NON-GERMINATION IN BARLEY FOR THE BREWING INDUSTRY: THE INFLUENCE OF GENOTYPE, TEMPERATURE, MOISTURE AND GAS EXCHANGE

FINAL REPORT – January 2014

1. BACKGROUND

This project was initiated by the brewing industry, where the non-germination of barley kernels causes large financial losses every year. We had a number of meetings both in Bloemfontein and in Caledon with dr. Idelet Meijering during the course of this project, to discuss different methods that can be used to determine what causes seeds to die while in storage. Prof. Labuschagne and dr. Potgieter (seed physiologist) and dr. Moloi (post-doctoral fellow responsible for the day-to day running of the project) also visited the malting plant in Caledon on three occasions, and on one occasion we visited storage facilities and spoke to silo managers on practices used during storage in the barley production areas. Dr. Moloi spent a week at the facility in Caledon early in 2012 where she was introduced to the laboratories and the methodology used in seed analysis. As the mechanism of loss of viability of seed is not well understood, we did not stick strictly to the methods proposed in the original application, but rather used a number of additional methods, with the input of our seed physiologist, dr. Potgieter who was involved in the project for the whole two year duration, after discussions with dr. Meijering. The moisture and gas exchange factors, as proposed in the original proposal, was therefore not included in this project, as it was felt that the alternative tests will give better answers, which in fact it did. All the results of the different tests are given in this report. The report is divided into two, with the first part reporting on the data from the seed received in 2012 (silo 12 and silo 13) and the second part on seed received in 2013.

2. INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the important crops (after wheat) in South Africa used for malt production (Ullrich 2002; Kotze 2009). Quality of malt is essential for the beer brewing industry and depends on the germination ability of barley. Alternatively, low quality barley may be used for animal feed (Kotze 2009, Kreisz 2009). The rate of seed viability and vigour loss is mainly a function of storage temperature and moisture content (MC) (Pukacka and Rajatszac 2005). However, seed viability and vigour differs with species and cultivars (Demirkaya et al. 2010).

The exact mechanisms of seed viability loss are not completely understood. Primarily, seed deterioration during storage is associated with free radical/ reactive oxygen species (ROS) accumulation such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($^{\cdot}OH$) etc. (McDonald 1999). One of the major sources of ROS is aerobic respiration, where electron leakage from transport chain generates O_2^- and subsequently H_2O_2 (Möller 2001). In support, it was discovered that the amount of H_2O_2 produced is directly proportional to the respiration activity. Estimates are that respiration rate ceases at seed water content lower than 0.25 g DW^{-1} (Vertucci and Farrant 1995), further reinforcing the effect of MC in seed viability.

If produced in high quantities, ROS initiate reactions with polyunsaturated fatty acids leading to lipid peroxidation and destruction of cellular membranes (Pukacka 1991), membrane leakage (Bailly 2004), protein structure and nucleic acid damage (McDonald 1999). This oxidative damage is irreversible (Bailly 2004). However if produced in moderate quantites, ROS may play an important signalling role (Mittler et al. 2004). To counteract ROS toxification, antioxidative mechanisms (enzymatic or non-enzymatic) are produced in plants. Enzymes include (among others) ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD) and guaiacol peroxidase (POD) (Asada 1999; Bailly 2004; Tian 2008). Superoxide dismutases catalyse the dismutation of O_2^- and H_2O into H_2O_2 (less toxic except in high concentrations) and O_2 (Zelko et al. 2002). Guaiacol peroxidases oxidize organic substrates with H_2O_2 producing an oxidized substrate and H_2O. Ascorbate perixidase and GR are enzymes of the ascorbate-glutathione cycle (also known as Halliwell-Asada cycle), which in concert with other enzymes successfully scavenge O_2^- and H_2O_2 (Asada 1999). The success of germination is related to ROS scavenging enzymes efficiency in dry seeds because this scavenging affects seed storage and vigour (Bailly 2004; Prodanovíc et al. 2007).

The purpose of the current study was to investigate the reasons behind the germination loss of barley seeds stored in the different silos at the depots. Managers of these depots, take precautions such as fumigating the silos before new intake,

testing the seeds for the correct moisture content, controlling the temperature inside the silos etc., to ensure that storage conditions favour high seed vigour and viability.

