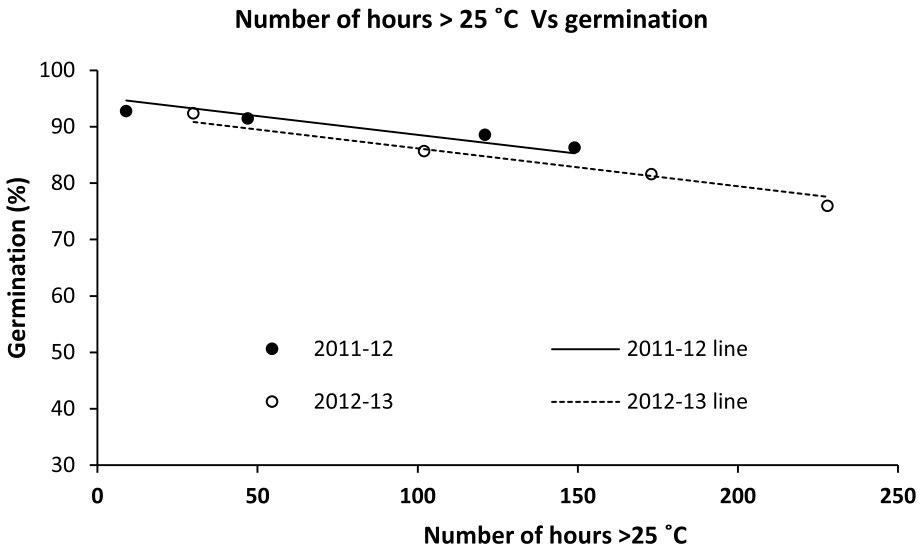


Figure 4.9 Correlation between numbers of hours >25 ˚C and seed germination of forage rape during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (number of hours >25 ˚C) and dependent variable over two seasons. The two lines revealed that there was no difference in seed germination reduction between the two seasons (*P=0.10*). The covariate (number of hours >25 ˚C) is significant (*P< 0.001*), showing that as the number of hours >25 ˚C increased , this significantly reduced seed germination (the common slope of parallel lines was -0.067).

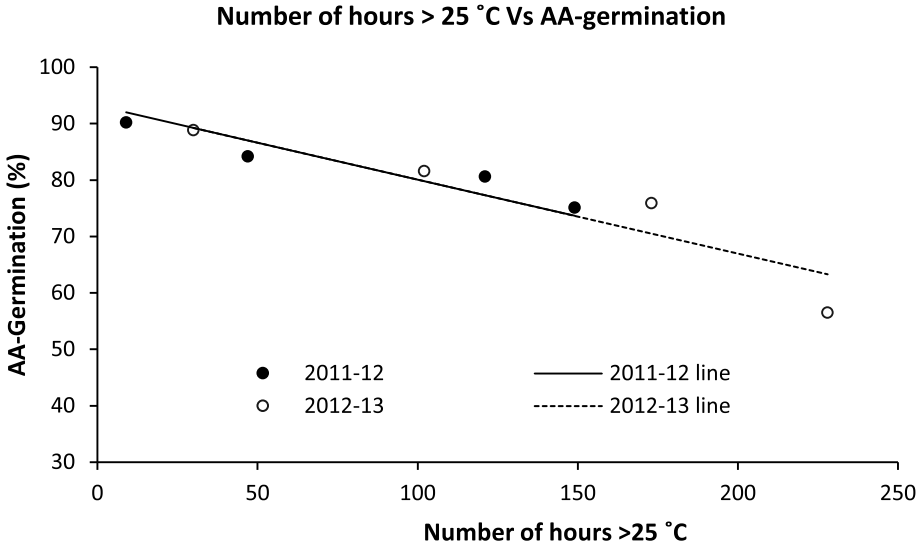


Figure 4.10 Correlation between number of hours >25 ˚C and AA- germination of forage rape during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (number of hours >25 ˚C) and dependent variable over two seasons. The two lines revealed that there was no difference in AA-germination reduction between the two seasons (*P=0.99*). The covariate (number of hours >25 ˚C) is significant (*P< 0.01*), showing that increasing number of hours >25 ˚C, significantly reduced AA-germination (the common slope of parallel lines was -0.131).

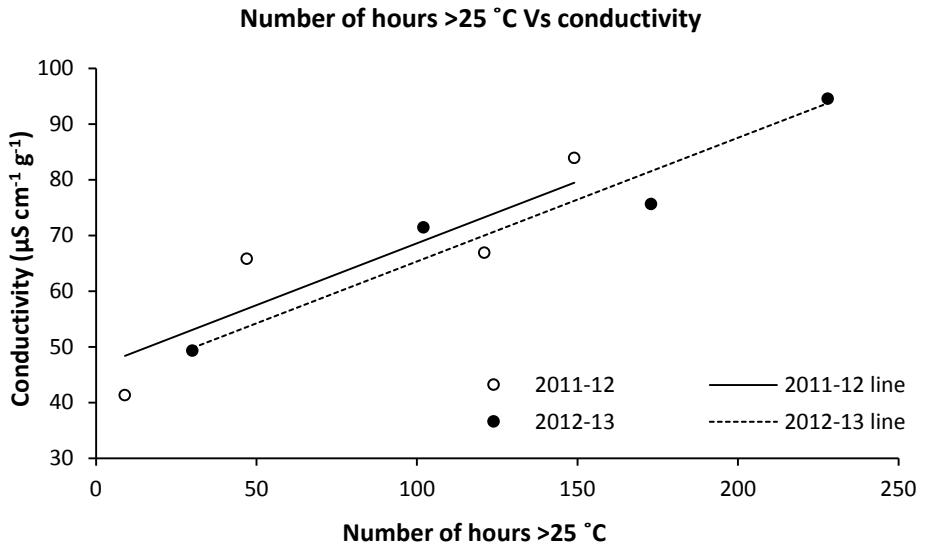


Figure 4.11 Correlation between number of hours >25 ˚C and electrical conductivity of forage rape during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (number of hours >25 ˚C) and dependent variable over two seasons. The two lines revealed that there was no difference in electrical conductivity reduction between the two seasons (*P=0.57*). The covariate (number of hours >25 ˚C) is significant (*P< 0.01*), showing that increasing number of hours >25 ˚C, significantly increased electrical conductivity (the common slope of parallel lines was 0.222).

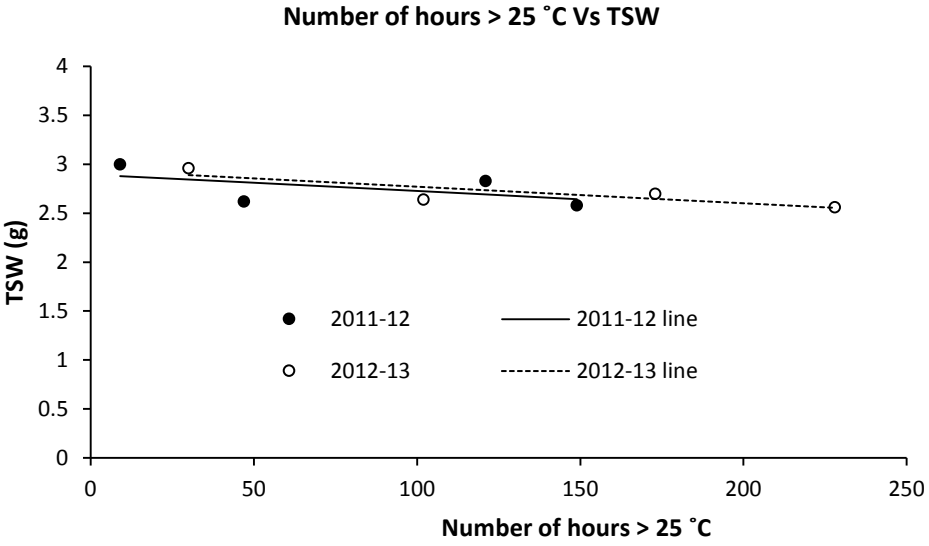


Figure 4.12 Correlation between number of hours >25 ˚C and TSW of forage rape during seed development over two seasons (using analysis of covariance). Solid and dotted lines are for the two seasons 2011-12 and 2012-13 respectively. Solid and hollow circles show means of the independent variable (number of hours >25 ˚C) and dependent variable over two seasons. The two lines revealed that there was a no difference in TSW reduction between two seasons (*P=0.77*). The covariate (number of hours >25 ˚C) was also non- significant (*P=0.11*), showing that increasing the number of hours >25 ˚C, had no effect on TSW (the common slope of parallel lines was -0.000169).

4.5 Discussion

In this field experiment, ambient day and night field temperatures were increased artificially by covering the plants with plastic sheet cages. These deliberate increases in temperature during seed development were used to mimic the possible effects of global warming on seed quality. The increase in temperature was due to natural solar radiation (greenhouse effect) and was dependent upon the intensity of solar radiation and outside ambient field temperature.

4.5.1 Effect of elevated temperature before physiological maturity on seed quality

Increasing mean air temperature can negatively affect the subsequent seed quality (Copeland & McDonald, 2001; Munir et al., 2001) . However, the intensity of losses in seed quality depends on when the heat stress occurs during seed development.

The seed filling stage is sensitive to high temperature stress, which can reduce seed mass and germination but most often seed vigour (Dornbos, 1995; Egli et al., 2005; Hampton et al., 2013; Shinohara et al., 2006a). Egli et al. (2005) demonstrated a decrease in soybean germination and vigour after exposure to high field temperature. Results of this field experiment agree with this report, in that by exposing forage rape plants to an elevated temperature during phase-I (seed filling to PM), seed quality was negatively affected. The analysis of seed quality i.e. seed germination, vigour and seed mass, showed a significant decrease in these traits, relative to control plants (exposed to only the ambient field environment). In this study, air temperature during phase-I (before PM) gradually increased inside the plastic cover cages. An increased number of hours > 25 ˚C, resulted in an increased HTT of 101.3 ˚C, producing a slight reduction in seed germination of 2% in the first season and 7% in the second season. However, the negative effect on seed vigour was larger, being 7% in the 2011-12 and 16% in the 2012-13 season. In the second season, HTT was more than double that of the first season (283 cf. 111 ˚Ch).

Seed mass estimated as 1000-seed weight, decreased with increasing HTT and hours exceeding 25 ˚C during phase-I. High temperature during seed filling has been reported to negatively affect seed mass (Mohammed & Tarpley, 2010; Sinsawat et al., 2004). A reduction in seed mass in response to increased temperature (higher HTT and hours > 25 ˚C) during seed filling may be related to a reduced seed growth period. Higher temperature during reproductive growth may shorten the time for seed to fully develop before maturity, resulting in decreased seed size (Young et al., 2004).The results of this experiment for heat stress during the seed filling stage further confirm the results of the previous experiment in controlled conditions (Chapter 3) and a possible mechanism of seed deterioration under these conditions has been explained (see Section 3.5 , Chapter 3).

4.5.2 Effect of elevated temperature after PM on seed quality

During the post-PM stage, after attaining maximum weight, seeds begin to enter into the maturation drying phase, and the connection between the mother plant is diminished (Bewley et al., 2012). An association between post-PM environmental conditions and seed germination and seed vigour loss has been reported by many researchers (Castillo et al., 1993; Castillo et al., 1994; Siddique & Wright, 2004; TeKrony & Hunter, 1995; Wang et al., 2012). Results of the present field study showed a linear decrease in both seed germination and vigour with increasing number of hours above 25 ˚C and increasing HTT; the incidence of abnormal seedlings increased when daily mean maximum temperature increased from 25 ˚C to 34˚C (range of increase during phase-II, was 33.4 -34 ˚C over the two seasons (Figure 4.2 (c) and 4.3 (c)). The elevated temperature during phase-II (PM →HM) in this study did not have a significant effect on seed mass because seeds had reached their maximum mass by PM.

Elevated temperature during phase-II (PM →HM), thus significantly reduced seed germination and vigour (AA) in both seasons. However, the effect was more prominent in the second season because of the exposure to higher temperature (HTT) during this season than in the 2011-12 season (605 cf. 340 ˚Ch). In contrast, seeds of commercially acceptable quality (>90%) levels of seed germination and seed vigour (AA) were harvested from control plants exposed to ambient phase-II (PM →HM) field temperature during this time. Hourly thermal time (HTT) (Tb= 25 ˚C) was significantly correlated with germination and vigour (AA) (Figure 4.5 & 4.6) and the correlation for two seasons indicated a consistent negative effect (Figure 4.5 & 4.6). Similarly, increasing number of hours above 25 ˚C also showed a significant correlation with seed germination and vigour (Figure 4.9 and 4.10). The decrease in seed vigour with temperature above 25 ˚C during phase-II (PM →HM) was also characterized by high seed conductivity, resulting in a significant linear negative relationship between (HTT) (Tb= 25 ˚C) and seed conductivity (Figure 4.7). A negative correlation between the two seed vigour tests (AA and conductivity test) indicated that either could be used for vigour testing of forage rape (Figure 4.4).

The temperature inside the plastic sheet cages was not controlled and it is acknowledged that by manipulating the ambient field temperature in this way, the duration, intensity and diurnal difference were substantially greater than normal field conditions. However, the response to heat stress during phase-II(PM →HM), was well described by linear regression, suggesting that around 300 ˚C h between PM and HM were required to produce a commercially unacceptable seed lot (seed vigour < 80%, as assessed by the AA test). It has been reported that seed vigour (AA-germination) of 80% can be taken as a minimum acceptable standard for planting seed in a wide range of field environment for acceptable emergence (Egli & TeKrony, 1995, 1996). Further, the significant correlation between numbers of hours above 25 ˚C and seed vigour also indicated that at least 100 hours above 25 ˚C were required to produce low vigour seed.

The present study demonstrated that seed conductivity increased as the degree hours (°Ch) increased, indicating that membrane integrity has been compromised due to this higher post-PM temperature. Signs of membrane destabilization as indicated by higher electrolyte leakage have been associated with the incidence of abnormal seedlings and attributed to the damage or death of some tissues in the seed parts, especially in the meristematic tissues (Marcos Filho, 2015).This relationship between conductivity and abnormal seedlings was also found in the present study; there was a significant relationship between conductivity and AA, with the reduction in AA-germination being caused by abnormal seedling production, not seed death. The conductivity test was able to detect this early seed deterioration (cellular membrane damage).In the present study, conductivity of non-stressed forage rape seeds was similar to the conductivity ranges proposed by Elias and Copeland (1997) for canola (*Brassica napus*). The integrity and functions of the membranes are sensitive to high temperature and as has been reported in earlier studies, heat stress disrupts cellular membrane stability and functions, thus enhancing the permeability of membranes to ions (Bailly et al., 2002b; Wahid & Shabbir, 2005). This phenomenon is the most cited cause of seed deterioration (McDonald, 1999). The changes in structure and functions of membranes are mostly because of an alteration in phospholipids' fatty acid composition (Larkindale & Huang, 2004).High temperature modifies the phospholipid compositions resulting in loss of membrane integrity due to changes in membrane configuration or structure and/or changes in properties of membrane-bound enzymes which lead to the leakage of ions in incubation water (Ren et al., 2009; Taiz & Zeiger, 1998).Heat stress after PM has also been reported to increase seed conductivity due to its effect on seed metabolism and the ability to maintain optimum metabolic activity, which leads to physiological changes (Dornbos et al., 1989; Grass & Burris, 1995b). Therefore, results of this study are consistent with these reports suggesting that heat stress may have modified the compositions of fatty acids in phospholipids leading to the increased electrolyte leakage and a reduction in seed vigour. Additionally, oxidative stress and overproduction of reactive oxygen species (ROS) by heat stress may also lead to lipid peroxidation of the cellular membranes and thus increase electrolyte leakage leading to loss of vigour and viability (Camejo et al., 2006; Rodríguez et al., 2005).This is confirmed in Chapter 5, where a reduction in seed germination and vigour due to heat stress was related with ROS (H_2O_2) accumulation and lipid peroxidation. The mechanism of H_2O_2 accumulation and lipid peroxidation is presented in Chapter 5.

A further, possible reason for reduction in seed vigour during this phase might be due to inactivation of membrane bound antioxidant enzymes which are necessary to detoxify excessive ROS production to minimize their deleterious effects on the cells and membrane lipid peroxidation; and/or reduced respiratory activity by reduction in ATP activity due to alteration in mitochondrial structure or functions as reported by Grass and Burris (1995b). They suggested that heat stress post PM reduced mitochondrial activity through ultrastructural changes in mitochondria and their functions. This is

confirmed in Chapter 6, where reduced activity of antioxidant enzymes and low ATP energy levels were recorded in low vigour heat stressed seeds.

In this study, the results confirmed those presented in Chapter 3, is that seeds from the top of the raceme position had slightly lower germination and vigour than those from the middle and basal raceme positions and this effect was more pronounced during post-PM heat stress. This raceme position effect was more profound with respect to seed vigour in both seasons. Reason for this have been discussed in Chapter 3.

Climatic conditions in Canterbury, New Zealand during December and the following January are very important for high quality forage rape seed production. In the present study, most of the temperature increase above 25 ˚C occurred from the last week of December until the middle of January in both seasons (Figure 4.2 and 4.3). Thus, a forage rape seed grower may avoid this situation of adverse climatic conditions during forage rape seed maturation by carefully selecting a region or area of production where temperature seldom rises above 25 ˚C during the last three weeks of seed maturation to avoid seed vigour loss.

Another possible option is to change sowing date to avoid the heat stress at this critical stage of seed development, and/or seeds may be harvested before they have reached HM and dried under controlled conditions to maintain high quality seeds, thereby reducing pre-HM seed deterioration.

4.6 Conclusions

- Exposure to elevated temperature of more than 100 ˚C h (Tb= 25 ˚C) during phase-I (before PM) when SMC was between 80-50% significantly decreased seed germination and seed vigour in both seasons and was correlated with increasing hourly thermal time (HTT) and the number of hours exceeding 25 ˚C.
- Seed mass was also decreased with higher temperature before PM but was not decreased after PM.
- A greater reduction in seed quality (seed germination and vigour) was found for exposure to elevated temperature after PM in both seasons, when SMC was between 50-14 % SMC (phase-II).
- Assuming an AA test result of 80% or greater is commercially acceptable, around 300 ˚Ch after PM was required to reduce seed vigour to an unacceptable level.
- Seeds harvested from the top of the raceme position were more sensitive to heat stress. Heat stress decreased seed germination, vigour and seeds mass from all three raceme positions but the largest decrease was found from the seeds harvested from the top of the raceme rather than those from the middle and basal raceme positions.

Chapter 5
Changes in malondialdehyde content and evaluation of reactive oxygen species (ROS) as a result of seed deterioration due to high temperature stress during seed development

5.1 Introduction

Seed development begins with rapid growth, which increases both fresh and dry weight, followed by a period of food reserve accumulation until maximum dry weight and seed physiological maturity are reached. Seeds then undergo desiccation until the seed moisture content reaches equilibrium with the relative humidity of the environment (Angelovici et al., 2010; El-Maarouf-Bouteau et al., 2011).During this process, the developing seed can face many environmental stresses involving temperature, photoperiod and humidity which can affect both germination and vigour of seeds (Cendán et al., 2013; Gutterman, 2000; Wang et al., 2012).High temperature and humidity can result in the production of excessive amounts of reactive oxygen species (ROS) which leads to oxidative stress and results in seed deterioration (Bailly, 2004; Sattler et al., 2004). After seeds reach physiological maturity, they may undergo physiological deterioration induced by pre-harvest climatic conditions such as high temperature and humidity during seed maturation. The greater the stress the more deterioration occurs. McDonald (1999) proposed four types of cell damage in his seed deterioration model: mitochondrial dysfunction, inactivation of enzymes, membrane degradation and genetic damage. Many cellular and metabolic alterations occur in seeds as a consequence of seed deterioration including loss of membrane integrity, degradation of DNA and reduced primary metabolism (El-Maarouf-Bouteau et al., 2011; Kibinza et al., 2006).One of the important mechanisms of seed deterioration is the production of ROS as a by-product during normal aerobic metabolism (Lee et al., 2010) and as a result of abiotic stress induced by heat (Chen et al., 2012).ROS include superoxide radicals (O^{-}_{2}), hydroxyl radicals (OH^{-}), hydrogen peroxide (H_2O_2) and single oxygen (O^{-}).ROS have been shown to oxidize most important macromolecules including lipids, proteins and nucleic acids and cause cell disruption and organism lesions (Chen et al., 2012).The free radical unpaired electron of ROS are known to be the cause of lipid peroxidation (McDonald, 1999).

Under normal physiological conditions, ROS production and removal is tightly controlled in cells by antioxidant enzyme activities without damaging the cells. When this balance is disrupted or oxidative stress is prolonged, ROS levels exceed antioxidant capability, allowing attack on membrane lipids in a chain reaction and damage to proteins (denaturation of enzymes) and nucleic acid (nicking, cross-linking and scission of DNA strands) (Lee et al., 2010). Over accumulation of ROS, and free radical attack on lipids and proteins is considered a significant cause of seed deterioration (Bailly, 2004). Degradation

and peroxidation of lipids as a consequence of ROS activity will eventually damage cellular membranes and reduce their integrity. The loss of membrane integrity is considered a first sign of seed deterioration, resulting in decreased membrane fluidity, increased permeability and loss of other functions related to the membranes (Lee et al., 2010; McDonald, 1999; Miller et al., 2008). Loss of membrane integrity as consequence of changes in phospholipid composition is well correlated with the loss of seed viability and vigour. Loss of membrane integrity can be estimated by electrolyte leakage through the conductivity test (Lee et al., 2012; Parkhey et al., 2012). Lipid peroxidation has been demonstrated to occur during physiological deterioration in soybean (Sung & Jeng, 1994), peanut (Sung & Chiu, 1995) and sunflower seeds (Kibinza et al., 2006) but it did not appear to be the main factor involved in seed deterioration in other species such as pigeon peas (Kalpana & Rao, 1996), wheat (Lehner et al., 2008) and maize (Lin & Pearce, 1990). The major cause of seed deterioration in pigeon peas was changes in total lipids and phospholipids (Kalpana & Rao, 1996), antioxidant enzymes and soluble sugars in wheat (Lehner et al., 2008) and an increase in phosphatidic acid in maize (Lin & Pearce, 1990). Malondialdehyde (MDA), a product of lipid peroxidation is a good indicator and its content is a method to measure the degree of lipid peroxidation in oil rich seeds (Sung & Jeng, 1994).