In order to understand the germination loss during storage, the first approach was to investigate the involvement of antioxidative (stress) enzymes. The following antioxidative enzyme activities were measured: superoxide dismutase (SOD), guaiacol peroxidase (POD), asscorbate peroxidase (APX) and glutathione reductase (GR) (i.e to see if there is any difference in activity of these enzymes between the ''bad and good'' seed batches). In addition, the differences in the activities of these enzymes among the dry seeds (to see if bad seeds can be detected before germination) and between the dry and germinated seeds were investigated.

This was done with the expectation that we could predict high risk seeds by using the activity/concentration level of the antioxidative compounds. Furthermore, electrical conductivity of the seeds was measured to investigate if membrane damage contributed to the germination loss of silo stored barley. In an attempt to investigate the contribution of membrane damage to germination loss, malondialdehyde (MDA) content was measured.

2. MATERIALS AND METHODS

2.1. Plant material: In March 2012, 3-5 month-old barley seed (cv. Erica) samples from two silo's (i.e. silo 12 and silo 13) were obtained from the SAB Maltings (Caledon, South Africa). The temperature inside the silos was controlled (25 ± 1 ^{o}C) and closely monitored. Sample pooling was done at intervals (1-10) for every 50 000 tons to get an estimate of inhomogeneity (e.g. sample from silo 12 pool interval 1, would be termed 12-1). The MC of the silo 12 and 13 seed samples at the time of enzyme extractions was 11.6 and 12.0 respectively. Another cultivar used was Puma (81062435, 86014552, 81034601, 81058683), of these two there were good seed samples and two bad seed samples.

In February 2013, another batch of seeds (3-5 month-old) from different places was obtained. All seeds were received in small paper packages with proper labelling. These consisted of different barley cultivars (cv. Nemesia, SSG564, S5,S6, S9,

Puma, Marthe, Cocktail and Kristalia) stored in different silos, at different locations/ places (Bredasdorp, Caledon, Hartswater, H/Berg, Rietpoel, Napier, K/Dale, K/Melk, OAB Protem). They indicated that these were from problem batches of seed in terms of germination, but that is all information that was given.

2.2. Germination tests: Barley seeds (20 seeds from each packaging) were placed in a 90 cm filter paper lined 90 cm glass petri dishes. The germination was performed under varying germination conditions (to obtain the maximal germination conditions) as follows: 4 mL or 6 mL or 8 mL water and 15^{o}C or 20^{o}C or 25^{o}C or 30^{o}C. Germination tests were performed over a 48, 72 or 96 hour period. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedlings included radicle and cotyledons.

2.3. Moisture content tests: Upon receipt of the seeds, a few packages were tested to confirm their moisture content (i.e. to see if there was a difference in moisture content between the good and bad seeds). To do that, the mass of 50 fresh seeds was recorded. The seeds were then placed in the oven at 73^{o}C for 3-5 days until a constant mass was obtained.

2.4. Extraction procedure for antioxidative enzymes: The method described by Pukacka et al. (2005) was used with some modifications. The mortar and pestle were pre-cooled on ice. Mass of twenty (20) seeds was weighed (in order to express the final enzyme activities per seed) and the seeds were ground to a fine powder in liquid nitrogen and allowed to boil dry. To this, 5 mL 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 2% PVPP, 0.1% Triton X-100, 1 mM ascorbate (to prevent inactivation of ascorbate peroxidase plastidic isoforms) was added. The mixture was homogenized on ice until thawed and then centrifuged at 15 000 x g for 20 minutes, 4^{o}C. The supernatant was used as the enzyme extract for the determination of antioxidative enzyme activity.

2.5.Glutathione reductase assay: The 1 mL reaction mixture consisted of 400 µL 100 mM potassium phosphate buffer (pH 7,8), 270 µL 0.5 mM GSSG, 250 µL 0.2 mM NADPH, 30 µL 2 mM EDTA and 40 µL enzyme extract. The GSSG-dependent

oxidation of NADPH was measured at 340 nm, 25^{o}C for 3 minutes. The activity was expressed as µmol $NADP^{+}$ min^{-1} $seed^{-1}$.