Seed deterioration and therefore the loss of seed vigour associated with ROS activity has been demonstrated in sunflower seeds by Kibinza et al. (2006), Bailly et al. (2008) and El-Maarouf-Bouteau et al. (2011). Accumulation of hydrogen peroxide as result of increased ROS activity and impairment of antioxidant enzyme activities has been previously reported to decrease seed vigour (Bailly et al., 2002a). Hydrogen peroxide is a relatively stable ROS molecule and can move from cellular synthesis sites to the surrounding cells (Bienert et al., 2006; Henzler & Steudle, 2000).The H_2O_2 becomes potentially dangerous for the surrounding cellular environment due to its oxidizing powers, as it can easily react with other molecules such as lipids, nucleic acids and proteins and eventually become damaging or fatal for cells (Halliwell, 2006; Mittler, 2002; Rajjou et al., 2012; Rao et al., 1997).
High temperature stress during seed development and maturation can often reduce seed vigour without affecting the seed germination (Hampton et al., 2013), which explain why high germinating seed lots can have low vigour. However, little is known of the effect of timing of this heat stress on the physiological processes resulting in seed deterioration.

5.2 Objectives

Most of the previous work on lipid peroxidation in deteriorating seed has focused on stored seeds, with limited literature available on lipid peroxidation and ROS production in seeds during seed development and maturation. The aim of the present study was to determine whether loss of firstly seed vigour and then seed germination of forage brassica seeds subjected to high temperature stress (240 ˚C hours) during seed development was related to changes in lipid peroxidation and ROS

production. It was hypothesised that seed deterioration due to high temperature stress before and at physiological maturity was due to the action of ROS, particularly H_2O_2, leading to lipid peroxidation.

5.3 Materials and methods

5.3.1 Experiment 1

Measurement of Malondialdehyde (MDA) content (a product of lipid peroxidation) in forage rape seeds subjected to high temperature stress during seed development.

5.3.1.1 Background

Seeds from the 2012-13 forage rape trial harvested after heat stress treatments, with known germination and vigour (data presented in Section 3.4.1 and 3.4.2) were used for this experiment. To investigate the level of lipid peroxidation, MDA a product of lipid peroxidation, was measured. MDA is produced by thiobarbituric acid reactive substances (TBARS), as described by Heath and Packer (1968). Determination of MDA content has proved to be a convenient method for quantifying the extent of lipid peroxidation (Matsushita, 1980).

5.3.1.2 Extraction of MDA

MDA content of forage rape seeds was measured according to Dhindsa et al. (1981) and Shi et al. (2006) . For MDA extraction 0.1 g of seed for each of the seed lots was ground in a mortar with 1.5 ml of 10% Trichloroacetic acid (TCA) and the homogenate product collected into a 2 ml eppendorf tube. The homogenate was then centrifuged at 10000 *X* g for 15 min and the supernatant was collected into another 2 ml eppendorf tube and centrifuged again @ 14000 x g for 15 mins. From the resulting supernatant, 0.6 ml was then mixed with 0.6 ml of thiobarbituric acid (TBA), (0.6 %(w/v) TBA in H_2O) and incubated for 30 mins at 95 ˚C and then quickly cooled on ice for 5 mins before centrifugation for 10 mins at 10000 x g. The absorbance of the solution was then measured using a cuvette/microplate reader spectrophotometer (SpectraMax® M2, Molecular Devices, Sunnyvale, California) at 450, 532 and 600 nm using 1ml cuvettes. The MDA concentration (μM) was calculated using the formula below (Chen & Arora, 2011)

[MDA] = 6.54 x (A 532 – A 600) - 0.56 x A450

where the A532, A 600 and A 450 represent the absorbance of the solution at 450 nm, 532 nm, and 600 nm respectively.

5.3.2 Experiment 2:

Quantification of hydrogen peroxide in heat stressed seeds.

5.3.2.1 Background

Hydrogen peroxide (H_2O_2), a ROS, is produced as a consequence of heat stress (Chen et al., 2012). Excessive production of H_2O_2 can cause oxidative stress under various abiotic stress conditions. The oxidative stress is associated with seed deterioration (Kibinza et al., 2006). The aim of this experiment was to quantify the level of H_2O_2 production in seeds subjected to heat stress during seed development. Details about the heat stress treatments have been given in Chapter 3 (Section 3.3). Seeds from the forage rape crop 2012-13 harvest with known germination and seed vigour were used in this experiment (see Section 3.4.1 and 3.4.2).

5.3.2.1 Extraction of Hydrogen peroxide (H_2O_2)

H_2O_2 contents of seeds were determined according to Bailly and Kranner (2011) except that a 96 microplate reader was used instead of a cuvette. For H_2O_2 extraction 0.1 g of seeds were ground in a mortar and homogenized with 2 ml of 0.2M perchloric acid. After 15 min of centrifugation at 14000 g, 1ml of the resulting supernatant was neutralized to pH 7.5 with 4M KOH. This turned the solution a yellow colour as it became alkaline. This was then centrifuged for 4 min at 14000 x g after sitting on ice to precipitate the insoluble potassium perchlorate. 20µl of the supernatant was loaded into 96 well flat bottomed polystyrene microplates with blanks and standards, all in triplicate. 200µl of Xylenol Orange reagent was added and left for 30 minutes at room temperature to develop the purple complex reaction product before reading in a SpectraMax M2 plate reader at a wavelength of 595 nm.

Xylenol orange reagent was prepared according to the method of Gay et al. (1999) and Bindschedler et al. (2001). Solution A contained 25 mM $FeSO_4$ and 25 mM $(NH_4)_2SO_4$ in 2.5 M H_2SO_4. Solution B contained 100 mM sorbitol and 125 µM Xylenol orange in water. 100µl of Solution A was added to 9.9 mls of Solution B just before use and used at 200µl per well.

Standards were prepared by diluting 14.7M H_2O_2 1 to 500 in de-ionized (DI) H_2O to make a stock solution. The actual concentration of this H_2O_2 solution was determined using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} at wavelength 240nm in a quartz cuvette. A typical reading was around 1.1, indicating a concentration of 25.2mM (1.1/43.6 = 0.0252M = 25.2mM). This stock solution was then diluted 1 to 100 with DI H_2O to make a working solution of 252 µM. The standards were prepared by taking 0, 25, 50, 75, 100, 125, 150 and 200µl of the working solution and adding water to 1ml in a 1.5ml eppendorf tube. This resulted in H_2O_2 concentrations of 0, 6.3, 12.6, 18.9, 25.2, 31.5, 37.8 and 50.4µM. New standards were prepared each day.

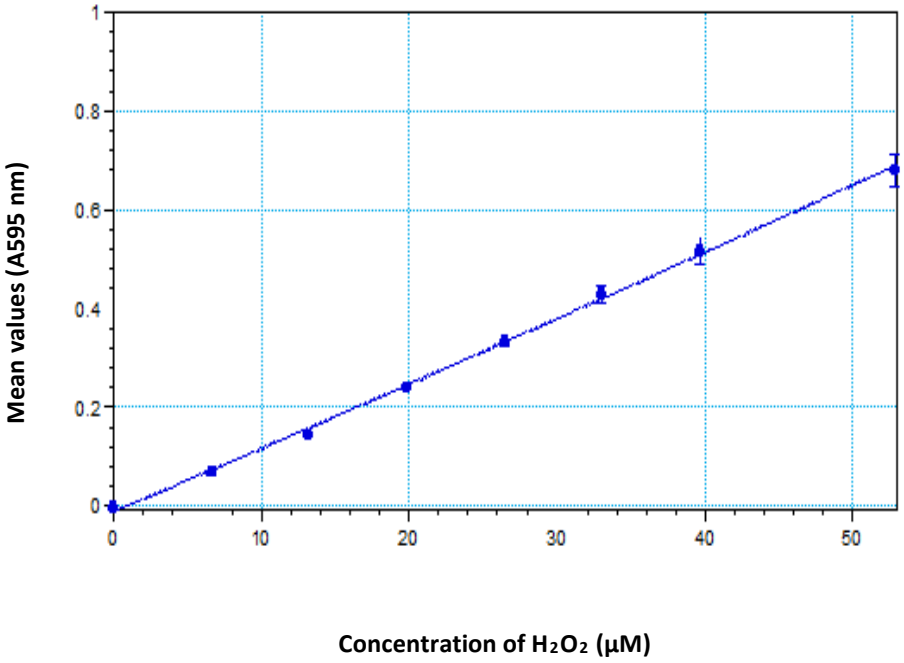


Figure 5.1 The relationship between concentration of H_2O_2 and its mean value at 595 nm after reaction with Xylenol Orange. The correlation co-efficient (R^2) was 0.999 for this linear fit. Slope was 0.0133, y intercept was -0.0191.

5.3.3 Statistical analysis

Statistical analyses of data were performed using Genstat software (16th Edition). Analysis of variance (ANOVA) was used to compare the means for seeds from each temperature stress treatment at a particular seed development stage. The impact of temperature stress at two seed development stages (80% and 50% SMC) and 80+50% SMC during seed development on MDA and H_2O_2 contents of seeds was analysed in a 2x2 factorial design with temperature stress at 80% SMC (+,-) and at 50% SMC (+, -) as treatment factors. Least significant difference (LSD 5%) was used to compare means of treatments between factors. The interaction between two treatment was calculated and Least significant interaction (LS interaction 5% = LSD X√2) was used to test interaction significance. Least Significant effect (LSE) was calculated [(LSE (5%) (Main Effect) = LSD (Main effect)/√2] to compare the differences in seed quality after treatment effects on the seeds between the racemes of any of three sections of the plant i.e. top, middle and base.

5.4 Results

5.4.1 Lipid peroxidation

MDA levels of seeds were determined to evaluate lipid peroxidation. Heat stress (240°C h, 70% R.H) induced lipid peroxidation in forage rape seeds as indicated by increased MDA contents (Table 5.1). Non-stressed (control) seeds had a MDA content of 12.27 nmoles g^{-1}. Temperature stress at both ≈80% SMC and 50% SMC significantly increased the MDA content, but the stress at 80% SMC plus 50% SMC, did not further increase the MDA content over that for either individual time of stress (Table 5.1). Heat stress at ≈80% SMC (as a main effect) significantly (*P<0.001*) increased the MDA content (Table 5.1), but the impact of heat stress was greater at physiological maturity with a significant increase (*P<0.001*) in MDA content. There was a highly significant (*P<0.001*) interaction for heat stress between the two seed development stages (80% SMC X 50% SMC) in terms of an increase in MDA content in forage rape seeds. The maximum accumulation of MDA was found in seeds harvested from plants subjected to high temperature stress at 80% SMC plus 50% SMC (PM) stage (Table 5.1).

MDA content in non-stressed (control) seeds, harvested from all three raceme positions was lower than in the heat stressed seeds (Table 5.1). Heat stress application significantly increased the MDA in seeds harvested from all three raceme positions of the forage rape plant (Table 5.1). The MDA levels in seeds from the top section of the raceme significantly (*P< 0.001*) increased with heat stress applied at ≈ 80% SMC and 50% SMC individually. Although there was a 34 % increase in MDA in seeds when heat stress was applied at 50% SMC, the MDA level of seeds stressed at 80% SMC was only 21 % more than that in non-stressed seeds (Table 5.2). There was a negative significant (*P<0.01*) interaction for heat stress treatments at the two seed development stages (80% SMC x 50% SMC) in seeds from the top of the raceme position (Table 5.2). However, seeds from the middle and basal sector of the raceme also had a highly significant (*P<0.001*) increase in MDA concentration following heat stress treatment applied either at seed filling stage (80% SMC) or at physiological maturity (50% SMC) stage (Table 5.1). The interaction between applying heat stress at the two seed development stages was significant (*P < 0.001*) for middle and base raceme position seeds (Table 5.1).

The differences in MDA levels between the seeds harvested from the three raceme positions (top, middle and base) were determined by the least significant effect (LSE 5%). The MDA levels of seeds from the pods of middle racemes did not differ from that of seeds harvested from top racemes for the non-stressed (control) or seeds heat stressed at ≈ 80% SMC (Table 5.2). Heat stress applied at physiological maturity (50% SMC) significantly increased the differences in the level of MDA between seeds from the top and middle sector of the raceme (*P<0.05*) and between top and base raceme seeds (*P< 0.001*) (Table 5.2). No significant differences in MDA concentration were found between the seed from the middle sector of the raceme and the basal raceme seeds with the heat stress either applied

at seed filling or at physiological maturity. No significant interaction between heat stress treatments was found for differences in MDA content between seeds of any raceme section of the plant.

MDA concentration was, however, greater in seeds from the top of the raceme than that of the middle and basal racemes for heat stress at 50% SMC and 80+50% SMC. The MDA concentration in seeds from the top racemes was greater than that of seeds harvested from the base racemes at 50% SMC, but no difference was found in control seeds or seeds exposed to heat stress during seed development at 80% SMC. No differences in concentration of MDA was found in seeds harvested from middle racemes and seeds from basal racemes with heat stress at 80% SMC but there was for heat stress at 50% SMC (Table 5.2).

Table 5.1 Effect of temperature stress during seed development on forage rape seed MDA content (nmoles g^{-1} seed).

Treatments	MDA (nmoles g^{-1} seed)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	15.38	16.18	15.26	14.71
Heat stress at 80% SMC	18.38	19.53	17.96	17.64
LSD (5%)	**0.68**	**1.14**	**1.09**	**0.82**
Significance of difference	***	***	***	***
Main effect of heat stress at 50% SMC				
Nil (no stress)	15.10	15.29	14.96	15.06
Heat stress at 50% SMC	18.66	20.42	18.26	17.30
LSD (5%)	**0.68**	**1.41**	**1.09**	**0.82**
Significance of difference	***	***	***	***
Treatment means				
Control	12.27	12.44	12.16	12.21
Heat stress at 80% SMC	17.93	18.13	17.75	17.90
Heat stress at 50% SMC	18.49	19.91	18.36	17.21
Heat stress at (80%SMC+50% SMC)	18.82	20.92	18.16	17.38
SEM	0.37	0.50	0.48	0.36
LSD (5%)	**0.96**	**1.61**	**1.54**	**1.17**
Interaction effect (80% x 50% SMC)	-5.33	-4.68	-5.79	-5.52
L.S. interaction (5%)	**1.35**	**2.27**	**2.17**	**1.64**
Significance of interaction	***	**	***	***

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001.*
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC=[(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 5.2 Comparison of MDA content (nmoles g^{-1} seed) in seeds harvested from three sections of the racemes of forage rape plants.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P <0.05*).

Treatment	T-M	T-B	M-B
Main effect of heat at 80% SMC			
Nil (no stress)	0.91	1.46	0.56
Heat stress at 80% SMC	1.57*	1.89*	0.32
LSD (5%)	**1.43**	**1.28**	**1.28**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	**1.00**	**0.91**	**0.92**
Main effect of heat at 50% SMC			
Nil (no stress)	0.33	0.24	-0.09
Heat stress at 50% SMC	2.15*	3.11*	0.97*
LSD (5%)	**1.43**	**1.28**	**1.28**
Significance of difference	*	***	*ns*
LSE (5%)	**1.00**	**0.91**	**0.92**
Treatment means			
Control	0.28	0.23	-0.04
Heat stress at 80% SMC	0.38	0.24	-0.14
Heat stress at 50% SMC	1.54*	2.69*	1.15
Heat stress at (80%SMC+50% SMC)	2.75*	3.53*	0.78
S.E.M	0.63	0.57	0.58
LSD (5%)	**2.01**	**1.81**	**1.84**
LSE (5%)	**1.43**	**1.28**	**1.30**
Interaction effect (80% x 50% SMC)	1.11	0.83	-0.27
L.S. interaction (5%)	**2.83**	**2.55**	**2.55**
Significance of difference	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001.*
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC=[(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC=[(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

5.4.2 Hydrogen peroxide (H_2O_2)

H_2O_2 concentration in heat stressed seeds was significantly higher than in non-stressed (control) seeds (Table 5.3). H_2O_2 concentration in control and heat stressed seeds ranged from 3.02 to 6.00 µmoles g^{-1} of seed. Heat stress applied at the seed filling (≈80% SMC) stage significantly (*P >0.05*) increased the H_2O_2 concentration in seeds by 35% more than the seeds in the absence of heat stress (Table 5.3). There was a larger (47% increase over non-stressed seeds) impact of heat stress at physiological maturity (≈50% SMC) than at seed filling (≈80% SMC) stage, which also significantly (*P<0.001*) increased hydrogen peroxide concentration in seeds. However, the interaction of heat stress application at the two seed development stages (80 x 50% SMC) was non-significant (*P= 0.652*) (Table 5.3). The largest increase in the H_2O_2 concentration in seeds was found after heat stress at both seed development stages, ≈80% SMC plus ≈50% SMC (Table 5.3).

Heat stress impacted on H_2O_2 concentration in seeds harvested from different racemes sections i.e. top, middle and base (Table 5.3). The lowest accumulated level of H_2O_2 was found in non-stressed (control) seeds harvested from all three racemes positions (Table 5.3). Heat stress significantly (*P<0.05*) increased H_2O_2 concentration at the seed filling stage (≈80% SMC) in seeds harvested from the top sector of the raceme but did not differ significantly in seeds from the middle and basal racemes positions. However, heat stress at physiological maturity (≈50% SMC) significantly increased H_2O_2 concentration (*P<0.05*) in seeds from all three raceme positions (Table 5.3). The interaction of heat stress between the two seed development stages (80 x 50% SMC) was non-significant in seeds harvested from all three raceme positions (Table 5.3). In general, the largest increase in the level of H_2O_2 accumulation in seeds was found after heat stress at both seed development stages (80% plus 50% SMC) from all three raceme positions (Table 5.3).

Heat stress at seed filling stage (≈80% SMC) significantly (*P<0.05*) increased differences in accumulated H_2O_2 level between the seeds of the top (T) section racemes and those in the middle (M) and base (B) of the raceme (Table 5.4), but there was no significant difference in H_2O_2 contents between any of the three racemes positions when heat stress was applied at physiological maturity (Table 5.4). The interaction between the two seed development stages (80 x 50% SMC) in terms of differences in H_2O_2 contents of any comparison between the three raceme positions was not significant.

Least significant effect (LSE) shows that H_2O_2 content in seeds from the top raceme section was higher than that in seeds from the middle and basal raceme positions in the presence of heat stress either at 80% SMC or 50% SMC. H_2O_2 concentration in middle raceme seeds did not differ from the seeds of basal raceme sections (Table 5.4). The seeds from the top sector of the raceme had a higher H_2O_2 level than did seeds from the middle and basal racemes for heat stress at both seed development stages (80% SMC + 50% SMC). However, H_2O_2 concentration in seed from the middle sector of the raceme did not differ with that in seeds from the basal raceme position with this treatment (Table 5.4).

Table 5.3 Effect of temperature stress during seed development on forage rape seed H_2O_2 content (µmoles g^{-1} of seed).

Treatments	H_2O_2 (µmoles g^{-1} of seed)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	3.74	4.72	3.24	3.28
Heat stress at 80% SMC	5.05	7.22	4.06	3.89
LSD (5%)	**1.10**	**1.79**	**1.35**	**0.92**
Significance of difference	*	*	*ns*	*ns*
Main effect of heat stress at 50% SMC				
Nil (no stress)	3.55	4.88	2.86	2.94
Heat stress at 50% SMC	5.23	7.06	4.43	4.23
LSD (5%)	**1.10**	**1.79**	**1.35**	**0.92**
Significance of difference	**	*	*	*
Treatment means				
Control	3.02	3.70	2.46	2.88
Heat stress at 80% SMC	4.09	6.06	3.26	2.99
Heat stress at 50% SMC	4.47	5.74	4.01	3.68
Heat stress at (80%SMC+50% SMC)	6.00	8.37	4.85	4.78
SEM	0.49	1.14	0.85	0.55
LSD (5%)	**1.55**	**2.52**	**1.91**	**1.30**
Interaction effect (80% x 50% SMC)	0.46	0.27	0.04	0.99
L.S. interaction (5%)	**2.19**	**3.55**	**2.69**	**1.83**
Significance of interaction	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001.*
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, **LS. Interaction** = LSD (Treatment means) x√2

Table 5.4 Comparison of H_2O_2 (µmoles g^{-1} of seed) in seeds harvested from different sections of the raceme of forage rape plants.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatment	T-M	T-B	M-B
Main effect of heat stress at 80%SMC			
Nil (no stress)	1.49*	1.45*	-0.04
Heat stress at 80% SMC	3.14*	3.32*	0.18
LSD (5%)	**1.45**	**1.57**	**1.55**
Significance of difference	*	*	*ns*
LSE (5%)	**1.03**	**1.11**	**1.05**
Main effect of heat at 50% SMC			
Nil (no stress)	2.00	1.93	-0.07
Heat stress at 50% SMC	2.63	2.84*	0.21
LSD (5%)	**1.45**	**1.57**	**1.48**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	1.03	1.11	1.05
Treatment means			
Control	1.24	0.82	-0.42
Heat stress at 80% SMC	2.76*	3.04*	0.28
Heat stress at 50% SMC	1.73*	2.08*	0.35
Heat stress at (80%SMC+50% SMC)	3.52*	3.59*	0.07
S.E.M.	0.91	0.98	0.92
LSD (5%)	**2.04**	**2.21**	**2.08**
LSE (5%)	**1.45**	**1.57**	**1.48**
Interaction effect (80% x 50% SMC)	0.27	-0.71	-0.98
L.S. interaction (5%)	2.88	3.12	2.93
Significance of difference	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= significant at *P<0.001.*
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) -(Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, **LS. Interaction** = LSD (Treatment means) x√2

5.5 Discussion

Seed quality in terms of subsequent germination can be negatively influenced by the temperature in which the parent plant is growing (Sanhewe et al., 1996). Among all the environmental factors, high temperature stress can reduce the production and quality of crops (Van der Merwe et al., 2015). Seed's physical, biochemical, physiological and genetic characteristics are influenced by its response to the parental environment and any stress can lead to oxidative stress (Daws et al., 2004; Pukacka & Ratajczak, 2005). Therefore , the disruption or alteration in oxidative metabolism of seed developed under stress conditions could be a reliable indicator of stress severity and its response to the stressful environment (Varghese et al., 2011).

The present study has shown that short periods of high temperature stress at specific seed development stages significantly altered metabolic activities in these seeds relative to the seeds developed under an ambient field temperature (control). In this study, a single heat stress treatment either before or at PM, significantly increased H_2O_2 and MDA accumulation in developing forage rape seeds by between 33-50%. However, when the heat stress was applied twice, before and at PM, MDA and H_2O_2 were not increased significantly over that for either single treatment. Pre-harvest seed deterioration (loss of seed vigour) was probably due to this oxidative stress which led to the accumulation of reactive oxygen species (ROS) (especially H_2O_2) and subsequently resulted in lipid peroxidation, as indicated by the enhanced accumulation of MDA in heat stressed seeds. The present observations are consistent with the results reported by Bhatia et al. (2010) and Wang et al. (2012). Bhatia et al. (2010) found that pre-harvest seed deterioration as a result of field weathering when harvested at or three weeks after PM, induced oxidative stress which subsequently resulted in the over accumulation of ROS and lipid peroxidation in soybean. Wang et al. (2012) in a field study, demonstrated that soybean plants exposed to high temperature and humidity stress (40 °C/30 °C, 100%/70% RH, light/dark) during seed development and maturity began to deteriorate due to the accumulation of ROS (especially H_2O_2) in the developing seeds. They concluded that the higher level of lipid peroxidation was the outcome of excessive accumulation of ROS in the developing heat stressed seeds relative to the control (unstressed) seeds.

ROS are generally produced as a by-product of normal metabolism during seed development, germination, desiccation and ageing. Any resultant cellular damage through oxidative stress leads to seed deterioration (Bailly, 2004; Bailly & Kranner, 2011; McDonald, 1999).ROS, despite their negative role through oxidative damage, also play a signalling role to regulate various physiological processes. There is a delicate equilibrium between ROS generation and redox signalling molecules for normal metabolic processes (Bailly, 2004; Sharma et al., 2012). This homeostatic condition of intracellular concentration of ROS signals can be altered through various environmental stresses which leads to enhanced production of ROS (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2015; Mittler, 2002).The

over production of ROS because of environmental stresses poses a serious threat to cells and causes damage to membrane lipids (peroxidation of unsaturated fatty acids), nucleic acids and proteins (through oxidation, denaturation and inactivation of enzymes) (Eltayeb et al., 2010; Mittler, 2002; Srivastava & Dubey, 2011). Seed deterioration due to oxidative stress is deemed to be irreversible with the cellular damage caused known to be a common cause of decreased structural integrity and increased mortality of seeds (Bailly, 2004; Repetto et al., 2012).

Among ROS, H_2O_2 is continuously produced during aerobic metabolic processes such as photosynthesis and respiration in mitochondria, chloroplasts, plasma membranes, cell walls and peroxisomes (glyoxysomes, in oily seeds). It has a dual role, as it may act as a signal transduction in cell growth and development, but is triggered in a wide range of stressful environments in low concentration and leads to programmed cell death (PCD) in high concentration (Bailly, 2004; Mittler et al., 2004; Mori & Schroeder, 2004; Quan et al., 2008; Wan & Liu, 2008). H_2O_2 has a longer half-life (half million of 1 ms) and is relatively stable as it has no unpaired electrons, compared to other ROS such as O_2^-, and $OH^\bullet$ which have a much shorter half-life. Unlike most other ROS, it can easily cross biological membranes (aquaporin) and damage other surrounding organelles within the cell, far from its production site, and consequently produce oxidative stress (Bienert et al., 2007; Neill et al., 2002). The disturbance between the balance in production and removal of H_2O_2 is generally the cause of this oxidative stress. Serious damage to the cell organelles can occur by over accumulation of H_2O_2 via lipid peroxidation. It can also induce damage to almost all the important macromolecules, including DNA and proteins (Jimenez et al., 2002). H_2O_2 can inactivate enzymes, such as those of the Calvin cycle, by oxidizing their thiol groups and can also disturb cellular functions by damaging transport proteins and ion channels (Halliwell & Gutteridge, 2015).

In this study, heat stress during the seed filling stage (≈80% SMC) increased both MDA and H_2O_2. At this stage, seeds are mostly undergoing histodiffrentaition, and the production of H_2O_2 is associated with high metabolism and respiratory activity in the developing seeds (Bailly et al., 2008; Bailly et al., 2004). In normal or homeostatic conditions, ROS is normally removed by the detoxifying enzymes of the ascorbate–glutathione cycle (Bailly et al., 2008). Heat stress probably disrupts, inactivates or alters the activity of those detoxifying enzymes which leads to the over accumulation of H_2O_2 and subsequently lipid peroxidation. This is also likely to be the reason for increase in H_2O_2 and MDA recorded from seeds subjected to heat stress at PM. Following PM when seeds are desiccating, ROS production is normally reduced as a result of a decrease in metabolic and respiratory activity. However, any hypoxic conditions of the internal seed tissues and metabolic imbalance during this maturation drying stage could result in the over production of ROS (Bailly et al., 2008; Borisjuk & Rolletschek, 2009).This is also supported by the argument that during drying, membrane permeability of plant tissues increases due to modifications of its functions and metabolic disorders followed by the

accumulation of ROS (Bewley & Black, 1994; Crowe et al., 1998; Li & Sun, 1999; Xu et al., 2006).The antioxidant enzymes of the ascorbate-glutathione cycle activity are normally increased during the early stages of seed development in homeostatic conditions but decrease at later stages of seed development. At the later stages of seed development, during reserve deposition and maturation drying, activity of many of the thiol dependent antioxidant enzymes such as glutathione reductase, glutathione peroxidase (GPX) and peroxiredoxin (Prx), increases (Nogueira et al., 2013; Xu et al., 2006). The possible mechanism by which the ROS accumulation and lipid peroxidation in seeds might occur following heat stress is due to the inactivation of thiol dependant and some other antioxidant enzymes of the ascorbate-glutathione cycle (Kumar et al., 2013; Tian et al., 2008).

Forage brassica seeds borne at different raceme positions and developed under different heat stress conditions exhibited differences in ROS accumulation and lipid peroxidation. Heat stress effects on the metabolic activity (higher accumulation of H_2O_2 and MDA) were greater in seeds collected from the top of the raceme position than those harvested from middle and lower raceme positions. This might be due to the age of the seeds produced at the top raceme position because there was a lag between the seed produced on the different racemes positions. The seeds produced on the top raceme position are 10-15 days older than the seeds produced on the lower racemes of the plants (Illipronti et al., 2000). Such differences in seed quality and metabolic activities related to the raceme position were also reported by Froud-Williams and Ferris (1987) in blue grass (*Poa trivialis*), Adam et al. (1989) in soybean and Natarajan and Srimathi (2009) in petunia.

Heat stress treatments resulted in increased levels of both H_2O_2 and MDA accumulation as shown in table 5.1 and 5.3. However, no significant relationship (*P=0.191*) was established between H_2O_2 and MDA accumulation in deteriorating seed for the heat stress treatments, when H_2O_2 was regressed against MDA (Figure 5.2). Though MDA levels were increased by all heat stress treatments, when overall MDA contents of all treatments were regressed against seed germination and vigour, no significant correlation was established, either between MDA and seed germination (*P=0.37*), AA-germination (*P=0.21*) or with conductivity (*P=0.108*). This clearly indicates that increased MDA accumulation due to heat stress did not have significant effect on seed germination and vigour (Figure 5.3-5.5). There was no direct relationship between lipid peroxidation and deterioration in membrane integrity, since the electrolyte leakage markedly increased in all heat stressed treatment whereas MDA levels were increased for seed filling stage (80% SMC) relative to the control, but only slightly increased further after the stress at the later stage of seed development (Figure 5.5). Bailly et al. (1996) also found no direct relationship between lipid peroxidation and electrolyte leakage in sunflower seeds stressed at 45 ˚C, and suggesting that lipid peroxidation is not the sole mechanism to contribute to seed deterioration. However, it has been reported that high concentration of H_2O_2 can damage the biomolecules and can modify the intrinsic properties of membranes, like fluidity (Creissen &

Mullineaux, 2002; Dat et al., 2000) leading to oxidative stress by losing the detoxifying potential necessary to complete germination (Bailly, 2004).

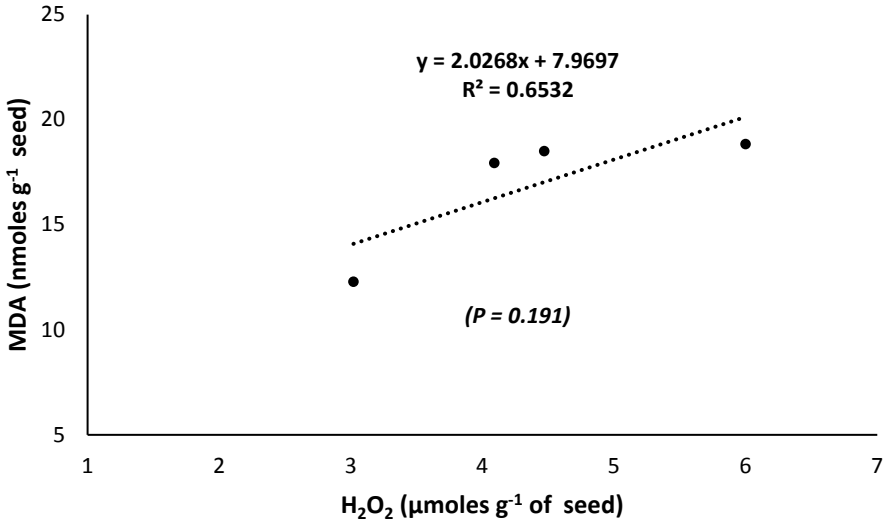


Figure 5.2 Correlation of H_2O_2 with MDA accumulation in heat stressed seed. The relationship did not appear to be linear and the regression was not fitted. No relationship existed between H_2O_2 and MDA accumulation in deteriorating seed as a result of temperature stress.

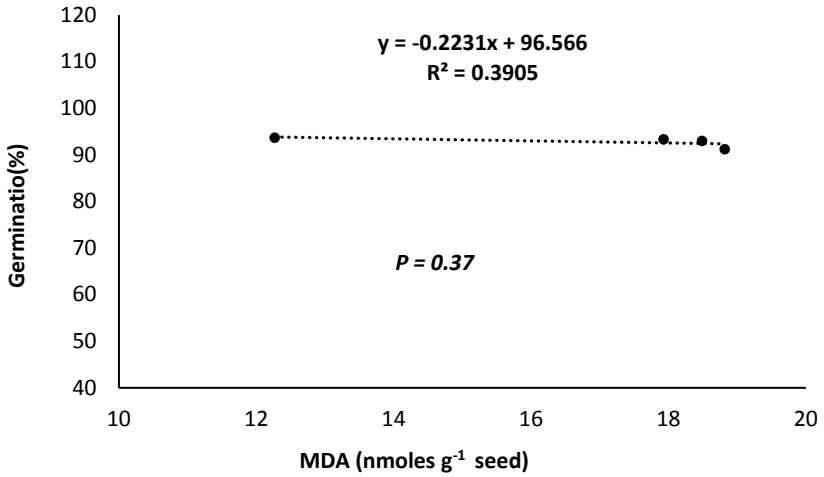


Figure 5.3 Correlation of MDA with standard germination in heat stressed seeds. There was no linear relationship and the regression was not significant (*P=0.37*). Increase in MDA did not reduce seed germination of heat stressed seeds (see Table 3.2 for germination data of 2012- 13 season.

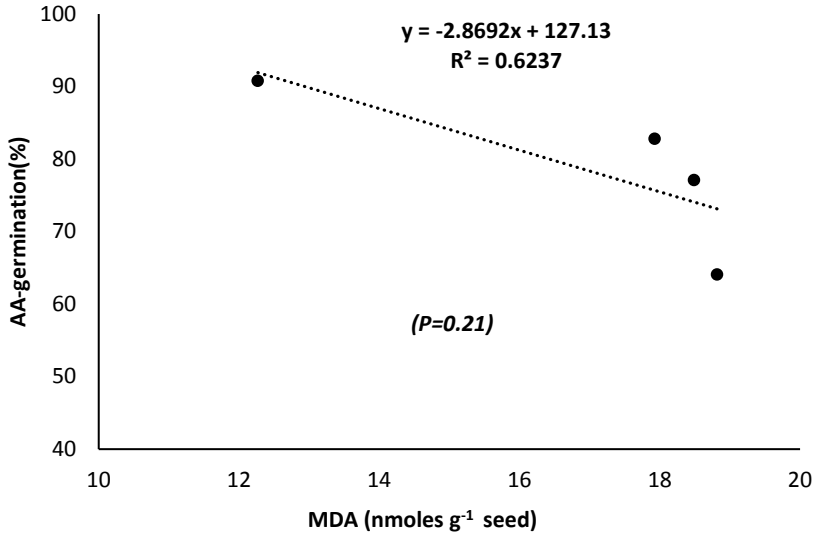


Figure 5.4 Correlation of MDA with AA- germination in heat stressed seeds. The relationship is not a linear fitted line and the regression was not significant (*P=0.21*).
Increase in MDA did not reduce AA-germination of heat stressed seeds (see Table 3.5 for AA-germination data of 2012- 13 season).

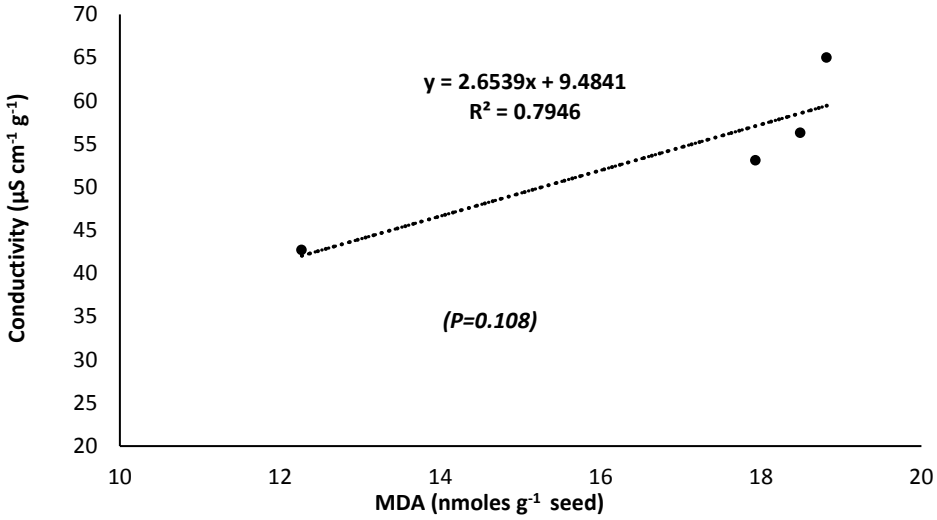


Figure 5.5 Correlation of MDA with seed conductivity in heat stressed seeds. The relationship is not a linear fitted line and the regression was not significant (*P=0.108*).
Increase in MDA did not increased conductivity of heat stressed seeds (see Table 3.8 for conductivity data of 2012-13 season).

Higher accumulation of H_2O_2 due to heat stress had a significant effect on seed quality attributes i.e. seed germination and vigour. There was a significant correlation (*P<0.05*) between H_2O_2 and seed germination for heat stress treatments (Figure 5.7). Similarly, there was a significant correlation

between H_2O_2 and the two seed vigour tests (conductivity and AA-test *P< 0.01* and *P <0.05* respectively) (Figure 5.6 - 5.8) indicating that increasing H_2O_2 level decreased both seed germination and seed vigour.

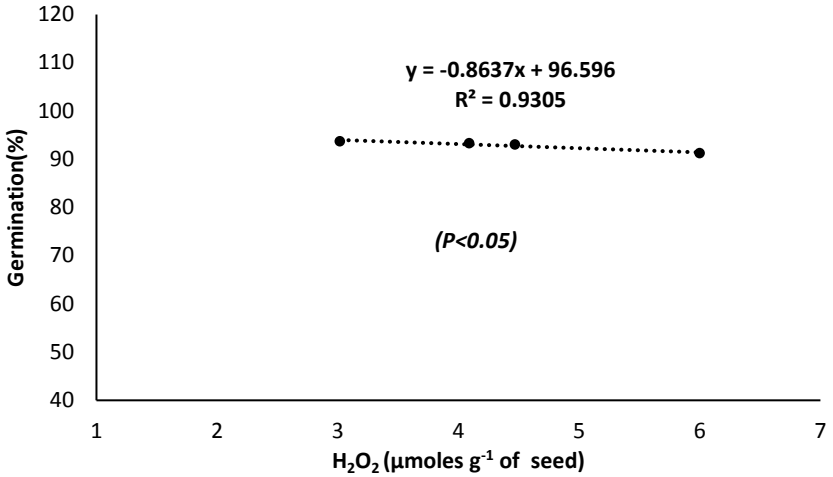


Figure 5.6 Correlation of H_2O_2 with standard germination in heat stressed seeds. There was a significant linear relationship and the regression was not significant (*P=0.05).* Increase in MDA did not reduce seed germination of heat stressed seeds (see Table 3.2 for germination data of 2012- 13 season.

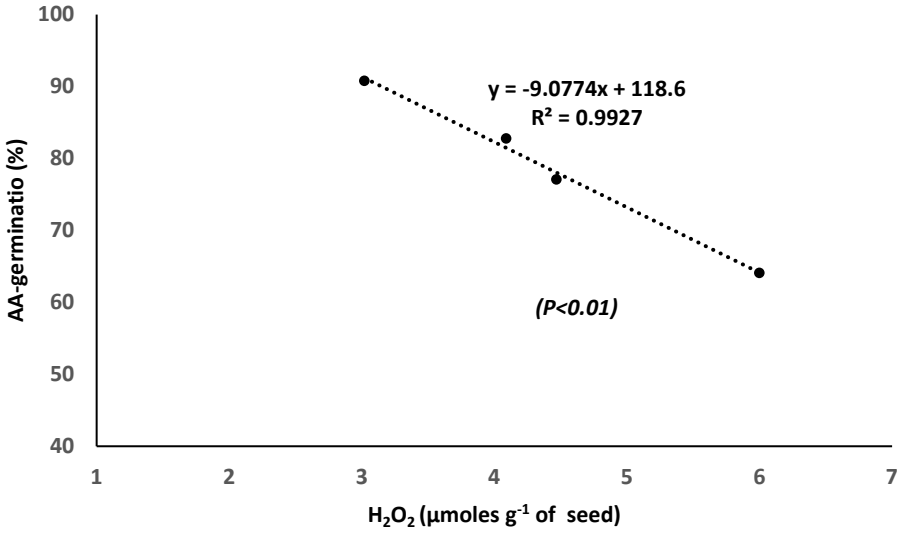


Figure 5.7 Correlation of H_2O_2 with AA-germination (%) in heat stressed seed. The relationship is linear and the regression was significant (*P<0.01*). Seed vigour decreased with the increase in the accumulation of H_2O_2 (see Table 3.5 for AA-germination data of 2012-13 season).

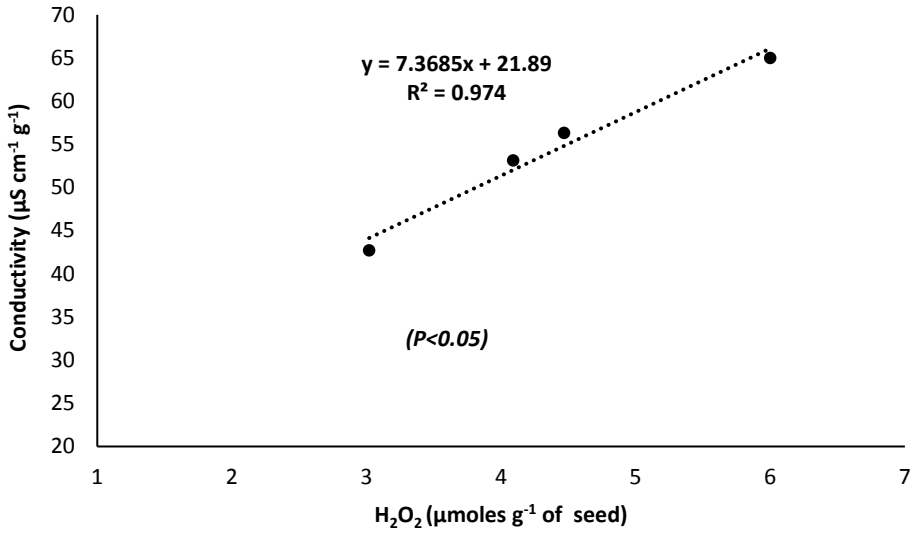


Figure 5.8 Correlation of H_2O_2 with Conductivity (µS cm^{-1} g^{-1}) in heat stressed seeds. The relationship is linear and the regression was significant (*P<0.05*).With increase in ROS, the conductivity of heat stressed seeds increased (see table 3.8 for conductivity data of 2012-13 season).

Kibinza et al. (2006) found a linear relationship between H_2O_2 content and sunflower seed viability but not for MDA, during induced seed deterioration at high temperature (35 ˚C).The results of the present study are partially consistent with this report as Kibinza et al. (2006) reported that lipid peroxidation occurred only after 50% of the seeds lost their viability, and associated seed ageing with H_2O_2 accumulation and loss of some detoxifying enzyme activities. However, our results are in agreement with Bailly et al. (2002a), who associated a marked increase in H_2O_2 accumulation with loss of seed vigour. Previous studies on sunflower (Bailly et al., 2004) and cotton (Goel & Sheoran, 2003) seeds, have revealed a significant relationship between H_2O_2 and MDA contents. However, in the present study, there was no significant relationship found between H_2O_2 and MDA accumulation after heat stress, suggesting that H_2O_2 alone is not responsible for lipid peroxidation and some other ROS such as $OH^{\bullet}$ O^{-}_2, $^1O_2$ are also involved (Bailly et al., 2008; Douglas, 1996) or H_2O_2 and/or its derivatives such as the highly reactive hydroxyl radical ($OH^{\bullet}$) might cause oxidative stress by reacting with molecules other than lipids such as proteins and nucleic acid (Beckman & Ames, 1997).

5.6 Conclusions

In this study, heat stress increased both ROS (H_2O_2) and MDA accumulation in seeds stressed both before and at PM. A relationship between H_2O_2 and MDA was not established but seed vigour declined as H_2O_2 and MDA accumulation in seeds increased. Seeds collected from the top of the raceme had a higher accumulation of H_2O_2 and MDA after heat stress than those from the bottom and middle raceme positions. Seed deterioration likely occurred due to the possible alteration in the equilibrium between ROS accumulation and its removal through detoxifying enzymes to maintain homeostatic condition. The implication of this mechanism was investigated and is discussed in Chapter 6.