2.6. Ascorbate peroxidase assay: A modified method described by Mishra et al. (1993) was used the the determination of ascorbate peroxidase (APX) activity. The reaction mixture (1 mL) consisted of 570 µL 50 mM phosphate buffer (pH 7.0), 200 µL 0.1 mM H_2O_2, 150 µL 0.5 mM sodium ascorbate, 50 µL 0.1 mM EDTA and 30 µL enzyme extract. The reaction was initiated by adding the enzyme. Absorbance decrease was measured for 5 minutes at 290 nm, 20^{o}C. The APX activity was expressed as µmol ascorbate min^{-1} $seed^{-1}$.

2.7. Guaiacol peroxidase assay: The guaiacol peroxidase activity was measured by following a method described by van der Westhuizen et al. (1998). The reaction mixture (1 mL) contained 50 µL 0.2M 30 % H_2O_2, 100 µL 50 mM guaiacol, 340 µL double distilled H_2O, 500 µL 80 mM phosphate buffer (pH 5,5) and 10 µL enzyme. Absorbance increase due to tetraguaiacol formation was measured at 470 nm for 3 minutes at 30^{o}C. Activity was expressed as µmol tetraguaiacol min^{-1} $seed^{-1}$

2.8. Superoxide dismutase assay: A method by Keppler and Novacky (1987) was used for superoxide dismutase (SOD) assay. The 1 mL reaction mixture consisted of 10 µL enzyme extract, 990 µL solution of 2 µM riboflavin, 0.1 mM EDTA, 75 µM NBT and 13mM Methionine. The reaction samples were placed below fluorescent light in the growth cabinet for 40 minutes. Another sample (at least three replications) without the enzyme extract was placed under similar conditions to measure the maximum attainable absorbance at 560 nm. The reference sample (blank) was not irradiated. The SOD activity was expressed as log (A_{560nm} with enzyme /mass/ A_{560nm} without enzyme)/ min.

2.9. *Electrolyte leakage assay*: 6 mL de-ionised water was added to 20 weighed seeds in a clean glass petri-dish and slightly shaken for 5 minutes to ensure that all ions on the seed surface are dissolved into the seed-soak-water. The first electrical conductivity (EC) measurement was taken, which represented the electrolyte leakage of non-germinated (i.e. dry) seeds. Afterwards, seeds were transferred to a temperature controlled chamber and were allowed to imbibe water for germination to

commence. After 48 h of imbibition, seeds had germinated and the conductivity of the seed soak water was again measured. Results were put into a formula:
Electrolyte leakage = EC (mS)/ Sample weight (g)/ Volume (mL).

2.10. Malondialdehyde assay: The MDA content was done according to a method described by Metwally et al. (2003). Twenty seeds were ground in 5 mL 0.1 % TCA to a fine paste on ice and centrifuged 10 000 x g, 15 min at 4^{o}C. Four mL 20 % TCA containing 0.5% TBA was added to 1 mL supernatant. The mixture was heated at 95^{o}C for 30 minutes, Cooled on ice and centrifuged for 5 minutes. Absorbance of the TBA-MDA complex was measured at 532 nm. To correct overestimation of MDA content, absorbance at 440 nm was taken.

2.11. Protein assay: Protein content was determined using the Bradford (1976) Bio-Rad protein dye method. SDS-PAGE was also performed to identify unique protein profiles between the good and bad seeds.

3. RESULTS AND DISCUSSION

The 4 mL test led to higher germination; however the root and coleoptile growth was not sustained by this volume at varying temperatures (15, 20, 25, 30 and 35^{o}C). In addition, the 8 mL test led the lowest germination percentage at varying temperatures (results not shown).

Moisture content of the received seeds was also tested and it ranged between 9-12% (results not shown). SDS-PAGE protein profiles showed no unique bands between the “good and bad” seed samples (results not shown).