Chapter 6
Effect of high temperature stress during seed development on antioxidant enzymes, nucleotides and ultrastructure of forage rape seeds

6.1 Introduction

6.1.1 Antioxidant enzyme system of seeds

One of the mechanisms of seed deterioration involves the impairment of physiological processes due to oxidative stress following the overproduction of reactive oxygen species (ROS) which are deleterious to plant tissues (Ahmad & Prasad, 2012; Ashraf, 2009; Siebers et al., 2015). Cell organelles such as mitochondria, chloroplasts, peroxisomes or glyoxysomes, plasma membranes, apoplasts and the nucleus have high metabolic activity. This involves a high rate of electron flow through the electron transport chain within the cell and is considered to be the major source of ROS production (Gill & Tuteja, 2010; Sandalio et al., 2013; Temple et al., 2005).Under normal environmental conditions, oxidative stress is minimal due to the efficient processing of ROS through the well-coordinated enzymatic and non-enzymatic antioxidant systems. However, during active physiological processes, or during abiotic stresses, the rapid generation of ROS such as H_2O_2, $O_2^{\bullet-}$, $OH^{\bullet}$, or $^1O_2$ occurs due to high oxidizing metabolic activity. If ROS production becomes greater than ROS detoxification, this causes severe damage to biomolecules such as lipids, proteins and DNA, so that finally cellular metabolism is arrested (Gill et al., 2015; Hasanuzzaman et al., 2013).

Heat stress, like other abiotic stresses, uncouples enzymes of different metabolic pathways, which ultimately results in the accumulation of unnecessary and harmful ROS (Asada, 2006). Among ROS, superoxide ($O_2^{\bullet-}$) is formed through the Mehler reaction in chloroplasts by the photoxidation reaction (flovoprotein, redox recycling). This also occurs in the electron transport chain of mitochondria and during the photorespiration reaction in glyoxysomes or in the plasma membrane through NADPH oxidase, xanthine oxidase and membrane polypeptides. The hydroxyl radical is formed by the reaction of hydrogen peroxide with $O_2^{\bullet-}$ through the Haber- Weiss reaction, by the reaction of hydrogen peroxide with Fe^{+2} through the Fenton reaction and by the decomposition of O_3 in apoplastic space (Karuppanapandian et al., 2011; Møller et al., 2007), while the singlets oxygen is generated by the photo inhibition, and electron transport chain (ETC) reaction in chloroplasts (Huang & Xu, 2008; Karuppanapandian et al., 2011).Hydrogen peroxide is formed either by the dismutation of the superoxide radical into hydrogen peroxide or by the inactivation of hydrogen peroxide scavenging enzymes, produced through normal metabolic process (Willekens et al., 1995).

Excessive and toxic ROS are, therefore, liable to cause damage to the cells. The cells must be equipped with efficient and robust machinery to create a balance between production and scavenging of ROS. ROS also play a positive role as signalling molecules to stimulate and control various biological processes such as response against various biotic and abiotic stresses, defence against pathogens and systemic signalling (Gill & Tuteja, 2010), but as already explained this equilibrium could be perturbed by many biotic and abiotic stresses leading to the production of excessive ROS. Plants have developed a wide range of endogenous systems to protect themselves from oxidative stress by scavenging ROS molecules to create a balance and keep the ROS to sub lethal levels (Foyer & Noctor, 2005).The antioxidant system is comprised of various enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include super oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). The non-enzymatic antioxidants are glutathione (GSH), ascorbic acid (AsA) and lipid soluble carotenoids and tocopherols (Khan & Singh, 2008; Mittler, 2002).

Superoxide dismutase has the ability to convert one form of ROS i.e. superoxide ($O_2^{\bullet -}$) into the equally toxic form of hydrogen peroxide (H_2O_2) (Bowler et al., 1992; Gill et al., 2015). Therefore, plants require the presence of effective antioxidant systems such as CAT and enzyme pathways such as the Halliwell-Asada Pathway enzymes (APX, DHAR, MDHAR, and GR) to detoxify ROS, especially H_2O_2. CAT is the principal enzyme for the removal of H_2O_2 localized in peroxisomes/ glyoxysomes. In the absence or inactivation of CAT, alternative enzymatic mechanisms can scavenge H_2O_2 (Willekens et al., 1997). Ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes are important enzymes of the ascorbate-glutathione cycle. This cycle efficiently eliminate ROS, especially H_2O_2, by successive oxidation and reduction of ascorbate, glutathione and NADPH by the APX and GR enzymes (Figure 6.1)(Noctor & Foyer, 1998). APX, localized in the cytosol, chloroplasts (Sharma et al., 2012), peroxisomes (Jiménez et al., 1998) and mitochondria (De Leonardis et al., 2000), is involved in the efficient elimination of H_2O_2 in the absence of CAT (Singh et al., 2010) by oxidation of ascorbate into dehydroascorbate (Noctor & Foyer, 1998; Smirnoff, 2000).

The activities of antioxidant enzymes not only depend upon the susceptibility or tolerance to stress of different crop varieties, their growth stages and growing season, but also on the temperature. The activation or inactivation of these enzymes is temperature dependent. Activities of SOD, APX and CAT declined with increasing temperature after an initial increase while the peroxidase (POX) and glutathione reductase (GR) enzymes were inactivated or showed a decline in activity in a range of temperatures from 20-50 ˚C (Chakraborty & Pradhan, 2011). However, other researchers have reported an increasing trend in APX and GR activities with increasing temperature to protect the cells from oxidative stress by ROS (Balla et al., 2009; Hasanuzzaman et al., 2012).Generally, increasing temperature results in upregulation of antioxidant enzymes until a critical level of temperature is

reached, after which a decline in the expression of these enzymes is observed (Rani et al., 2013). The temperature range required to maintain increased level of the antioxidant enzymes varies between stress tolerant and intolerant crop varieties. Tolerant varieties have upregulated activities of some antioxidant enzymes relative to susceptible varieties during heat stress (Chakraborty & Pradhan, 2011).

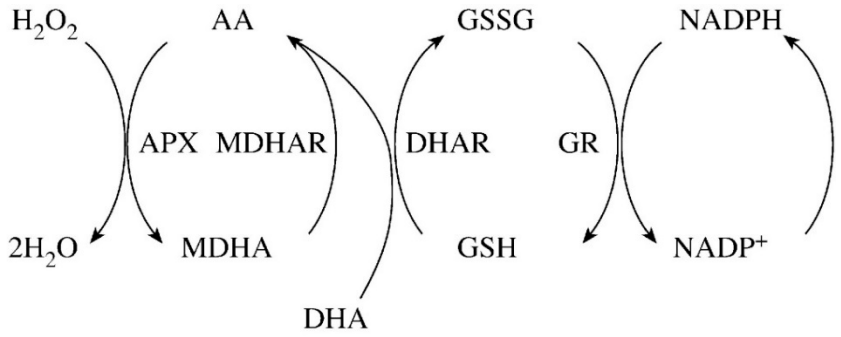


Figure 6.1. Schematic diagram of the Ascorbate-glutathione cycle also called the Halliwell–Asada pathway (May et al., 1998).
In this reaction cycle, Hydrogen peroxide (H_2O_2) can be eliminated by ascorbate peroxidase (APX). Ascorbate in reduced form (Asc) is oxidized to generate monodehydroascorbate (MDH), which is reduced either by monodehydroascorbate reductase (MDHAR) to Asc or reacts with dehydroascorbate (DHA) due to its instability. A reduction reaction then again reduces DHA by dehydroascorbate reductase (DHAR) to Asc using GSH (glutathione) as the reductant. Oxidation of GSH generates glutathione disulfide (GSSG) dimer which is then reduced by NADPH-dependent glutathione reductase (GR) to GSH. The electron acceptor NADP is regenerated during the reduction of MDHA and GSSG by the respective enzymes.

Many studies have reported that seed deterioration is associated with the loss of antioxidant enzyme activity (Bailly, 2004; Bailly et al., 2002a; Demirkaya, 2013; Demirkaya et al., 2010; Pukacka & Ratajczak, 2005) but most of these studies and the information published on seed deterioration at the physiological level have either been conducted on stored seed or by artificially accelerating the ageing of seed. However, the physiological mechanisms of pre-harvest seed deterioration are still poorly understood. No substantive data are available on the physiological and biochemical changes which occur during pre-harvest seed deterioration due to adverse environmental conditions, particularly high temperature stress during seed development. It was hypothesized that high temperature stress during seed development inactivates or decreases antioxidant enzyme activities or the rate of their synthesis.

6.1.2 Objectives

The present study was conducted to study the effect of high temperature during forage rape seed development on the expression of ROS scavenging antioxidant enzyme activity.

6.1.3 Materials and methods

6.1.3.1 Enzyme extraction

Enzyme extraction was carried out as described by Bailly and Kranner (2011) with some minor modifications. For the extraction of enzymes, 0.1 g of forage brassica seeds were ground with liquid nitrogen in a mortar, defatted with hexane to remove most of the oil from the seed material, and homogenised with 1 ml of extraction buffer containing, 0.1M potassium phosphate buffer (pH 7.8), 2 mM dithiothreitol, 0.1mM EDTA, 1.25mM PEG-4000 and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged for 15 min at 14,000 g. The supernatant was then desalted by applying 75µl to the top of a spin column containing Bio-Gel P-DG gel equilibrated with 0.1M potassium phosphate buffer (pH 7.8), and centrifuged for 4 min at 1000 g. This sample clean up removed biomolecules below 6,000 Daltons and improved the reproducibility of the readings (Bio-Rad, USA). This extract was then used for enzyme assays.

6.1.3.2 Measurement of antioxidant enzyme activity

(i) Superoxide dismutase (EC 1.15.11)

Superoxide dismutase activity (SOD, EC 1.15.11) was measured, as described by Bailly & Kranner (2011), by the inhibition of Nitro blue tetrazolium (NBT) photo reduction, with some modifications. For the 3 ml assay the reaction mixture contained 2.5 ml of 0.1 M potassium phosphate buffer (pH 7.8), 0.03 mL (30 µl) of 6.3mM NBT (final concentration 63 µM), 0.3 ml of 130 mM Methionine (final concentration 13 mM) and 10 µl of enzyme extract. The reaction was allowed to start after adding 30 µl of 130µM riboflavin (final concentration of 1.3 µM). Identical tubes were also established with the reaction mixture but not the sample enzyme extract. These served as 100% standards, while tubes containing neither sample nor riboflavin acted as 0% blanks. All tubes were illuminated with a 500 W fluorescent lamp for 15 minutes. After illumination 250 µl in triplicate of each sample with standards and blanks were loaded into flat bottomed 96 well polystyrene microplates and the absorbance was measured at 560 nm with a SpectraMax M2 Microplate Reader. One unit of SOD is defined as the amount of enzyme that inhibits NBT photo-reduction to blue formazan by 50%, (the formation of blue formazan is evaluated by a reaction carried out without enzyme extract) and SOD activity of the extracts was expressed as units SOD (mg seed weight)$^{-1}$.

(ii) Catalase (EC 1.11.1.6)

Catalase (EC 1.11.1.6) was measured according to the method of Bailly and Kranner (2011).The reaction mixture contained 10 µl of enzyme extract, 430 µl of 50 mM phosphate buffer (pH 7.0), 60 µl of 26 mM H_2O_2 (final concentration 3.125 mM) to make the total volume 500 µl in a 1 cm path length cuvette. After adding the extraction buffer and enzyme extract to the cuvette, H_2O_2 was added to start

the reaction and the decrease in absorbance at 240 nm for 1 min was recorded. The molar extinction coefficient ε=43.6 mM^{-1} cm^{-1} was used to calculate the CAT activity. Specific activity was defined as the degradation of 1 nano mole H_2O_2 in 1 min at 240 nm mg^{-1} seed weight.

(iii) Ascorbate peroxidase (EC 1.11.1.11)

Seeds of forage brassica from the various heat stress treatments (see Chapter 3) were imbibed for 8 hours at 20 ˚C on germination paper before extraction. Enzyme was extracted according to the method of Bailly and Kranner (2011). APX (EC 1.11.1.11) activity was measured by the method of Chen & Arora (2011) by estimating the rate of oxidation of ascorbic acid (AsA) to dehydroascorbate. Enzyme activity was determined by a decrease in absorbance at 290 nm. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.1 mM EDTA, 10 µl of enzyme extract and 50 µl of H_2O_2 (final concentration 0.1 mM) in a final volume of 500 µl. The reaction was initiated after the hydrogen peroxide addition and the decrease in absorbance at 290 nm recorded. One unit of APX activity was defined as the conversion of 1 µM AsA to dehydroascorbate per minute at 290 nm. The molar extinction coefficient (ε=2.8 mM^{-1} cm^{-1}) was used to calculate ascorbate peroxidase activity.

(iv) Glutathione reductase (EC 1.6.4.2)

Glutathione reductase (GR) activity was measured by the method of Bailly and Kranner (2011). Glutathione reductase activity was determined as the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.8, 10 mM glutathione (oxidized), 3 mM $MgCl_2$ and 10 µl of enzyme extract in a final volume of 500 µl. Absorbance of the blank rate was recorded and then 10 µl of 10 mM NADPH was added to initiate the reaction with GR activity recorded as the decrease in absorbance of NADPH per minute at 340 nm (ε=6.22 mM-1 cm-1). The enzyme activity was determined as nmoles of NADPH min^{-1} g FW^{-1}.

6.1.3.3 Statistical analysis

Statistical analysis was same as described in detail in Chapter 5, Section 5.3.3.

6.1.4 Results

6.1.4.1 Superoxide dismutase acivity

High temperature (heat) stress at the seed filling stage (≈80% SMC) did not significantly reduce SOD enzyme activity, but there was a highly significant *(P<0.001)* reduction when the temperature stress occurred at PM (≈50% SMC) (Table 6.1). There was no significant interaction between heat stress at the two seed development stages (80% SMC and 50% SMC). The reduction recorded for the double stress was explained by the latter stress, not the former (Table 6.1).

In seeds from the top section of the raceme, temperature stress at 80% SMC did not reduce SOD enzyme activity , but temperature stress at 50% SMC and at 80% SMC + 50% SMC did so *(P<0.05).* Once again, as there was no significant interaction between the two times of temperature stress, the reduction for the double stress was explained by the stress applied at 50% SMC (Table 6.1). SOD enzyme activity was not affected by any temperature stress treatment for seeds from the middle (M) and base section of the raceme (Table 6.1).

When SOD enzyme activity between different sections of the raceme was compared (Table 6.2), activity was higher in seeds from the top (T) of the raceme than those from the middle of the raceme (M) *(P<0.05)* for both temperature stress and non-stressed plants, but activity differences between T or M and seeds from the base (B) of the raceme were not significant (Table 6.2).

Table 6.1 Effect of high temperature during forage rape seed development on superoxide dismutase enzyme (nmoles g^{-1} seed weight) activity in hand harvested seeds from the bulk and from the different raceme positions.

Treatment	SOD activity (nmoles g^{-1} seed weight)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	1.35	1.48	1.24	1.33
Heat stress at 80% SMC	1.30	1.39	1.27	1.24
LSD (5%)	**0.06**	**0.13**	**0.11**	**0.18**
Significance of difference	*ns*	*ns*	*ns*	*ns*
Main effect of heat stress at 50% SMC				
Nil (no stress)	1.39	1.52	1.29	1.37
Heat stress at 50% SMC	1.26	1.35	1.23	1.20
LSD (5%)	**0.06**	**0.13**	**0.11**	**0.18**
Significance of difference	***	*	*ns*	*ns*
Treatment means				
Control	1.43	1.62	1.24	1.45
Heat stress at 80% SMC	1.34	1.41	1.33	1.29
Heat stress at 50% SMC	1.26	1.34	1.24	1.20
Heat stress at 80% +50% SMC	1.25	1.36	1.21	1.19
SEM	0.03	0.06	0.04	0.08
LSD (5%)	**0.08**	**0.19**	**0.16**	**0.25**
Interaction effect (80% x 50% SMC)	0.08	0.23	-0.12	0.15
L.S. interaction (5%)	**0.11**	**0.27**	**0.23**	**0.35**
Significance of difference	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P < 0.05*; ** = Significant at *P< 0.01*;
***= Significant at *P <0.001*.
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC = [(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC = [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 6.2 Comparison of SOD (nmoles g^{-1} seed weight) in seeds harvested from three sections of the racemes of forage rape plants.
An * indicates the difference (e.g. T- M) is significantly different from zero (i.e. M and T differ significantly a *P<0.05*).

Treatment	T-M	T-B	M-B
Main effect of heat stress at 80 % SMC			
Nil (No stress)	0.24*	0.16	-0.09
Heat stress at 80% SMC	0.12	0.14	0.02
LSD (5%)	**0.23**	**0.24**	**0.22**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	**0.16**	**0.91**	**0.16**
Main effect of heat stress at 50% SMC			
Nil (No stress)	0.23*	0.15	-0.09
Heat stress at 50% SMC	0.13	0.15	0.02
LSD (5%)	**0.23**	**0.24**	**0.22**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	**0.16**	**0.91**	**0.16**
Treatment means			
Control	0.37*	0.17	-0.20
Heat stress at 80% SMC	0.08	0.12	0.03
Heat stress at 50% SMC	0.10	0.14	0.03
Heat stress at (80%SMC+50% SMC)	0.15	0.16	0.01
S.E.M	0.09	0.11	0.09
LSD (5%)	**0.32**	**0.34**	**0.31**
LSE (5%)	**0.23**	**0.24**	**0.22**
Interaction effect (80% x 50% SMC)	0.34	0.07	-0.25
L.S. interaction (5%)	**0.45**	**0.48**	**0.44**
Significance of difference	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P < 0.05*; ** = Significant at *P< 0.01*;
***= Significant at *P < 0.001*; [1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(50% SMC)]/2
Heat stress at 80% SMC=[(80%) + (80+50% SMC)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(80% SMC)]/2
Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2
Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) - (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD(Treatment means) x√2,
LSE (5%) (Treatment means)=LSD (Treatment means)/√2.
LSE (5%) (Main Effect) = LSD (Main effect)/√2

6.1.4.2 Catalase

Seeds harvested from control plots had higher catalase (CAT) enzyme activity than these from heat stressed seeds (Table 6.3). Heat stress at the seed filling stage (≈80% SMC) significantly reduced CAT enzyme activities (*P<0.01*), but the effect of heat stress was larger at the physiological maturity stage (≈50 SMC) (*P<0.001*). There was no significant interaction between heat stress at the two seed development stages (80% SMC and 50% SMC) on CAT activity (*P=0.108*), which is explained by the larger effect at the physiological maturity stage than that at the seed filling stage (Table 6.3).

Non-stressed seeds harvested from all three raceme positions had a higher CAT activity than the heat stressed seeds. Catalase activity was reduced for all heat stressed seeds irrespective of raceme position, except for heat stressed seed harvested from the basal position of the raceme at 80% SMC (Table 6.3). The impact of heat stress at physiological maturity (≈50% SMC) was larger than at the seed filling stage. Heat stress at physiological maturity significantly reduced the catalase enzyme activity in seeds from the top, base (*P<0.01*), and middle (*P<0.001*) raceme sections of the raceme. Heat stress at the seed filling stage (≈80% SMC) significantly reduced catalase enzyme activity in seeds from pods on the top and middle raceme (*P<0.05*) but did not reduce it significantly in seeds from the basal raceme position (Table 6.3). There was a significant interaction between heat stress treatment at the two seed development stages (80% SMC and 50% SMC) in seeds from the top section of the raceme but not for the seeds harvested from the middle and basal raceme positions (Table 6.3).

Heat stress at the seed filling stage (≈80% SMC) did not produce any significant differences in CAT activity between any of the raceme positions. However, heat stress at physiological maturity (≈50% SMC) significantly increased the differences in CAT activity between the seeds from the middle and top raceme portions (*P<0.05*) but this difference was not significant between seeds from the basal raceme position and those from the top and middle of the raceme (T-B and M-B respectively) (Table 6.4). There was no significant interaction between the two heat stress treatments in CAT activity between raceme positions (Table 6.4).

The mean differences in seed catalase activity between raceme positions were compared to the effect of heat stress in seeds from each individual raceme position (Table 6.4). Middle raceme seeds had a higher catalase activity than seeds from the top raceme position both in the non-stressed and stressed treatments at both the seed filling and physiological maturity stages. This difference in catalase activity was larger in non-stressed seeds than that in heat stressed seeds at both times (Table 6.4). Seeds from the top of the raceme did not differ in catalase activity with those from the basal raceme position for either heat stress treatment, nor was there any significant interaction. Seeds from the middle raceme position did not differ with basal raceme seeds in catalase activity with heat stress at the seed filling

stage (80% SMC) but had higher catalase activity in non-stressed seeds compared to stressed seeds at physiological maturity (50% SMC) (Table 6.4).

Table 6.3 Effect of high temperature during seed development on Catalase enzyme (nmoles H_2O_2 min^{-1} mg^{-1} seed weight) activity in hand harvested seeds from different raceme positions.

Treatments	Catalase activity (nmoles H_2O_2 min^{-1} mg^{-1} seed weight)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	0.17	0.15	0.21	0.16
Heat stress at 80% SMC	0.13	0.1	0.15	0.13
LSD (5%)	**0.023**	**0.04**	**0.05**	**0.05**
Significance of difference	**	*	*	*ns*
Main effect of heat stress 50% SMC				
Nil (no stress)	0.21	0.17	0.26	0.20
Heat stress at 50% SMC	0.09	0.09	0.1	0.09
LSD (5%)	**0.02**	**0.04**	**0.05**	**0.05**
Significance of difference	***	**	***	**
Treatment means				
Control	0.24	0.19	0.31	0.22
Heat stress at 80% SMC	0.18	0.15	0.21	0.18
Heat stress at 50% SMC	0.11	0.11	0.11	0.10
Heat stress at (80% SMC+ 50% SMC)	0.08	0.06	0.09	0.09
SEM	0.01	0.02	0.02	0.02
LSD (5%)	**0.03**	**0.06**	**0.08**	**0.07**
Interaction effect (80% x 50% SMC)	0.04	0.00	0.08	0.03
L.S. interaction (5%)	**0.04**	**0.08**	**0.11**	**0.10**
Significance of interaction	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P<0.001*

Values represent the average of four replicates.

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(SMC 50%)]/2

Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(SMC 80%)]/2

Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2

Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) – (Control)].

LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 6.4 Comparison of CAT (H_2O_2 decomposed µmoles per min^{-1}) in seeds harvested from three sections of the raceme of forage rape plants.
An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatment	M-T	T-B	M-B
Main effect of heat stress at 80% SMC			
Nil (no stress)	0.06*	-0.01	0.06
Heat stress at 80% SMC	0.04	-0.03	0.01
LSD (5%)	**0.08**	**0.07**	**0.08**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	**0.05**	**0.05**	**0.06**
Main effect of heat stress at 50% SMC			
Nil (no stress)	0.09*	-0.03	0.06
Heat stress at 50% SMC	0.01	-0.01	0.01
LSD (5%)	**0.08**	**0.07**	**0.08**
Significance of difference	*	*ns*	*ns*
LSE (5%)	**0.05**	**0.05**	**0.06**
Treatments means			
Control	0.12*	-0.03	0.10*
Heat stress at 80% SMC	0.06	-0.03	0.03
Heat stress at 50% SMC	0.00	0.01	0.01
Heat stress at (80%SMC+50% SMC)	0.02	-0.02	0.00
S.E.M	0.03	0.03	0.04
LSD (5%)	**0.11**	**0.10**	**0.12**
LSE (5%)	**0.08**	**0.07**	**0.08**
Interaction effect (80% SMC x 50% SMC)	0.08	-0.13	0.05
L.S. interaction (5%)	**0.15**	**0.14**	**0.16**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*;

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(50% SMC)]/2

Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(80% SMC)]/2

Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2

Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) – (Control)].

LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD(Treatment means) x√2,

LSE (5%) (Treatment means)=LSD (Treatment means)/√2,

LSE (5%) (Main Effect) = LSD (Main effect)/√2

6.1.4.3 Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was highest for non-heat stressed (control) seeds but not always significantly so (Table 6.5). The impact of heat stress was highest on physiologically mature seeds. For the bulk seeds heat stress at the seed filling stage (≈80% SMC) did not reduce APX peroxidase activity but heat stress at PM (≈50% SMC) significantly (*P < 0.01*) decreased APX activity (Table 6.5). There was no significant interaction for APX activity between the stress applied at the two seed development stages (80% SMC and 50% SMC) (*P=0.275*) (Table 6.5).

APX activity ranged from 1.87 to 4.97 μmol min^{-1} mg^{-1} FW, the latter being for the non-heat stressed seeds (Table 6.5). APX activity decreased with the heat stress treatments (Table 6.5). With the heat stress at PM, APX activity was reduced by 51% compared to the non-stressed control seeds. The largest reduction (62%) in APX activity was found in seeds subjected to the double heat stress at PM (≈50% SMC) following the heat stress at the seed filling (≈80% SMC) stage (Table 6.5).

At 80% SMC, heat stress reduced APX activity in seeds from the top and middle racemes but not in seeds from the bottom raceme (Table 6.5). Heat stress at PM reduced APX activity in seeds from all three raceme positions ((*P<0.05*) for the top and middle racemes and *P<0.01* for the base raceme (Table 6.5)). The highest APX activity was recorded in seeds from all three raceme positions for non-stressed seeds. The largest significant reduction in APX activity was found for heat stress at both seed development stages (≈80% SMC and ≈50% SMC) in seeds from all three raceme positions. However, the interaction of applying stress at the two seed development stages (80% SMC and 50% SMC) was not significant in seeds from all three raceme positions i.e. top, middle and base (*P=0.418, P= 0.237 and P= 0.367* respectively) (Table 6.5).

There were no significant differences in APX enzyme activity in response to heat stress in seeds between any two raceme positions. However, in the non-stressed (control) seeds there was a significant difference between the middle and basal raceme positions, where the APX scavenging activity was significantly higher (*P > 0.05*) in seeds from the middle of the raceme than in seeds from the basal raceme position at both the seed filling (80% SMC) and PM (≈ 50% SMC) stages (Table 6.6). The interaction effect was non-significant for any comparison between raceme positions for APX activity (Table 6.6).

Table 6.5 Effect of high temperature stress during forage rape seed development on Ascorbate peroxidase (APX) (µmol min^{-1} mg^{-1} FW) activity in hand harvested seeds from the bulk sample and from different raceme positions.

Treatments	APX activity (µmol min^{-1} mg^{-1} FW)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	3.69	3.59	4.29	3.20
Heat stress at 80% SMC	2.53	2.30	2.65	2.65
LSD (5%)	**1.21**	**1.27**	**1.60**	**1.15**
Significance of difference	*ns*	*	*	*ns*
Main effect of heat stress at 50% SMC				
Nil (no stress)	4.09	4.62	3.67	3.98
Heat stress at 50% SMC	2.14	2.32	2.19	1.91
LSD (5%)	**1.21**	**1.60**	**1.15**	**1.27**
Significance of difference	**	*	*	**
Treatment means				
Control	4.97	4.86	5.88	4.18
Heat stress at 80% SMC	3.20	3.10	3.35	3.15
Heat stress at 50% SMC	2.41	2.31	2.69	2.22
Heat stress at (80% SMC+ 50% SMC)	1.87	1.50	1.95	2.15
SEM	0.53	0.56	0.71	0.51
LSD (5%)	**1.70**	**1.79**	**2.26**	**1.62**
Interaction effect (80% SMC x 50% SMC)	1.23	0.95	1.79	0.96
L.S. interaction (5%)	**2.38**	**2.52**	**3.19**	**2.28**
Significance of interaction	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*;

***= Significant at *P <0.001*

Values represent the average of four replicates.

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(SMC 50%)]/2

Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(SMC 80%)]/2

Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2

Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].

LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 6.6 Comparison of Ascorbate peroxidase (µmol min^{-1} mg^{-1} FW) activity in seeds harvested from the top (T), middle (M) and base(B) of the racemes of forage rape plants. An * indicates the difference (e.g. T-M) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatment	M-T	T-B	M-B
Main effect of heat stress at 80% SMC			
Nil (no stress)	0.70	0.38	1.08*
Heat stress at 80% SMC	0.35	-0.35	0.00
LSD (5%)	**1.10**	**1.25**	**0.82**
Significance of difference	*ns*	*ns*	*
LSE (5%)	**0.78**	**0.89**	**0.58**
Main effect of heat stress at 50% SMC			
Nil (no stress)	0.64	0.31	0.96*
Heat stress at 50% SMC	0.42	-0.28	0.13
LSD (5%)	**1.10**	**1.25**	**0.82**
Significance of difference	*ns*	*ns*	*
LSE (5%)	**0.78**	**0.89**	**0.58**
Treatment means			
Control [1]	1.02	0.67	1.70*
Heat stress at 80% SMC	0.26	-0.05	0.21
Heat stress at 50% SMC	0.38	0.09	0.46
Heat stress at (80% SMC+ 50% SMC)	0.45	-0.65	-0.20
S.E.M	0.49	0.55	0.36
LSD (5%)	**1.55**	**1.76**	**1.15**
LSE (5%)	**1.10**	**1.25**	**0.82**
Interaction effect (80% SMC x 50% SMC)	0.83	-0.02	0.83
L.S. interaction (5%)	**2.19**	**2.48**	**1.62**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05;* ** = Significant *at P< 0.01*; ***= significant at *P< 0.001*;

[1] Plants in the pots left in the field.

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(50% SMC)]/2

Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(80% SMC)]/2

Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2

Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].

LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

6.1.4.4 Glutathione reductase (GR)

The highest activity of glutathione reductase (GR) was recorded in the seeds of non-heat stressed (control) plants (Table 6.7). A decline in GR activity was recorded in bulk seeds with application of temperature stress at both seed development stages (Table 6.7). Heat stress significantly reduced GR activity during seed filling (80% SMC) and at physiological maturity (*P <0.001*) (Table 6.7). GR activity was further decreased when heat stress was applied at both seed filling (80% SMC) and PM (≈ 50% SMC) (Table 6.7). However, there was no significant interaction for GR activity between the stress applied at these two seed development stages (*P= 0.708*) (Table 6.7).

The main effect of temperature during seed filling (80% SMC) was a significant 20% reduction in GR enzyme activity (*P > 0.001*) and a similar significant 26% reduction was also recorded at PM (*P>0.001*) compared to seeds from the non-heat stressed control. GR activity declined by 41% for the double heat stress (Table 6.7)

Non-stressed seeds harvested from the three raceme positions had the highest GR activity (Table 6.7), with the greatest activity occurring in seeds harvested from the middle sector of the raceme (Table 6.7). At the seed filling stage (80% SMC), GR activity was not affected in seeds from the basal racemes but was significantly reduced in seeds from the top and middle raceme positions (*P<0.001* and *P <0.01* respectively) (Table 6.7). Heat stress at PM significantly reduced the enzyme activity in seeds harvested from all three raceme positions (*P>0.001*) (Table 6.7). These decreases were 24%, 28% and 23% for the top, middle and basal raceme positions respectively. There was no significant interaction for GR activity between the two temperature stress treatments in seeds harvested from any of the raceme positions (Table 6.7).

The responses to heat stress on GR activity of seeds harvested from the three raceme positions was compared with least significant effect values (Table 6.8). GR activity was higher in seeds from the pods on the middle sector of the raceme than that of the seeds from the top raceme section both in non-stressed and the seeds stressed either during seed filling or at PM (Table 6.8). The GR activity in seeds from the top and middle racemes did not differ with that of basal raceme seeds when the temperature stress was applied during seed filling, but there was a higher GR activity in seeds from both the top and middle racemes than the basal raceme with temperature stress application at PM (Table 6.8).

Though non-stressed seeds had a higher GR activity at the top and middle raceme position than those of seeds from the basal racemes, the main effect of heat stress during seed filling (80% SMC) did not differ. However for heat stress applied during the seed filling stage the GR activity was significantly higher from seeds from the top raceme than the basal raceme position (*P>0.05*) (Table 6.8).

Table 6.7 Effect of high temperature stress during forage rape seed development on Glutathione reductase (GR) (nmol NADPH min^{-1} mg^{-1} FW) activity in hand harvested seeds from the bulk sample and from different raceme positions.

Treatments	GR activity (nmol of NADPH min^{-1} mg^{-1} FW)			
	Raceme bulk	Raceme positions		
		Top (T)	Middle (M)	Base (B)
Main effect of heat stress at 80% SMC				
Nil (no stress)	0.94	0.95	1.07	0.79
Heat stress at 80% SMC	0.75	0.70	0.83	0.73
LSD (5%)	**0.07**	**0.10**	**0.13**	**0.07**
Significance of difference	***	***	**	*ns*
Main effect of heat stress at 50% SMC				
Nil (no stress)	0.97	0.94	1.10	0.86
Heat stress at 50% SMC	0.72	0.71	0.79	0.66
LSD (5%)	**0.07**	**0.10**	**0.13**	**0.07**
Significance of difference	***	***	***	***
Treatment means				
Control	1.06	1.05	1.24	0.90
Heat stress at 80% SMC	0.87	0.84	0.96	0.81
Heat stress at 50% SMC	0.81	0.85	0.89	0.68
Heat stress at (80%SMC+50% SMC)	0.63	0.57	0.69	0.65
SEM	0.03	0.04	0.06	0.03
LSD (5%)	**0.08**	**0.14**	**0.19**	**0.10**
Interaction effect (80% x 50% SMC)	0.01	-0.07	0.08	0.06
L.S. interaction (5%)	**0.11**	**0.20**	**0.27**	**0.14**
Significance of interaction	*ns*	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*;
***= Significant at *P <0.001*
Values represent the average of four replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)]/2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)]/2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)]/2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)]/2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%)- (Control)].
LSD (Main Effect) = LSD (Treatment means)/√2, LS**. Interaction** = LSD (Treatment means) x√2

Table 6.8 Comparison of Glutathione reductase (GR) (nmol of NADPH min^{-1} mg^{-1} FW) activity in seeds harvested from the top (T), middle (M) and base (B) racemes of forage rape plants. An * indicates the difference (e.g. M-T) is significantly different from zero (i.e., M and T differ significantly at *P<0.05*).

Treatment	M-T	T-B	M-B
Main effect of heat stress at 80% SMC			
Nil (no stress)	0.12*	0.16*	0.28*
Heat stress at 80% SMC	0.12*	-0.03	0.09
LSD (5%)	**0.11**	**0.15**	**0.18**
Significance of difference	*ns*	*	*ns*
LSE (5%)	**0.07**	**0.11**	**0.13**
Main effect of heat stress at 50% SMC			
Nil (no stress)	0.16*	0.09	0.25*
Heat stress at 50% SMC	0.08*	0.05	0.13
LSD (5%)	**1.11**	**0.15**	**0.18**
Significance of difference	*ns*	*ns*	*ns*
LSE (5%)	0.07	0.11	0.13
Treatments			
Control	0.19*	0.14	0.34*
Heat stress at 80% SMC	0.13*	0.03	0.15
Heat stress at 50% SMC	0.04	0.17*	0.22*
Heat stress at (80%SMC+50% SMC)	0.12*	-0.08	0.04
S.E.M	0.05	0.07	0.09
LSD (5%)	**0.15**	**0.22**	**0.27**
LSE (5%)	0.10	0.15	0.19
Interaction effect (80% x 50% SMC)	0.14	-0.14	0.01
L.S. interaction (5%)	**0.21**	**0.31**	**0.38**
Significance of interaction	*ns*	*ns*	*ns*

ns= Non significant; *= Significant at *P< 0.05*; ** = Significant at *P< 0.01*; ***= Significant at *P< 0.001*;

Main effect of heat stress at 80% SMC

Nil (no stress) = [(Control) + [(50% SMC)]/2

Heat stress at 80% SMC= [(80%) + (80+50% SMC)]/2

Main effect of heat stress at 50% SMC

Nil (no stress) = [(Control) + [(80% SMC)]/2

Heat stress at 50% SMC= [(50% SMC) + (80+50% SMC)]/2

Interaction Effect = [(80+50% SMC)-(50% SMC)] - [(80% SMC) - (Control)].

LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD(Treatment means) x√2,

LSE (5%) (Treatment means)=LSD (Treatment means)/√2

LSE (5%) (Main Effect) = LSD (Main effect)/√2

6.1.5 Discussion

Environmental stresses such as low or high temperature are likely to induce ROS accumulation in cells and decrease the capacity to eliminate or detoxify the excessive ROS to maintain an optimum level for efficient participation in metabolic processes. This accumulation subsequently causes oxidative damage to cells and even cell death (Bailly, 2004; Lee et al., 2010; McDonald, 1999; Xu et al., 2013).

In the present study, a significant decrease in SOD activity was recorded in forage rape seeds only when heat stress was applied at PM, suggesting that SOD function as a first line defence against ROS scavenging had been perturbed and its activity declined, presumably through a reduced ability to convert O_2^- into H_2O_2 (Castillo, 1996; Gill et al., 2015). These reductions in SOD activity are similar to those reported by Sung (1996) for soybean and Goel and Sheoran (2003) for cotton. The decline in SOD activity under heat stress is possibly either due to reduced synthesis or degradation of enzymes, through changes in assembling enzyme units (Castillo, 1996; Garnier et al., 2006).

The Catalase (CAT) enzyme has the features of high capacity and low affinity in the peroxisomes/glyoxysomes of the plant cells, and is a principle scavenging enzyme of the antioxidant system through its ability to destroy and eliminate the H_2O_2 produced by dismutation of superoxide (Ali & Alqurainy, 2006; Willekens et al., 1997) and other metabolic processes such as photo-respiration and β-oxidation of fatty acids (Graham, 2008; Hu et al., 2012).The response of catalase activity to stressful conditions varies. Catalase activity has been reported to increase, decrease and sometimes does not show any significant change under heat stress conditions (Scandalios et al., 2000; Yin et al., 2008). The results of the present study showed a marked decrease in CAT activity with heat stress at both seed development stages, and the combined stress at both stages. These decreases in catalase activity also corroborate the findings of Bhatia et al. (2010), who reported that catalase activity was decreased in seed during pre-harvest seed deterioration. Catalase is sensitive to heat stress. A possible mechanism involved in the reduction of catalase activity is believed to be due to the photoinactivation of the catalase enzyme (Feierabend et al., 1992) and inhibition of enzyme synthesis in dark conditions, which may favour the accumulation of H_2O_2 and cause cellular damage (Bailly, 2004; Bailly et al., 2008). Liu and Huang (2000) and Jiang and Huang (2001)observed a similar result in grasses resulting from shading of photosynthetically active tissues. However, in the absence of lodging, this is unlikely to occur in the upright racemes of forage rape. Another possible mechanism for the decline in CAT activity may be due to the accumulation of H_2O_2 and its reaction with O^-_2 to produce the hydroxyl radical (OH^-) through via the Herbert-Weiss reactions (Bowler et al., 1992).

Both APX and GR enzyme activity declined in heat stressed seeds (the only exception being APX activity at 80% SMC). The decline in APX could be directly associated with the decrease in catalase activity (Chen & Asada, 1992; Hernandez et al., 2010; Lee et al., 2007). Alternatively, it could possibly be due to less regeneration of ascorbate (Asc) and reduced glutathione (GSH) from oxidized glutathione

(GSSG) using NADPH because APX requires ascorbate and glutathione reductase for the regeneration system for its activation (Apel & Hirt, 2004; McDonald, 1999). There was also a decrease in GR activity due to heat stress. GR can also play an important role in detoxification of endogenous H_2O_2 within chloroplasts by oxido-reduction in the ascorbate-glutathione cycle by maintaining an optimum concentration of reduced glutathione (GSH) and oxidized glutathione (GSSG). However, it has also been reported to provide protection from peroxidation damage of cellular membranes by trapping oxygen radicals (Alscher, 2006; Barclay, 1988).The continued decrease in GR activity under heat stress would also allow H_2O_2 accumulation. Jiang and Huang (2001) also reported the over accumulation of hydrogen peroxide after high temperature stress due to a decrease in GR activity. Siebers et al. (2015) demonstrated that heat stress during the reproductive phase in soybean significantly decreased the ability of the ascorbate system to detoxify the toxic ROS.

The response of both substrates i.e. glutathione and ascorbate, required for the glutathione-ascorbate cycle was similar for the different temperature stress treatments. Under heat stress, plants generate and increase the production of glutathione and ascorbate in different forms to be used as a substrate to empower the cells to detoxify the different ROS produced. However, he

at stress possibly inactivated the principal enzymes of this pathway. This may explain why the heat stress increased the levels of both glutathione and ascorbate (oxidized or reduced), or in total forms in higher concentration, since these substrates were not being used by the APX or GR, and therefore H_2O_2 would not be detoxified, as indicated by the higher concentration of H_2O_2 recorded (see Chapter 5) (Rivero et al., 2004).

6.2 Effect of high temperature stress on nucleotide pool and adenylate energy charge (AEC)

6.2.1 Introduction

Dry seeds contain metabolically inactive organelles and enzymes, formed during the maturation phase. Re-activation of these metabolic processes is necessary and this can only occur when seeds start to imbibe. A burst of energy is released during the early hours of hydration to initiate the seed germination process (Hourmant & Pradet, 1981; Rajjou & Debeaujon, 2008; Rosental et al., 2014). The generation and rapid increase of this energy is mainly in the form of adenosine triphosphate (ATP). The metabolic energy status of seeds can be quantified in the form of the adenylate energy charge (AEC) , which refers to the ratio of (ATP + 0.5 ADP) / (ATP + ADP + AMP) (Atkinson, 1968). AEC expresses the saturation of the adenylate pool in phosphoanhydride bonds (Atkinson, 1968; Kibinza et al., 2006; Pradet & Raymond, 1983). The ATP level and AEC are very low in quiescent seeds (<0.5), but abruptly increase during the imbibition process (Bewley et al., 2012; Moreland et al., 1974). AEC controls the

seed's metabolic activity and is directly correlated with the ATP energy pool by the action of adenylate kinase (Borisjuk et al., 2003; Pradet & Raymond, 1983).

Environmental stress is known to influence metabolic activity in plants; high temperature, low light and drought can all reduce the level of ATP and AEC (Mangat, 1982; McKee & Mendelssohn, 1984). Madden and Burris (1995) reported that high temperature reduced the maize nucleotide energy pool during the early hours of germination. Although ATP as an energy source is well correlated with seed germination and vigour, the mitochondria are the origin of the ATP energy produced during the early hours of germination (Grass & Burris, 1995a; Madden, 1992). Quiescent seeds have very low, almost negligible ATP energy, and mitochondria are functionally and structurally deficient to the extent that they can hardly be differentiated (Pradet & Raymond, 1983) . Although, the evidence of heat stress influence on ATP and or AEC is strong (Standard et al., 1983; Wahid et al., 2007; Zidenga, 2005), most studies have focused on plant growth and development, and very little literature is available on seeds. For that which is available, most studies have been carried out on post-harvest temperature stress during seed storage and its effects on seed viability (Kibinza et al., 2006; Smith & Berjak, 1995) and only limited studies have been carried out on heat stress effects on seeds before harvest.

The objective of the present study was to investigate the effects of heat stress during seed development on nucleotide levels in seeds.

6.2.2 Materials and methods

The experiment was conducted to assess the nucleotide energy charge in forage rape seeds of known vigour harvested after heat stress treatments (see Section 3.3, Chapter 3). Nucleotide extraction and determination was carried out according to the method of Smolders (2011).Before nucleotide extraction, 2.0 to 2.5 g (≈ 400 seeds) were weighed, imbibed in deionized water and placed on moistened germination paper at 20 ˚C for 8 hours to initiate the germination process. After 8 hours, seeds were ground with liquid nitrogen in a mortar and pestle, defatted with 5 ml dichloromethane by stirring for 10 min in a 15 ml tube and then dried in a micro Modulyo freeze dryer (with a FDP120 freeze dry pump). Dried seed powder (1 g) was weighed and nucleotides extracted with 4 ml of perchloric acid (8% v/v) and homogenised with a vortex mixer. The homogenate was then centrifuged at 4000 rpm at 4 ˚C for 4 min, after which the supernatant was cooled for 10 min and neutralized with 5.2 ml of KOH (1.33 M). The neutralized material was then vortexed and centrifuged (8 min @ 14000 rpm at 4 ˚C). The supernatant after cooling was then filtered through a 0.5µm filter to remove the perchloric acid. 720µl of filtered supernatant, containing adenine nucleotide was transferred into a 1.5 ml tube and 80 µl NaAc/HAc buffer (5.3 ml 1M NaAc + 14.7 ml 1 M HAc) was added to bring the pH to 4.2. The final solution was then reacted with 30 µl of chloroacetaldehyde (Sigma-Aldrich®, Saint Louis, Missouri, USA) for 4 min at 80˚C under gentle stirring using a thermomixer (Eppendorf) in a fume hood,

to convert the adenine nucleotide to fluorescently detectable etheno-adenine nucleotides and incubated in a thermomixer (Eppendorf) under a fume hood for 4 min at 80˚C. After derivatization, the samples were centrifuged for 8 min at 14000 rpm at 4 ˚C, transferred to HPLC tubes and stored at -75 ˚C until use. Standards of 10, 20 and 60 ppm of ATP, ADP and AMP were also prepared individually and as a mixture of all three nucleotides using the same procedure described above.

For the nucleotide separation and quantification, the HPLC analysis was carried out on a Dionex HPLC system (Ultimate 3000). Separation was achieved using a reversed-phase C18 column (Grace Smart, 250 x 4.6 mm, 5 µ, 120 Å). The fluorescent etheno(Ɛ)-adenine nucleotides were detected at an excitation wavelength of 340 nm and an emission wavelength of 420 nm using a Dionex RS Fluorescence Detector (Ultimate 3000). Data acquisition was performed using Chromeleon 7.2 Chromatography Data System software (Thermo Scientific).