3.1. The germination tests for barley seeds in 6 mL and varying temperatures

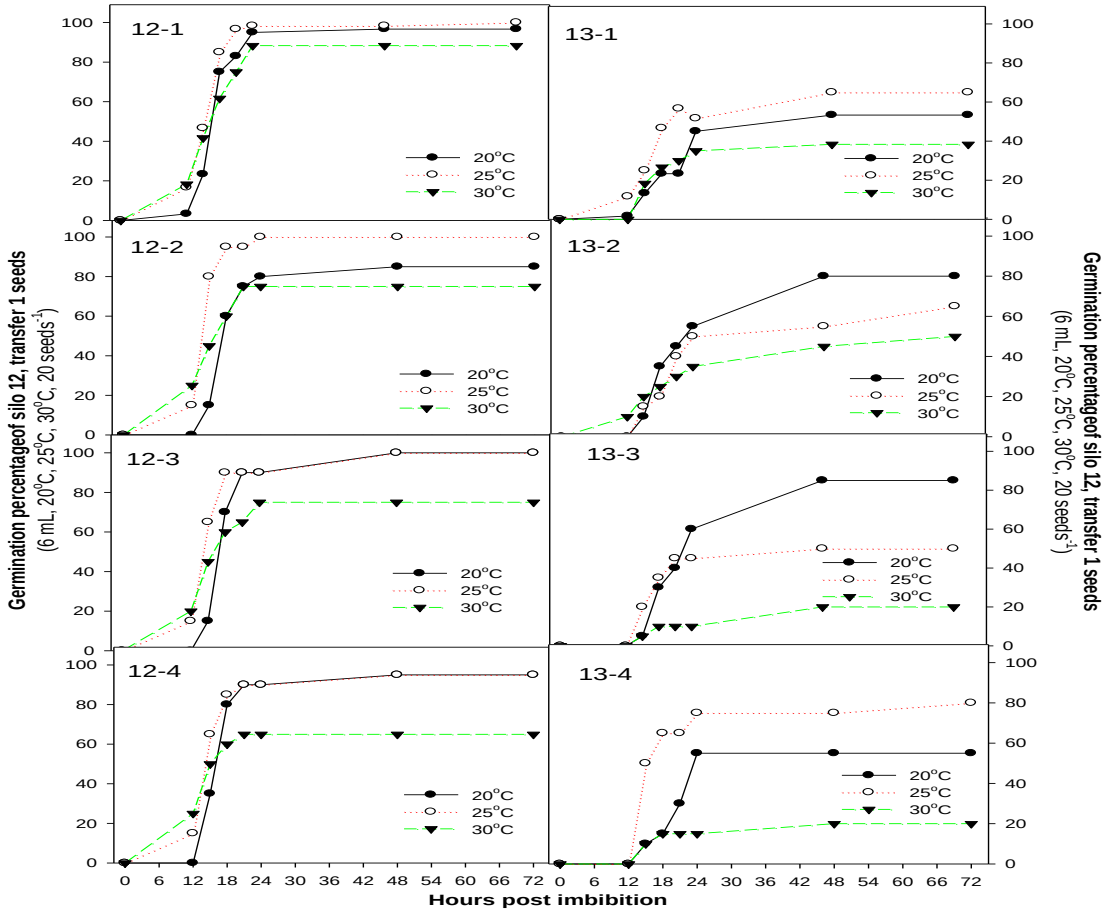

Figure 3.1.1. The effect of varying temperature on the germination ability of barley (cv., Erica) stored in silo12 and silo 13 (2012), transfers 1-4.

The silo 12 stored seeds at transfers 1 to 4 had higher germination percentages than the silo 13 stored seeds at the same transfers (2012). Temperature difference had a considerable impact on the germination ability of seeds. The silo 12 stored seeds had a higher germination percentage at 20^{o}C and 25^{o}C as opposed to the silo 13 stored seeds. 30^{o}C seemed to be the worst germination temperature for both silo 12 and silo 13 stored seeds. The silo 12 and silo 13 seeds responded in a different manner to temperature difference. Although the silo 13 stored seeds did not reach

germination percentage of at least 95%, the lower temperature (20^{o}C) was more favourable than higher temperature 25 and 30^{o}C (Fig. 3.1.1).