For the separation of etheno(Ɛ)-adenine nucleotides, two buffers of an aqueous solvent and organic solvent base were used. The aqueous solvent phosphate buffer A consisted of 0.1M K_2HPO_4, pH 6.0. Organic based buffer B solvent consisted of: 50% buffer A/50% methanol (v/v). Before the first run, the column was equilibrated with buffer A and a buffer blank was run. After the last run, the column was cleaned and flushed with HPLC grade water and stored in 50/50 methanol/ water.

20 µl each of the etheno (Ɛ)-adenine nucleotide (-ATP, -ADP and -AMP) standards were injected by an auto sampler to obtain a standard curve. A gradient elution was performed at a flow rate of 300 µl/min (Table 6.9). Etheno (Ɛ)-adenine nucleotides move faster with buffer in the column due to their polar nature. Ɛ –ATP moves more rapidly than Ɛ-ADP in phosphate buffer due its high solubility in aqueous buffers. Ɛ-AMP interacts more with the stationary phase due to lower solubility in an aqueous buffer. This interaction takes place by weak Van der Waals dispersion forces* (Israelachvili & Tabor, 1973). As a result of less solubility in the mobile phase and a greater interaction with the stationary phase, Ɛ-AMP takes the longest time to pass through the column. Based on the indifferent solubility, Ɛ-ATP elutes first and is then followed by Ɛ-ADP and Ɛ-AMP.

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*Sum of attractive or repulsive forces between dipoles. Compared to chemical bonds they are relatively weak. These forces define the solubility of organic substances in polar and non-polar media.

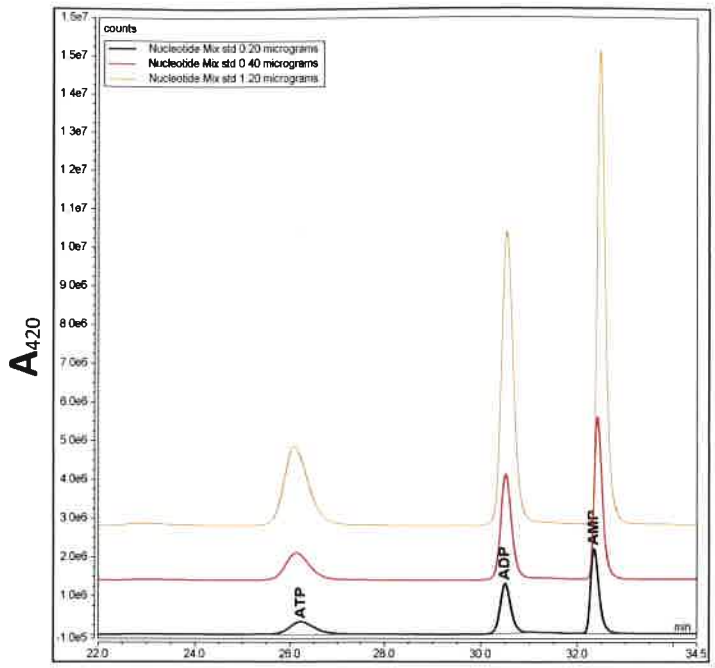

Figure 6.2 HPLC chromatogram of pure three mixed nucleotide standards (AMP, ADP and ATP). The peaks were used to identify the specific nucleotide with their retention time and peak areas were used to quantify the concentration of nucleotides.

The peaks of ATP, ADP and AMP were identified by comparing the retention times with chromatographic peaks of the standards. The eluted (Ɛ)-adenine nucleotides were excited by the fluorescent detector at a wavelength of 340 nm. The emitted signals were captured at 420 nm wavelength and plotted on a function of time to obtain the chromatogram. Peak retention time and peak area were quantified by the Chromeleon 7.2 software. Component identification is based on retention time while peak area under a curve is proportional to the concentration of the component. Mixed standards of AMP, ADP and ATP were derivatised and known amounts (0.2, 0.4 and 0.12 µg (20 µl each)) were injected and analysed by HPLC to establish specific retention times and to correlate the peak areas. Peak retention times of the standard curve peak areas were used to correlate the peak areas from injected samples to an absolute amount of ATP, ADP and AMP (Figure 6.2).

Table 6.9 Schedule of gradient flow

Step	Time (min)	Flow (µl/min)	Buffer A (%)	Buffer B (%)
0	0.5	0.3	100	0
1	15.5	0.3	100	0
2	25.5	0.3	0	100
3	30.5	0.3	0	100
4	35.5	0.3	100	0

6.2.2.1 Statistical analysis

For the analysis of quantified values of nucleotides, seed samples from three blocks were used with three replications. Statistical analyses of data were performed using Genstat software (16th Edition). Analysis of variance (ANOVA) was used to compare the means for seeds from each temperature stress treatment at a particular seed development stage. The impact of temperature stress at two seed development stages (80% and 50% SMC and 80+50% SMC) during seed development on nucleotides (AMP, ADP and ATP) content of seeds was analysed in a 2x2 factorial design with temperature stress at 80% SMC (+,-) and at 50% SMC (+, -) as treatment factors. Least significant difference (LSD 5%) was used to compare means of treatments between factors. The interaction between two treatment was calculated and Least significant interaction (LS interaction 5% = LSD X√2) was used to test interaction significance.

6.2.3 Results

The peaks of ATP, ADP and AMP were eluted at 26, 30.5 and 32.5 minutes respectively (Figure 6.2).The peaks were detected by their correspondent absorbance at 420 nm and identification of peaks were done by comparing with their respective peaks of eluted nucleotide standard at the same time. All eluted peaks of ATP, ADP and AMP nucleotides extracted from both control and heat stressed seeds were clearly identified without any distinct interference due to excitation by the chloroacetaldehyde and detection by the florescent detector. The ATP peak for non-heat stressed control seeds was identified to show variation between the stressed and non-stressed seed samples. The ATP and ADP profile were almost similar, whereas the AMP concentration was higher (Figure 6.3 a). The AMP peak exhibited interference with one or more unidentified compound present in the extract.

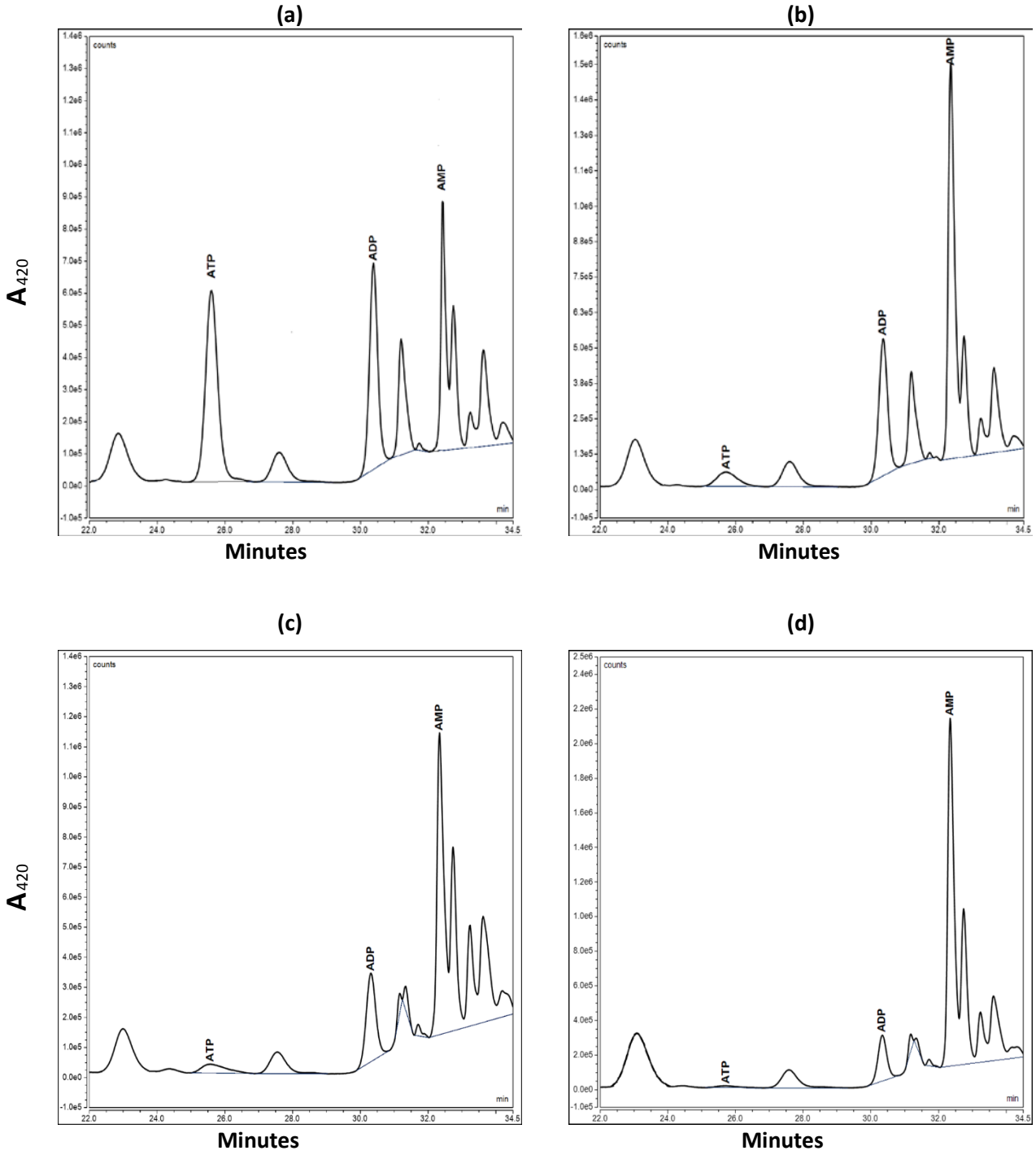

Figure 6.3 HPLC chromatogram of (a) Control (non-stressed) (b) heat stressed at seed filling stage (≈80% SMC) (c) heat stressed at PM (d) heat stressed at both (b) + (c).

All three heat stress treatments reduced ATP and ADP concentration but increased AMP concentration (Figure 6.3 b, c, d). ATP concentration was severely reduced, being almost non-detectable from the double stress treatment (Figure 6.3 d). At the seed filling stage (≈80% SMC), the eluted profile of ATP and ADP had declined while the AMP concentration had increased (Figure 6.3 b). Similarly, heat stress

at PM also reduced nucleotide ATP, ADP and also increased AMP (Figure 6.3 c). For the double stress treatment AMP had increased, a small amount of ADP was present but the ATP was negligible (Figure 6.3 d).

For quantitative estimation of nucleotide levels, standard curves were used. A clear difference in ATP and ADP pool size was detected between the control and heat stressed seeds (Table 6.10). All three heat stress treatments significantly *(P<0.001)* decreased the concentration of both ADP and ATP. The largest reduction in concentration of both nucleotides was recorded for the double stress at the seed filling and PM stages (Table 6.10). There was a significant interaction for heat stress between seed development stages (80% SMC and 50% SMC) for ATP concentration *(P < 0.01)* but not for ADP concentration *(P= 0.175)* (Table 6.10). In contrast, AMP concentration was lowest in the control seeds and increased significantly *(P<0.001)* with the heat stress treatments. There was a significant (*P< 0.01*) interaction between the two heat stress treatments for AMP (Table 6.10).

The adenylate energy charge (AEC) was also affected by the heat stress during seed development (Table 6.10). The AEC value of non-stressed seeds was 0.6 nmol g^{-1}**,** because of the high concentration of ATP and lower concentration of AMP and ADP. After the heat stress, the AEC values in all treatments were significantly *(P< 0.001)* decreased because of the decreases in ATP and ADP and the increase in AMP values. The AEC values of heat stress at seed filling and PM stage were almost similar (0.25 nmol $g^{-1)}$ due to a low concentration of ATP and a higher concentration of AMP. However, the AEC values were decreased to the greatest extent (0.12 nmol $g^{-1)}$ for the double heat stress. This was because of the high AMP value and very low ATP content (Table 6.10). The interaction between the two seed development stages (80% SMC and 50% SMC) was significant *(P<0.001)* (Table 6.10).

For treatment means, all heat stress treatments significantly increased AMP content compared to the control. The largest increase in AMP was with the double heat stress treatment (Table 6.10).However, there was no difference between heat stress at PM and for the double stress (Table 6.10). ADP content was significantly decreased in all heat stress treatments and the largest decrease was with the double heat stress (Table 6.10). Similarly, ATP content was also decreased with all heat stress treatments with the highest decrease being with the double heat stress treatment (Table 6.10).

Table 6.10 Nucleotide contents (nmol g^{-1})* and adenylate energy charge (AEC) of forage rape seeds stressed at a high temperature (240˚Ch) during seed development.

Treatments	AMP	ADP	ATP	AEC
Main effect of heat stress at 80% SMC				
Nil (no stress)	57.9	48.4	44.0	0.41
Heat stress at 80% SMC	91.7	35.2	6.7	0.18
LSD (5%)	**11.2**	**4.9**		**0.02**
Significance of difference	***	***	***	***
Main effect of heat stress at 50% SMC				
Nil (no stress)	76.5	50.4	45.9	0.39
Heat stress at 50% SMC	73.1	33.2	4.7	0.19
LSD (5%)	**11.2**	**4.9**	**2.8**	**0.02**
Significance of difference	*ns*	***	***	***
Treatment means				
Control [1]	46.37	58.56	80.97	0.60
Heat stress at 80% SMC	106.55	42.22	10.91	0.25
Heat stress at 50% SMC	69.35	38.32	7.03	0.23
Heat stress at (80%SMC+50% SMC)	76.86	28.18	2.45	0.12
LSD (5%)	**15.8**	**6.9**	**3.9**	**0.03**
SEM	4.6	2.0	1.2	0.01
CV%	10.6	8.3	7.9	5.9
Interaction effect (80% SMC X 50% SMC)	-52.6	6.2	65.9	0.24
L.S. interaction (5%)	**22.2**	**9.7**	**5.5**	**0.04**
Significance of interaction	**	*ns*	***	***

ns= Non significant; *= Significant at *P< 0.05*; ** =Significant at *P< 0.01*; ***= Significant at *P< 0.001*
*Dry- weight basis, Values are average of three replicates.

Main effect of heat stress at 80% SMC
Nil (no stress) = [(Control) + [(SMC 50%)] /2
Heat stress at 80% SMC= [(SMC 80%) + (SMC 80+50%)] /2
Main effect of heat stress at 50% SMC
Nil (no stress) = [(Control) + [(SMC 80%)] /2
Heat stress at 50% SMC= [(SMC 50%) + (SMC 80+50%)] /2
Interaction Effect = [(SMC 80+50%)-(SMC 50%)] - [(SMC 80%) - (Control)].
LSD (Main Effect) = LSD (Treatment means) /√2, **LS. Interaction** = LSD (Treatment means) x√2

6.2.4 Discussion

The parent plant environment has a major impact on the physiological processes in the seeds during their development. High temperature stress during seed development decreased the nucleotide pool size. Corbineau et al. (2002) reported that ATP levels in sunflower seeds were decreased by high temperature stress (45 ˚C) and that 24 hours of this heat stress treatment was sufficient to kill seeds. Similarly, Kibinza et al. (2006) demonstrated that high temperature stress resulted in a decrease in total adenine nucleotide level and ATP level. Within the cell mitochondria are the main source of the energy required during active metabolism. Seeds which develop under non-stress conditions have efficient oxidative phosphorylation and functioning mitochondria (Raymond et al., 1985).However, under heat stress, respiratory activity is reduced, probably due to damage to mitochondria at an ultrastructural level and/or a reduction in mitochondrial differentiation or development(Smith & Berjak, 1995). This alteration in energy pathways and energy metabolism in deteriorated seeds has been demonstrated by McDonald (1999) and Corbineau et al. (2002) in sunflower seeds. Grass and Burris (1995b) also demonstrated that during seed development, high temperature stress resulted in reduced adenosine triphosphate (ATP) synthesis and accumulation, and reduced nucleotide pool size and energy levels in wheat cultivars. The results of this study are in accordance with those reported by Grass and Burris (1995b), Corbineau et al. (2002) and Kibinza et al. (2006).

Heat stress during seed development also therefore resulted in a large reduction in the adenylate energy charge (AEC). The AEC of control (non-heat stressed) forage rape seed was much higher than that of the heat stressed seeds. AEC is the best gauge for the metabolic energy in the stored adenine nucleotide pool (Atkinson, 1968). Its values range from 0-1 depending upon the level of oxidative phosphorylation (Wiese & Seydel, 1995). In an efficient metabolic system under normoxic conditions (normal oxygen concentration), AEC values are usually ≥ 0.8 (Bewley & Black, 1994; McKee & Mendelssohn, 1984). The AEC values in this experiment were very low after heat stress because of a low concentration of ATP and higher values of adenosine mono phosphate (AMP). Heat stress during seed development decreased the metabolic activity, which was reflected in the lower AEC values. Grass and Burris (1995b) reported a decrease in AEC values due to high temperature stress during wheat seed development and maturation. These results are in agreement with this report. The decrease in AEC was mainly due to the decrease in mitochondrial ATP levels, suggesting that probably, in the respiratory chain, some dissociation of oxidative phosphorylation occurred, and as a consequence, AEC was decreased (Abrahim et al., 2003).This decrease in ATP or AEC is likely to be associated with reduced activity of mitochondria and/or structural changes in mitochondria. This will be discussed in the next section.

6.3 Impact of heat high temperature during seed development on ultrastructure of forage rape seeds as determined by electron microscopy

6.3.1 Materials and methods

For the electron microscopy, forage rape seeds from the different heat stress treatments were imbibed for 6-8 h on wet tissues, then were dissected by using a scalpel in a fixative solution (0.05 M sodium cacodylate buffer pH 6.8, containing 2.5% glutaraldehyde and 1% tannic acid) and maintained in fixative overnight at 4 ˚C. After removal from the fixative solution, the fragments were washed three times for 10 minutes each in the same buffer and post-fixed in 1% osmium tetroxide for 2 hours in the same buffer at room temperature. After a brief rinse in the buffer they were dehydrated through an acetone series (30, 50, 70, 90, 100% (twice)) for 30 min each and then transferred to 1:1 acetone and epoxy resin overnight at room temperature. Samples were transferred to neat epoxy resin on a rotator for 48 hours with three resin changes, then polymerized for 48 hours at 60 ˚C in flat moulds. Thin sections of embryonic axis approximately 80 nm wide were cut with a Diatome diamond knife on a Leica EM UC6 ultra microtome, mounted on 400-mesh, copper grids and stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate for 20 min and for 3 min, respectively. The sections were examined and images were taken by using an FEI Tecnai^{TM} 12 transmission electron microscope (TEM) operating at 120 kV, equipped with a Gatan Ultrascan 1000, 4 Megapixel digital camera.

6.3.2 Results

The analysis of TEM images of forage rape seeds heat stressed at two seed development stages i.e. during seed filling and PM, and at both stages, showed evidence of seed deterioration in comparison with the TEM images of control (non-heat stressed) seed. In the control seeds all cell organelles i.e. protein bodies (PB), lipid bodies (LB), mitochondria (M), ribosomes (R), endoplasmic reticulum, and nuclei were well distributed and visible (Figure 6.4 A-F). The structure of the plasma membranes and mitochondrial membranes was normal and the nuclear membrane with its nucleolus was visible. The bilayer of mitochondria with internal cristae was clearly visible. The cell wall of the plasma membrane was intact and continuous (Figure 6.4 A & B).

Signs of seed deterioration were evident in seeds after all three heat stress treatments. In seeds stressed at the seed filling stage, the outer membrane of organelles such as the nucleus was not fully developed. Some cells were shrunken and flattened (Figure 6.5 A-D). The nuclear membrane was not clearly visible and the structure of the plasma membrane was porous and broken in places (Figure 6.5 E-F). The outer membrane of the mitochondria was not fully developed and the cell wall was wavy (Figure 6.5 D). The cell organelles and structure of seeds heat stressed at PM, were damaged and deformed (Figure 6.6 A-F). Large intracellular spaces were visible between cells.

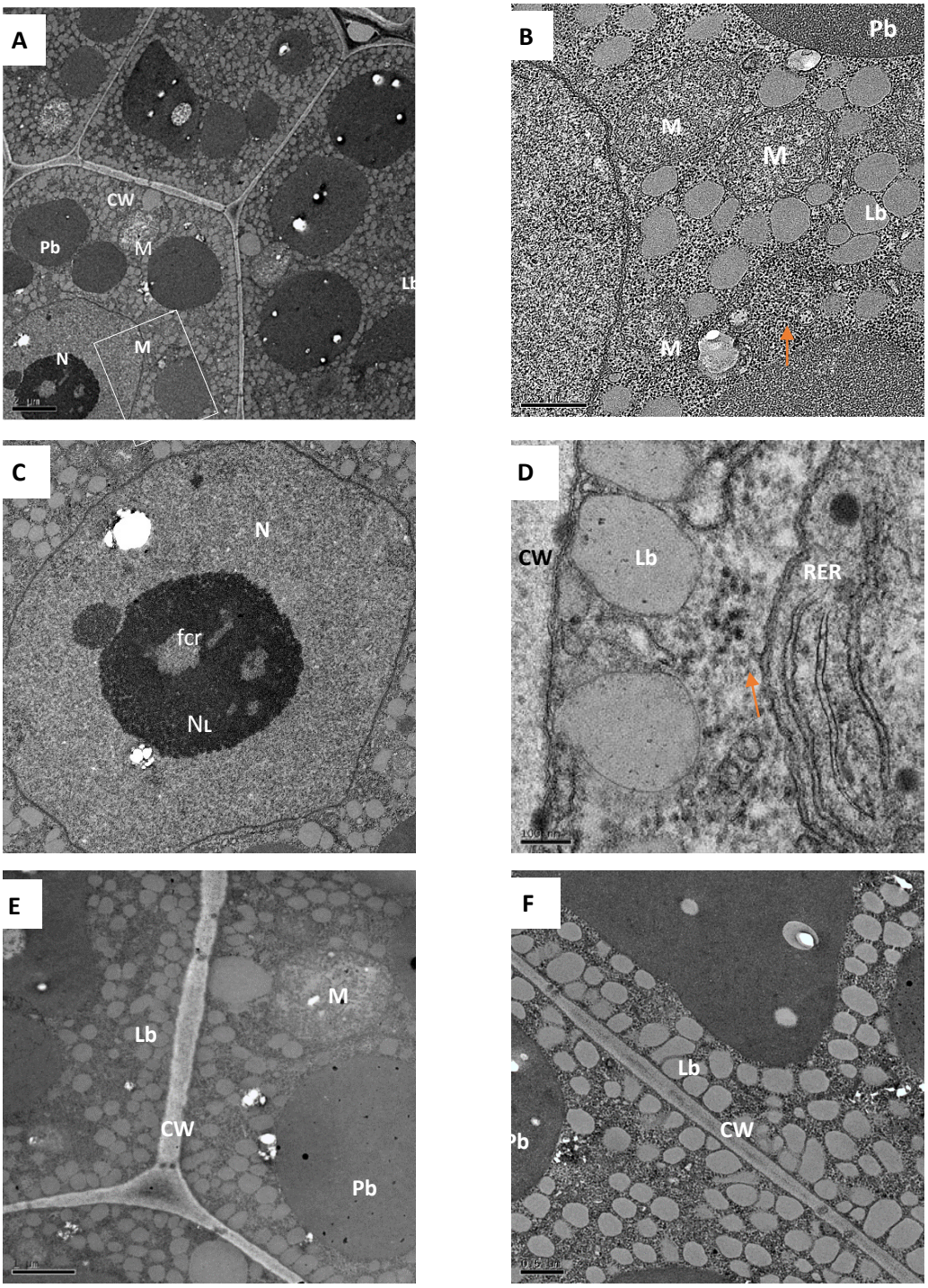

Figure 6.4 Transmission electron microscope images of mature (control) forage rape seed embryo axis.

(A) Cell wall (CW) and plasma membranes of organelles are well differentiated. Lipid bodies (Lb) and protein bodies (Pb) are visible and well distributed throughout the cell (Scale= 2 µm). (B) Mitochondria (M) with cristae and cytoplasm with ribosomes (dark spots-arrows) are visible (Scale=0.5 µm). (C) Nucleus with distinct border with nuclear membrane (NM) and nucleolus (NL) and fibrillar centre (fcr) visible in the nucleus (Scale= 100 nm). (D) Rough Endoplasmic Reticulum (RER). The dark spots are ribosomes (100 nm). (E-F) Cell wall is intact around cells; no pores or any diffusions were found and lipid bodies (Lb) are evenly distributed around the cell wall (Scale = E, 1 µm, F, 0.5 µm).

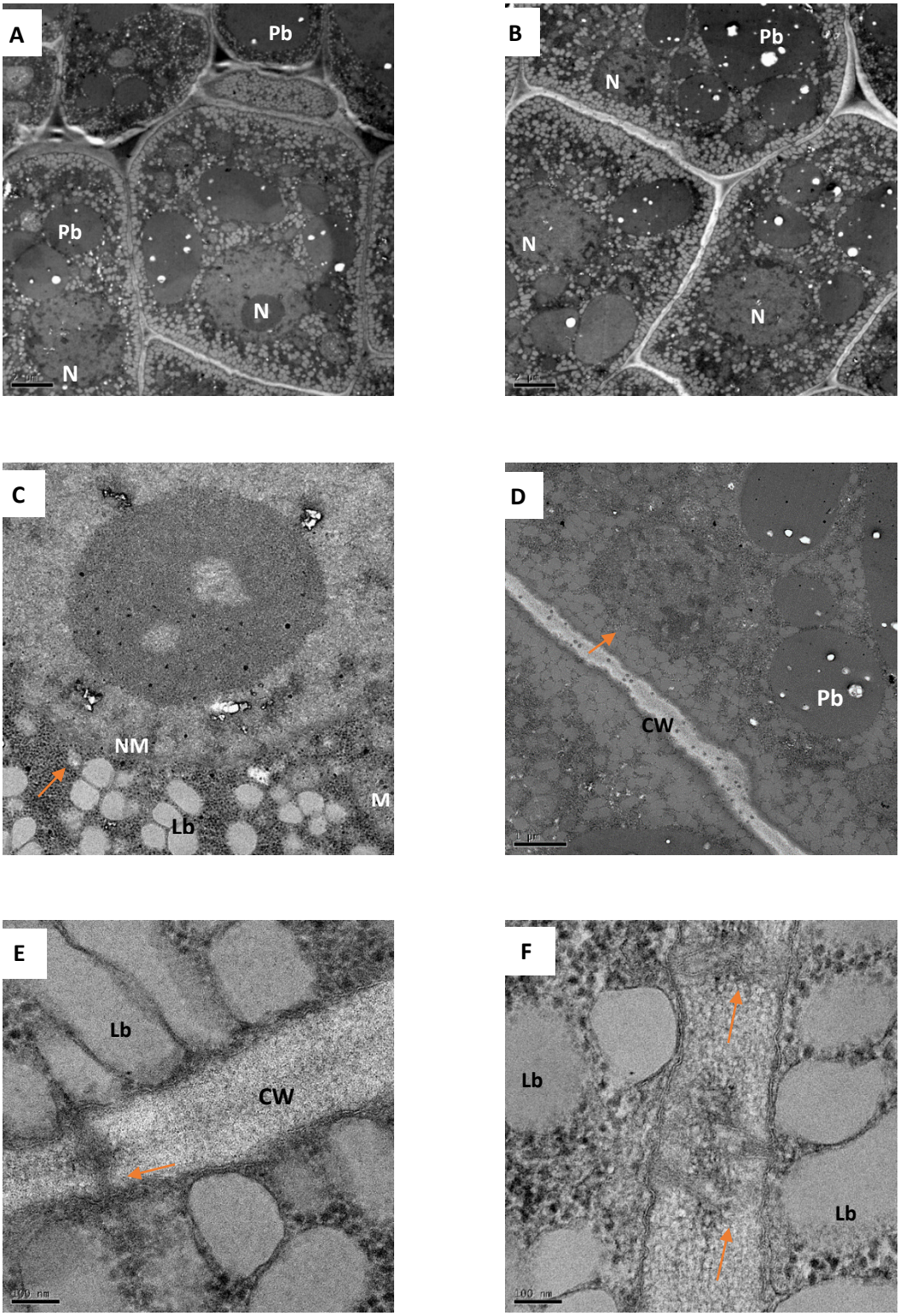

Figure 6.5 Transmission electron microscope images of forage rape seed heat stressed at the seed filling stage (≈80% SMC).

(A-D) Lipid bodies (Lb), protein bodies (Pb), and nucleus (N) are visible but not well developed, nuclear membrane (NM) in some cells is indistinct, and nucleolus is absent (arrows). (A-B) Protein bodies (Pb) are large in some cells, probably fused together to make large protein bodies. Cell wall is wavy and some cells are shrunken (Scale= 2 µm) (C-D) Plasma membrane of nucleus is indistinct (arrows) and cell wall (CW) is wavy. Mitochondria (M) are not clearly visible. (E-F). Cell wall is porous (arrow), plasma membrane is not continuous and outer membranes of lipid bodies (Lb) are not clearly visible.

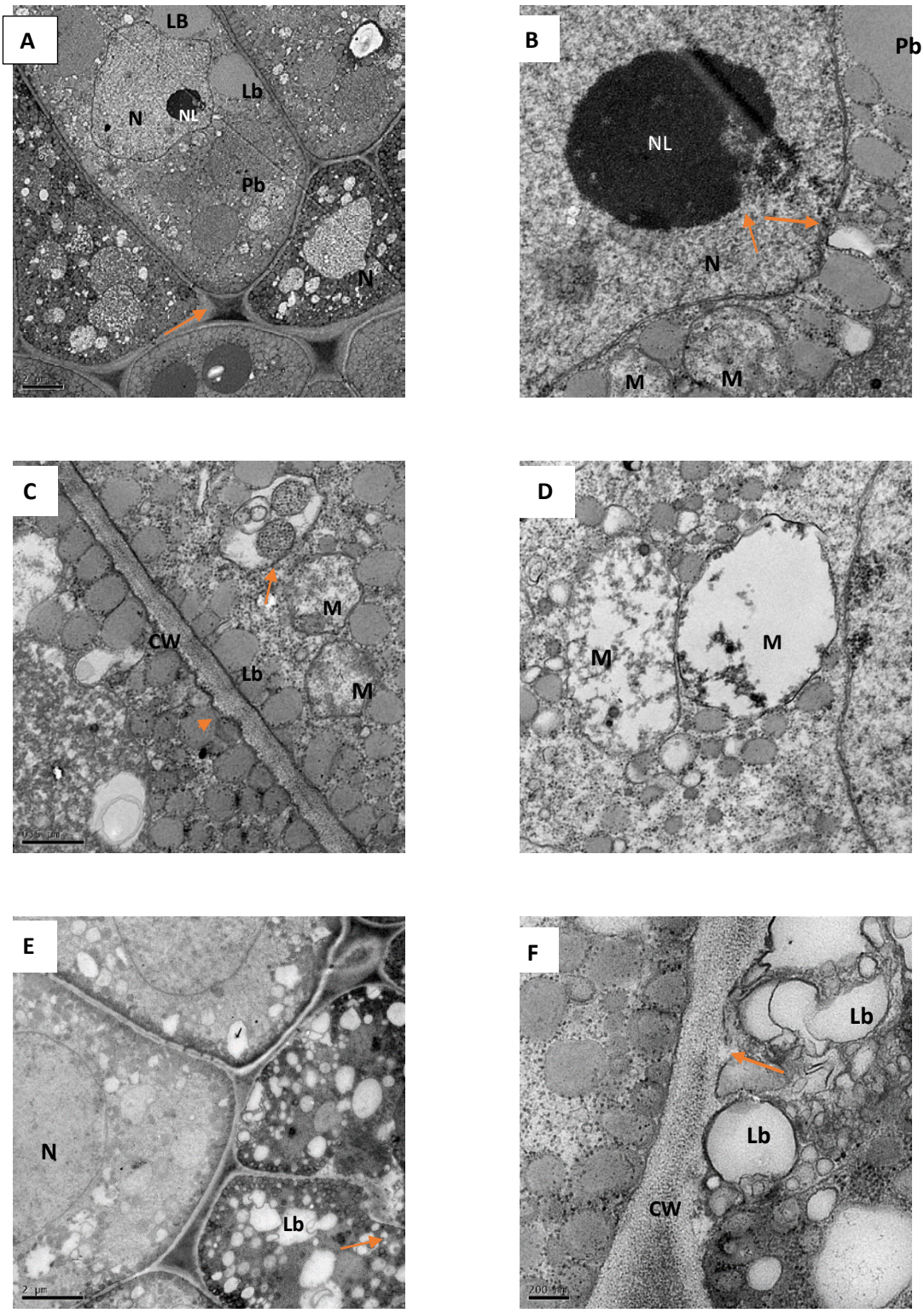

Figure 6.6 Electron micrograph of forage rape seeds, heat stressed at PM (≈50% SMC).
(A) Transparent nucleus with wavy contour and diffused nucleoplasm. Lipid bodies (Lb) fused to form large bodies. Large intercellular spaces are visible (arrows) (A, Scale=2 µm). (B) Nucleus (N) with nucleolus (NL); nuclear membrane and margin of nucleolus are not continuous and porous at some places (arrows). (C) Undeveloped mitochondria (M) without clearly visible cristae. Some mitochondrion appear to be engulfed by large vacuoles (arrows), plasma membrane is not continuous and is wavy on one side of the cell wall (arrow head). (D) Undeveloped mitochondria without distinct outer membrane, internal matrix is transparent and no cristae are visible. (E-F) Cell wall is broken at different places (plasma membrane is not continuous) and lipid vesicles are distorted and are fused together to form large lipid vesicles and are also fused with plasma membrane.

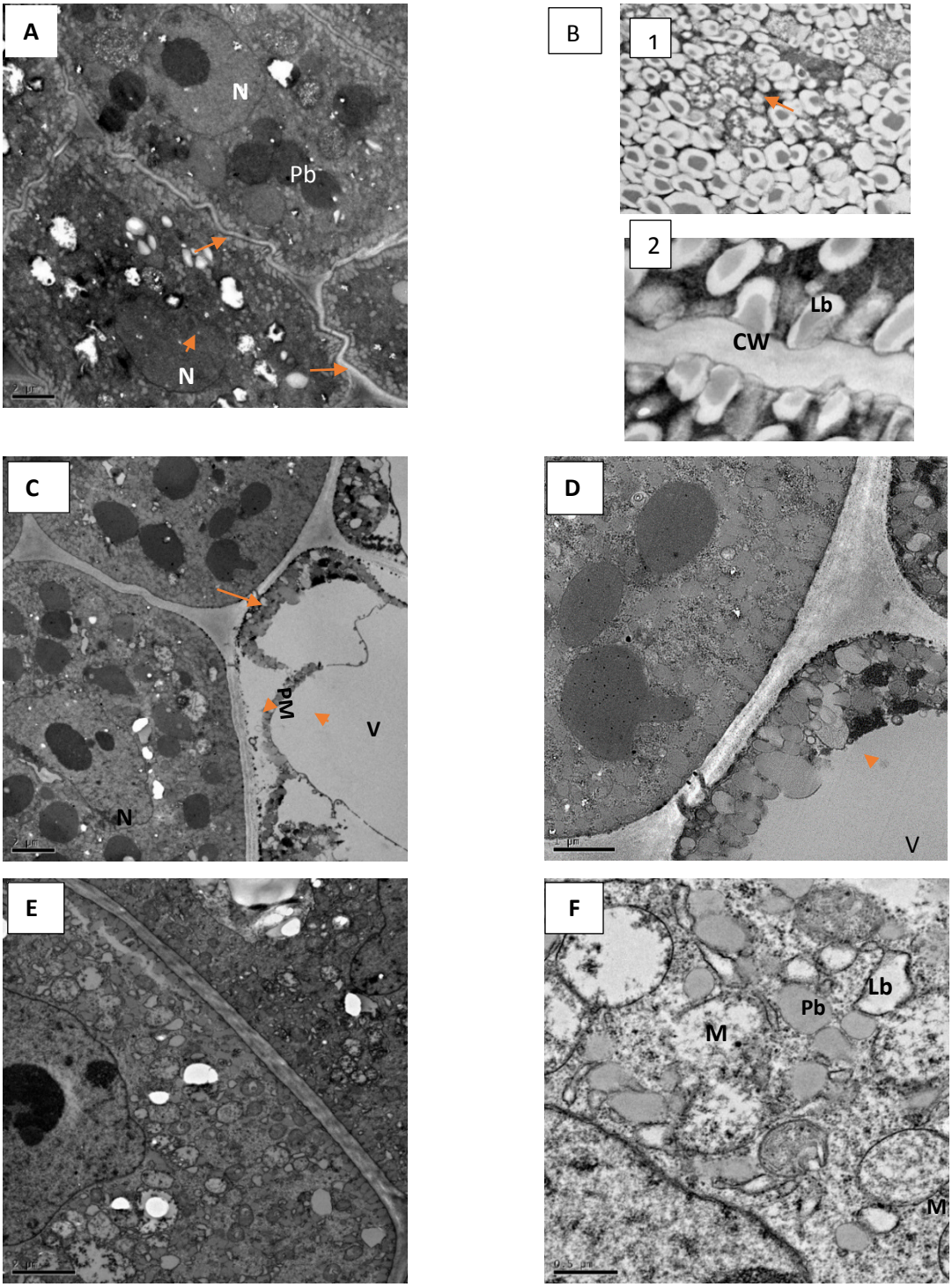

Figure 6.7 Electron microscope images of seed of forage rape heat stressed at both seed development stages (80% SMC + 50% SMC).
(A) Wavy cell wall (arrow), cell organelles are visible, i.e. protein bodies are fusing, some cells have nucleus without nucleolus and disrupted nuclear membrane (arrowhead).
(B1) Lipid bodies (Lb) are fusing together and underdeveloped mitochondria (M) with transparent matrix are visible between lipid bodies (arrows).(B2) Wavy cell wall and plasma membrane are not fully differentiated, lipid bodies are fusing with plasma membrane. (C-D).) Vacuolization in cell, flattened nucleus with nucleolus, outer membrane is not smooth. Plasma membrane (PM) is not differentiated at some places. Cell wall is broken at different places (arrow) .Lipid bodies (Lb) are merging and stretched away from cell wall (arrowheads). (E-F) Cell organelles i.e. lipid bodies (Lb), protein bodies (Pb) and mitochondria (M) are not distributed regularly and lack developed outer membranes.

The plasma membrane of the cell was indistinct and mitochondria were undeveloped and misshapen (deformed), with transparent matrix lacking internal structures (Figure 6.6 C-D).The cell walls were curvy (arrow) and indistinct at some places, and lipid bodies were fused with the plasma membrane (Figure 6.6 E-F).

The defects in cellular organelles and structures were greater in seed that was heat stressed at both the PM and seed filling stages (Figure 6.7 A-F). The plasma membrane showed major injury, the nuclear membrane had disappeared in some of the organelles, and nucleoli were absent. Breakdown of cell organelles and structures was observed and the cell wall was wrinkled and deformed. Lipid bodies were deformed and fused in the wrinkled cell wall and mitochondria were closely surrounded by lipid bodies (LB) (Figure 6.7 A-B). Large vacuoles were found and the lipid bodies were shrunken away from the plasma membranes to leave large spaces between the cell wall and plasma membrane. The plasma membrane was ruptured in some places (Figure 6.7 C-D).The breakdown of the organelle structures (lipid bodies, protein bodies and mitochondria) made them difficult to identify (Figure 6.7 E-F).

6.3.3 Discussion

Heat stress has been reported to induce ultrastructure changes in cellular organelles and their functions (Bita & Gerats, 2013; Richter et al., 2010; Wahid et al., 2007) including chloroplasts (Gao et al., 2010; Zhang et al., 2010), mitochondria (Kong et al., 2014), cellular membranes (Liu & Huang, 2000; Saidi et al., 2009; Savchenko et al., 2002) and the nucleus and endoplasmic reticulum (Khokhlova et al., 1987; Kislyuk et al., 1992; Suzuki et al., 2001).Most of these changes induced by heat stress are due to oxidative stress (Gill & Tuteja, 2010; Howarth, 2005; Wang et al., 2012).

In this study, electron microscope image analysis showed cellular damage to forage rape seeds following heat stress during seed development at PM. Heat stress damage to cellular organelles such as cell wall and plasma membranes, lipid bodies and mitochondria was clearly visible in heat stressed seeds relative to the non-heat stressed control. Heat stress damage to the cellular membranes in this study, including broken plasma membranes and thick, wavy and ruptured cell walls might be due to lipid peroxidation via ROS accumulation (described in Chapter 5) which reduced cellular membrane stability as indicated by increased electrolyte leakage. High electrolyte leakage indicated that deterioration in cellular structure of seeds had occurred (Shinohara et al., 2006b). Disorganization of cellular membranes may also reduce the mitochondrial efficiency which allow the peroxidative enzymes to cause damage to surrounding cellular organelles after imbibition (McDonald, 1999).

The mitochondria are the most sensitive organelles during seed deterioration (Kong et al., 2014; Wang et al., 2015).In these studies the TEM images of heat stressed seeds exhibited poorly differentiated, deformed mitochondria, and the internal matrix was less dense and transparent, and lacking internal structures such as cristae. Cheng et al. (1991) and Wang et al. (2015) reported that the outer and inner

membranous boundaries were not easily distinguishable. Thus in the present study, the mitochondria of heat stressed seeds were also severely damaged.

Generally, dry and quiescent seeds have poorly differentiated mitochondria. During imbibition rapid resumption of the mitochondrial membrane system which plays a vital role in seed vigour (Bewley, 1997; Bita & Gerats, 2013; Taylor et al., 2010) occurs. It has been reported that during imbibition the seed axis does not have chloroplasts, and therefore mitochondrial ATP synthesis is considered the main source of energy (Grass & Burris, 1995b; Xin et al., 2014). Imbibed seeds produce sufficient energy in the form of ATP and intermediates for activation of metabolic activities to start the germination process (Carrie et al., 2013; Howell et al., 2006; Logan et al., 2001; Taylor et al., 2010). Structural damage of mitochondria during seed deterioration negatively affects their function, mainly by decreasing cellular membrane integrity indicating that it was difficult for the heat stressed deteriorated seeds to recover their mitochondrial membrane system to allow oxidative phosphorylation (Frey & Mannella, 2000; Logan et al., 2001).The results of the present study demonstrate that due to structural damage, the mitochondria were unable to provide sufficient energy in the form of ATP to allow the germination process to occur uninterrupted (Benamar et al., 2003; Grass & Burris, 1995b; Yin et al., 2009). These results are consistent with the results of Kong et al. (2014) for oat seeds and Xin et al. (2014) for soybean seeds, who both demonstrated that mitochondria damaged by high temperature stress subsequently caused seed deterioration. Generally, damage to mitochondria occurs first, followed then by the overall deterioration of cell structures (Zhao & Li, 2000).

6.4 Conclusions

- In this study, the activity of ROS scavenging antioxidant enzymes, SOD, CAT, APX and GR activities following heat stress during forage rape seed development was examined. All of these antioxidant enzymes were negatively influenced by heat stress, as their detoxifying activity was either decreased, or their synthesis was inhibited.
- A significant negative effect of heat stress on the adenine nucleotides pool and AEC was recorded. This provides evidence that heat stress during forage rape seed development, altered the metabolic system by inducing changes in adenine nucleotide levels and the adenylate energy charge (AEC). This was mainly due to a decrease in cellular adenosine triphosphate (ATP), resulting in a decrease of AEC.
- Significant cellular damage occurred in seeds experiencing heat stress during seed development, especially at the cellular membrane and mitochondrial level. These observations explain the higher electrolyte leakage from heat stressed seeds (Chapter 3) and decreased level of nucleotides and reduced energy levels due to structural changes in mitochondria.

Chapter 7

Outcome, General Discussion and Future Research

The major objective of this study was to examine the effect of the production environment on seed quality, specifically looking at sowing date and short periods of high temperature stress during seed development of forage rape. After confirming the significant negative effect of heat stress on forage rape seed quality, particularly seed vigour in both controlled and field environmental conditions, a follow up study was conducted to investigate the physiological changes and ultrastructural deterioration in seeds resulting from this heat stress at the cellular level, particularly membrane degradation, enzyme inactivation and mitochondrial dysfunction. As a complete discussion is included for each of the experimental chapters, this chapter only provides a brief summary of research outcomes, general discussion and future research direction.

The seed industry has concerns over climate change and its potential impacts on seed production and quality. Loss of seed quality, particularly seed vigour is likely to increase with global environmental changes (Hampton et al., 2013). However, some consider that extreme and unexpected variability in localized weather could pose more of a challenge overall for the seed industry than long term climate shifts (Friis, 2011; Rahmstorf & Coumou, 2011; Singh et al., 2015). All aspects of crop production are likely to be affected by climate change, particularly reproductive growth (Farooq et al., 2011; Wahid et al., 2007).There is a need to produce seed of improved cultivars better able to cope with abiotic stress, particularly drought and temperature stress (Singh et al., 2015). There is also a need to minimize the adverse effects of climate change on seed production by changing management systems, by adjustments of crop calendars and moving to environments more suitable for high quality seed production (Singh et al., 2015), if that is possible.

By the end of this century, the predicted rise in global mean temperature of between 1.6 ˚C to 6.9 ˚C, will be very likely to reduce seed quality (Betts et al., 2011; Hampton et al., 2013; Solomon, 2007).The current study was conducted to investigate the effects of high temperature on forage rape seed quality. New Zealand forage rape seed production is centred in Canterbury, where daily mean temperature during forage rape seed development and crop maturation is around 20 ˚C and daily maximum temperature can exceed 30 ˚C (Hampton, 2004). This scenario is likely to become worse with the predicted rise in global temperature.

7.1 Summary of outcome and discussion

Forage rape *(Brassica napus*) is grown as an alternative feed in winter for grazing animals (Trethewey, 2009). New Zealand produced forage rape seed lots usually have high germination (>90%) but are

variable in their vigour status. It is a cool season crop and sensitive to heat stress, but seed can be maturing in field conditions which vary in temperature and humidity in the Canterbury environment. The previous seven year's weather data for the site of the sowing date study showed large variability in environmental conditions, particularly mean daily, mean maximum and mean minimum temperature from October to January, the time during which forage rape seed develops and matures (Figure 2.1-2.7, Chapter 2). Forage rape seed was sown in late autumn (25th March and 13th April 2011), with the intention that because of the three week difference in sowing time, the seeds would develop in contrasting environments. Seeds from each of the sowings were then hand harvested and tested for quality. Germination did not differ between two sowings when tested at and after seeds had reached physiological maturity (PM). However, while there were no difference in vigour at PM, differences in seed vigour between the two sowings became apparent as indicated by the conductivity test, for seeds harvested at the pre-desiccation final stage (25 % SMC) and at harvest maturity (14 % SMC).Results were not significant for the AA-vigour test. This perhaps suggests that in brassicas, the conductivity test is more sensitive in detecting seed deterioration than the AA-test, although for subsequent experiments the two vigour test results were significantly correlated. The environments differed little between two sowings in this season. The only difference was rainfall and relative humidity (R.H) which was probably the cause of the increased seed conductivity recorded due to weathering of seeds for the March sowing (Chapter 2). However, as earlier stated, previous environmental data show large variation in environmental conditions at the site of this study. For example, mean temperature during the seasons 2008-09 and 2010-11 was 20.3 ˚C (range 17.3-24.5 ˚C) and 20 ˚C (range 16.4-21.5 ˚C) respectively during the forage rape seed development and maturation period (October to the following January). The mean maximum temperatures during these seasons were 30 ˚C (range 26.8 - 35 ˚C) and 29.2 ˚C (range 23.4-32.6 ˚C) respectively. The mean minimum temperatures during these seasons were 9.5 ˚C (5.6-13.5 ˚C) and 10 ˚C (range 6.1-12.6 ˚C) respectively. By chance the season in which the trial was conducted was cooler than average. Repeating the trial in another season may have produced a different response in terms of seed quality, particularly if warmer temperatures had prevailed. Regional and seasonal variation in environmental conditions in New Zealand and its impact on seed quality has been reported by Shinohara et al. (2008).These authors demonstrated that low vigour garden pea seed lots were produced in regions and seasons where high temperatures occurred.

The environment during seed development and maturation can reduce seed vigour significantly (Gusta et al., 2004; Shinohara et al., 2008). Results from the controlled environment experiment clearly indicated that forage rape seed quality (i.e. seed germination and vigour) was reduced by the heat stress treatment of 240 ˚Ch (30/25 ˚C day and night 12 h cycle, R.H 70%), both at the seed filling stage (≈80% SMC) and at PM (≈50% SMC) in both seasons. The results in Chapter 3 indicate that seed vigour was more substantially reduced by this heat stress than seed germination in both seasons. Morrison and Stewart (2002) proposed a heat stress index of 29.5 ˚C for all brassica species, after finding that

above this temperature seed yield was decreased by up to 70% .The temperature stress during the flowering stage or early reproductive stage during embryogenesis reduced the number of seeds because of pollen sterility or ovary damage. This study targeted a heat stress of 30/25 ˚C (day and night, 240 ˚Ch) which did not kill the seeds but reduced germination through the production of abnormal seedlings. The impact of heat stress at PM on seed vigour was marginally higher than stress at the seed filling stage, suggesting that seed deterioration had already started because of the environment encountered by plants before PM. Previous studies also suggested that seed vigour loss occurred after PM (Coolbear, 1995; Hampton, 2000; TeKrony & Egli, 1997).This was also indicated by the higher electrical conductivity of seeds lots harvested after heat stress at PM (Chapter 3).The strong negative correlation between the two vigour tests (AA-germination and conductivity test) also supports this hypothesis.

As noted above, the vigour loss was lower following heat stress during seed development than it was from heat stress at PM. It is probable that seeds were able to undergo some self-repair as the seed development progressed. This is because the plants were returned to ambient field conditions after the heat stress treatment and remained there until harvest maturity. This has also been reported by Shinohara et al. (2006b) in pea seeds.

This self-repair process can only occur provided the cells are not dead or too severely damaged. In this experiment seeds were not killed following the heat stress, but germination was disrupted resulting in the production of abnormal seedlings. This self-repair presumably occurred because seeds were still on the mother plant and continuing to receive assimilates until they reached PM. This self-repair process terminated or ceased at the PM stage, when the connection between the seed and plant was disrupted. This self-repair process is initiated by the synthesis of DNA repair enzymes (Kranner et al., 2010).However if the damage is too great, the repair process may be impaired, resulting in the loss of vigour and viability (Elder et al., 1987) .

Reduction in seed quality i.e. seed germination, seed mass and especially vigour also increased in the artificially created high temperature field environment when applied during phase-I (seed filling to PM), which confirms the results of the previous experiment in a controlled environment (Chapter 3).The results indicate that seed deterioration depends upon the air temperature exceeding a threshold. For example before PM when the time above 25 ˚C was less than 50 ˚Ch, there was no reduction in germination and vigour. The data suggested that at least 100 ˚C h above 25 ˚C were required before the end result was a commercially unacceptable seed lot. Note, however, that the greatest reduction in seed mass occurred when heat stress was before PM. These results also confirm the pervious results from the controlled environment study (Chapter 3), in which seed mass, germination and vigour were all reduced when heat stress was applied during seed filling. High temperature stress (> 25 ˚C) during phase-II (PM-HM) had no effect on seed mass but did significantly

reduce both seed germination and seed vigour (Chapter 4). In both experiments seed conductivity was increased. Heat stress has been reported to destabilize the membranes of cells and organelles and this loss of membrane integrity is the most quoted cause of seed deterioration (Howarth, 2005; McDonald, 1999; Wahid et al., 2007). Membrane destabilization is generally attributed to lipid peroxidation which is induced by the free radical ROS and its derivatives (Wang et al., 2004). This free radical induced lipid peroxidation has the potential to damage the membranes, enzymes and nucleic acid, and is likely to be the major cause of seed deterioration in storage (MacFarlane, 2003; Małecka et al., 2001; Schwember & Bradford, 2010). No substantial data were available on lipid peroxidation during pre-harvest seed deterioration. In this study, heat stress significantly increased the MDA (a product of lipid peroxidation) content in heat stressed seed lots. This is believed to be due to the activity of reactive oxygen species (ROS). This increase in MDA was associated with increased electrolyte leakage (membrane integrity) and the subsequent reduction in seed vigour. However, H_2O_2 as ROS were not correlated with MDA (Figure 5.2, Chapter 5), suggesting that there might have been other ROS species, such as OH, $^1O_2$, O^-_2 which were involved with inducing lipid peroxidation, but other ROS species were not measured in this experiment.

Oxidative stress and lipid peroxidation have been widely reported as a reason for seed deterioration in oil seeds under stress conditions (Bailly, 2004; Bailly et al., 1998; Hendry, 1993; McDonald, 1999). Bailly et al. (1998) reported that loss of seed vigour and viability in sunflower seeds during ageing was associated with a decrease in the enzymatic antioxidant defence system in cells which lead subsequently to lipid peroxidation. The antioxidant enzyme defence mechanism was also affected during ageing in cotton (Goel & Sheoran, 2003), soybean (Murthy et al., 2002) and beech (Pukacka & Ratajczak, 2005). The results of this study on forage rape clearly indicated that heat stress reduced seed vigour due to impairment of the antioxidant enzyme system i.e. SOD, GR, CAT and APX (Chapter 6), which subsequently resulted in lipid peroxidation (Chapter 5). Higher hydrogen peroxide contents in heat stressed seeds were strongly correlated with loss of seed vigour (AA-germination) (Figure 5.7, Chapter 5). Seed deterioration after high temperature (45 ˚C, RH 100%) stress in sunflower induced both loss of seed vigour and viability and an increase in MDA accumulation (Kibinza et al., 2006). However, a correlation between lipid peroxidation and electrolyte leakage has not previously been established (Figure 5.5, Chapter 5). Though the reduction in seed vigour is believed to result from the impairment of the antioxidant enzyme system (Chapter 6), other mechanisms may also be involved. Heat stress due to high temperature leads to a marked increase in lipid peroxidation as shown by the MDA data (Chapter 5), but the subtle relationship with conductivity does not necessarily mean that lipid peroxidation was the only reason for the higher electrolyte leakage.

Cellular damage can be attributed to the accumulation of H_2O_2 .This compound and its derivatives like the hydroxyl radical ($OH^{\bullet}$) are very deleterious and known to react with nucleic acid and proteins,

causing oxidative stress to the surrounding molecules and structures (Beckman & Ames, 1997). Impairment of antioxidant enzymes or their inactivation by ROS occurs when amino acids close to the active sites are degraded. H_2O_2 reacts with the thiol group which leads to the inactivation of some enzymes in the Calvin cycle (Charles & Halliwell, 1980) and thus damage to cellular functions (Halliwell & Gutteridge, 2015). Bailly et al. (2004) reported an inability of seeds to complete the germination process due to a decrease in the detoxifying potential of the antioxidant enzyme by H_2O_2.

Moreover, H_2O_2 can also cause alteration in mitochondrial functions through the disruption of the electron transport chain (Bailly, 2004; Puntarulo et al., 1988). The present study clearly indicates the changes in the mitochondrial functions which lead to the marked decrease in ATP and AEC due to heat stress treatments (Table 6.10, Chapter 6).The ATP and AEC were high in non-stressed seeds but a marked decrease was observed with heat stress and both were almost negligible after the double stress at 80% SMC and 50% SMC (Chapter 6). The value reached indicated that metabolism was markedly altered. The activation of metabolism energy is linked with the aerobic cyanide-sensitive respiratory pathway (Raymond et al., 1985). Heat stress treatment at 30/25 ˚C resulted in the depletion of ATP production in heat stressed seeds and an increase in AMP which lead to a decrease in AEC (Chapter 6). The increased AMP levels in stressed seeds suggest that activity of adenylate kinase (EC 2.7.4.3) was probably sufficient to allow this increase, even when the ATP regeneration system was reduced (Smith & Berjak, 1995). High metabolic energy with high ATP in non-stressed seeds indicated a high rate of phosphorylation which in turn indicated that the metabolism in non-stressed seeds was not altered. Heat stress would probably induce an irreversible alteration in ATP synthesis because of damage at the ultrastructure level to mitochondria (Smith & Berjak, 1995) as found in the present study (Chapter 6, Section 6.3.2). Mitochondria are known to participate in the production of ROS. Under normal physiological conditions around 1-2% of the oxygen consumed by the mitochondria is transformed into H_2O_2 (Kroemer, 1997; Puntarulo et al., 1988). Kibinza et al. (2006) demonstrated a positive relationship between energy metabolism and H_2O_2 in aged sunflower seeds.

Tiwari et al. (2002) showed that even mild oxidative stress could lead to an increase in electron leakage from the electron transport chain, which lead to an overproduction of ROS, and resulted in the depletion of ATP. On the basis of the results in this study, it is proposed that H_2O_2 accumulation during the seed deterioration which occurred because of heat stress during seed development may be involved in the depletion of ATP content, leading to the loss of seed vigour. The microscopy images of ultrastructure damage to mitochondria in heat stressed seeds support this proposal.

7.2 Future research

In this study, seed vigour was reduced by high temperature induced heat stress during forage rape seed development and maturation. Low seed vigour can influence field emergence and potential storability of forage rape seed lots. However, there were some aspects which were not investigated in this study, and need further investigation.

- In this study, only one location with two sowing dates was used to investigate the variation in seed quality in contrasting natural environments. More locations or regions of forage rape production area and sowings could be included for further study to help in determining why the variation in seed vigour occurs among both locations/regions and cropping seasons. This would allow the identification of the most suitable forage rape seed production region and an adjustment of the sowing calendar to avoid heat stress during the forage rape seed development and maturation period.
- Further research with a longer interval between sowings is required to determine any differences in seed quality due to sowing time within a season.
- Use of more cultivars to screen for heat resistant genotypes. In this study, only one late flowering cultivar of forage rape "Greenland" was used. There is no information as to whether current forage rape cultivars have any tolerance to heat stress.
- Because of space limitations, it was not possible to use more than one controlled temperature regime for this study, or to determine how many ˚Ch were required to initiate the seed deterioration process. Further work is required.

- In this study, differences in seed quality of forage rape harvested from different positions of the raceme was ascribed to differences in seed maturity level and the differences in seed quality became greater after heat stress. Whether this is the only reason for differences in seed quality between different positions of the raceme is not clear. Further research is required to investigate the source sink relationship and competition for assimilates between seed produced at different positions of the raceme as well as the effect of heat stress on the source sink relationship. High interception of photosynthetically active radiation (PAR) is known to have a role in seed quality, particularly seed mass (Mandal & Sinha, 2004). Differences in seed quality might be due to differences in light interception at the different position on the raceme, but this needs further investigation as in this study PAR was not measured.
- This study demonstrated the role of lipid peroxidation and ROS activity in heat stressed seeds. Localization of ROS in damaged seed embryo tissue can be used to further investigate the extent of seed deterioration and tissue damage. Techniques and protocols as described by

Bailly and Kranner (2011) can be used to localize hydrogen peroxide in seed tissues and visualize this through electron microscope and/or fluorescent or confocal laser scanning. This will provide further evidence of the role of ROS and lipid peroxidation in seed deterioration.

- In this study, loss of seed vigour was attributed to the reduced mitochondrial ATP energy levels and ultrastructural changes in mitochondria of heat stressed seeds. Impaired ATP production is related to reduced mitochondrial respiratory activity (Grass & Burris, 1995b). However, respiratory activity of mitochondria was not measured. Further research could be conducted by extracting mitochondria of heat stressed seeds to measure the respiratory activity and to correlate it with changes in mitochondrial ATP levels.

Appendices

Appendix 1 Phenological growth stages and BBCH-identification keys of *Brassica napus*

Code	Description
Principal growth stage 0: Germination	
00	Dry seed
03	Beginning of seed imbibition
05	Radicle emerged from seed
07	Hypocotyl with cotyledons emerged from seed
08	Hypocotyl with cotyledons growing towards soil surface
09	Emergence: cotyledons emerge through soil surface
Principal growth stage 1: Leaf development	
10	Cotyledons completely unfolded
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
1.	Stages continuous till . . .
19	or more leaves unfolded
Principal growth stage 2: Formation of side shoots	
20	No side shoots
21	Beginning of side shoot development: first side shoot detectable
22	2 side shoots detectable
23	3 side shoots detectable
2.	Stages continuous till . . .
29	End of side shoot development: 9 or more side shoots detectable
Principal growth stage 3: Stem elongation	
30	Beginning of stem elongation: no internodes (“rosette”)
31	1 visibly extended internode
32	2 visibly extended internodes
33	3 visibly extended internodes
3.	Stages continuous till . . .
39	9 or more visibly extended internodes

Principal growth stage 5: Inflorescence emergence	
50	Flower buds present, still enclosed by leaves
51	Flower buds visible from above (“green bud”)
52	Flower buds free, level with the youngest leaves
53	Flower buds raised above the youngest leaves
55	Individual flower buds (main inflorescence) visible but still closed
57	Individual flower buds (secondary inflorescences) visible but still closed
59	First petals visible, flower buds still closed (“yellow bud”)
Principal growth stage 6: Flowering	
60	First flowers open
61	10% of flowers on main raceme open, main raceme elongating
62	20% of flowers on main raceme open
63	30% of flowers on main raceme open
64	40% of flowers on main raceme open older petals falling
65	Full flowering: 50% flowers on main raceme open,
67	Flowering declining: majority of petals fallen 69 End of flowering
69	End of flowering
Principal growth stage 7: Development of fruit	
71	10% of pods have reached final size
72	20% of pods have reached final size
73	30% of pods have reached final size
74	40% of pods have reached final size
75	50% of pods have reached final size
76	60% of pods have reached final size
77	70% of pods have reached final size
78	80% of pods have reached final size
79	Nearly all pods have reached final size

Principal growth stage 8: Ripening	
80	Beginning of ripening: seed green, filling pod cavity
81	10% of pods ripe, seeds dark and hard
882	20% of pods ripe, seeds dark and hard
83	30% of pods ripe, seeds dark and hard
84	40% of pods ripe, seeds dark and hard
85	50% of pods ripe, seeds dark and hard
86	60% of pods ripe, seeds dark and hard
87	70% of pods ripe, seeds dark and hard
88	80% of pods ripe, seeds dark and hard
89	Fully ripe: nearly all pods ripe, seeds dark and hard
Principal growth stage 9: Senescence	
97	Plant dead and dry
99	Harvested product

Weber and Bleiholder, 1990; Lancashire et al., 1991

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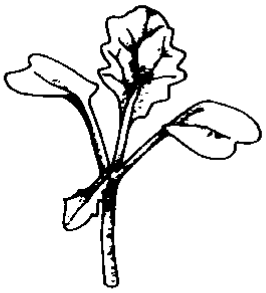

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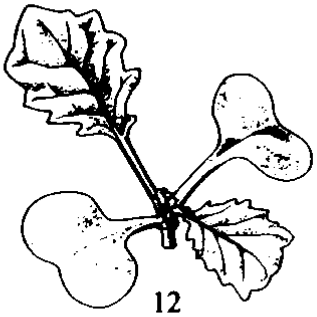

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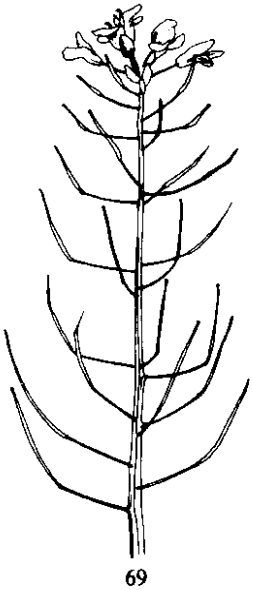

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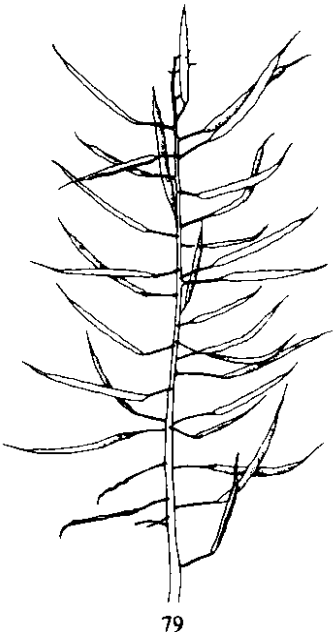

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Appendix 2 Maximum tolerated ranges between four replicates of 100 seeds

This table indicates the maximum range (i.e. difference between highest and lowest) in germination percentage tolerable between replicates, allowing for random sampling variation only at 0.025 probability. To find the maximum tolerated range in any case calculate the average percentage, to the nearest whole number, of the four replicates: if necessary, form 100-seed replicates by combining the sub-replicates of 50 or 25 seeds which were closest together in the germinator. Locate the average in column 1 or 2 of the table and read off the maximum tolerated range opposite in column 3.

Average percentage germination		Maximum range	Average percentage germination		Maximum range
1	2	3	1	2	3
99	2	5	87-88	13-14	13
98	3	6	84-86	15-17	14
97	4	7	81-83	18-20	15
96	5	8	78-80	21-23	16
95	6	9	73-77	24-28	17
93-94	7-8	10	67-72	29-34	18
91-92	9-10	11	56-66	35-45	19
89- 90	11-12	12	51-55	46-50	20

(extracted from Table 5 B, ISTA 2016)

Appendix 3 Maximum tolerated range for conductivity

This table indicates the maximum range (i.e. difference between highest and lowest) in conductivity reading that is tolerable between replicates. To find the maximum tolerated range in any case, calculate the average conductivity from four replicates. Locate the average in column 1 or 2 of the table and read off the maximum tolerated range in column 3.

Average conductivity ($\mu S\ cm^{-1}\ g^{-1}$)		Maximum range ($\mu S\ cm^{-1}\ g^{-1}$)	Average conductivity ($\mu S\ cm^{-1}\ g^{-1}$)		Maximum range ($\mu S\ cm^{-1}\ g^{-1}$)
From	To		From	To	
1	2	3	1	2	3
10	10.9	3.1	32	32.9	8.5
11	11.9	3.3	33	33.9	8.8
12	12.9	3.6	34	34.9	9.0
13	13.9	3.8	35	35.9	9.3
14	14.9	4.1	36	36.9	9.5
15	15.9	4.3	37	37.9	9.8
16	16.9	4.6	38	38.9	10.0
17	17.9	4.8	39	39.9	10.3
18	18.9	5.1	40	40.9	10.5
19	19.9	5.3	41	41.9	10.8
20	20.9	5.5	42	42.9	11.0
21	21.9	5.8	43	43.9	11.3
22	22.9	6.0	44	44.9	11.5
23	23.9	6.3	45	45.9	11.8
24	24.9	6.5	46	46.9	12.0
25	25.9	6.8	47	47.9	12.3
26	26.9	7.0	48	48.9	12.5
27	27.9	7.3	49	49.9	12.8
28	28.9	7.5	50	50.9	13.0
29	29.9	7.8	51	51.9	13.3
30	30.9	8.0	52	52.9	13.5
31	31.9	8.3	53	53.9	13.8

(Extracted from table 15B, ISTA, 2016)

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