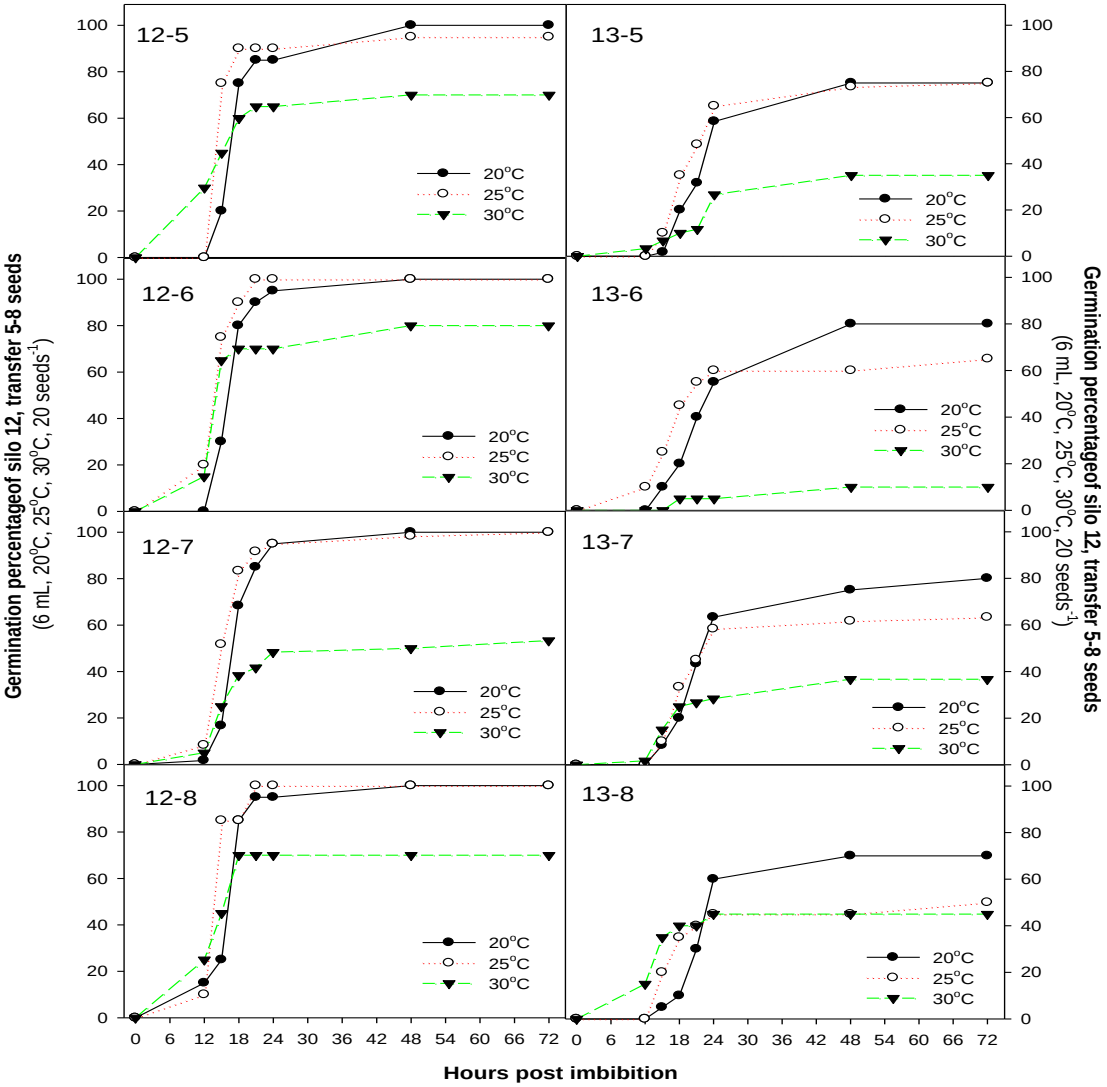

Figure 3.1.2. The effect of varying temperature on the germination ability of barley (cv., Erica) stored in silo12 and silo 13 (2012), transfers 5-8.

The results of Figure 3.1.2 were similar to those of Figure 3.1.1, with silo 12 stored seeds having higher germination ability than silo 13 stored seeds. The silo 13 stored seeds still showed higher germination potential at 20^{o}C than 25^{o}C.

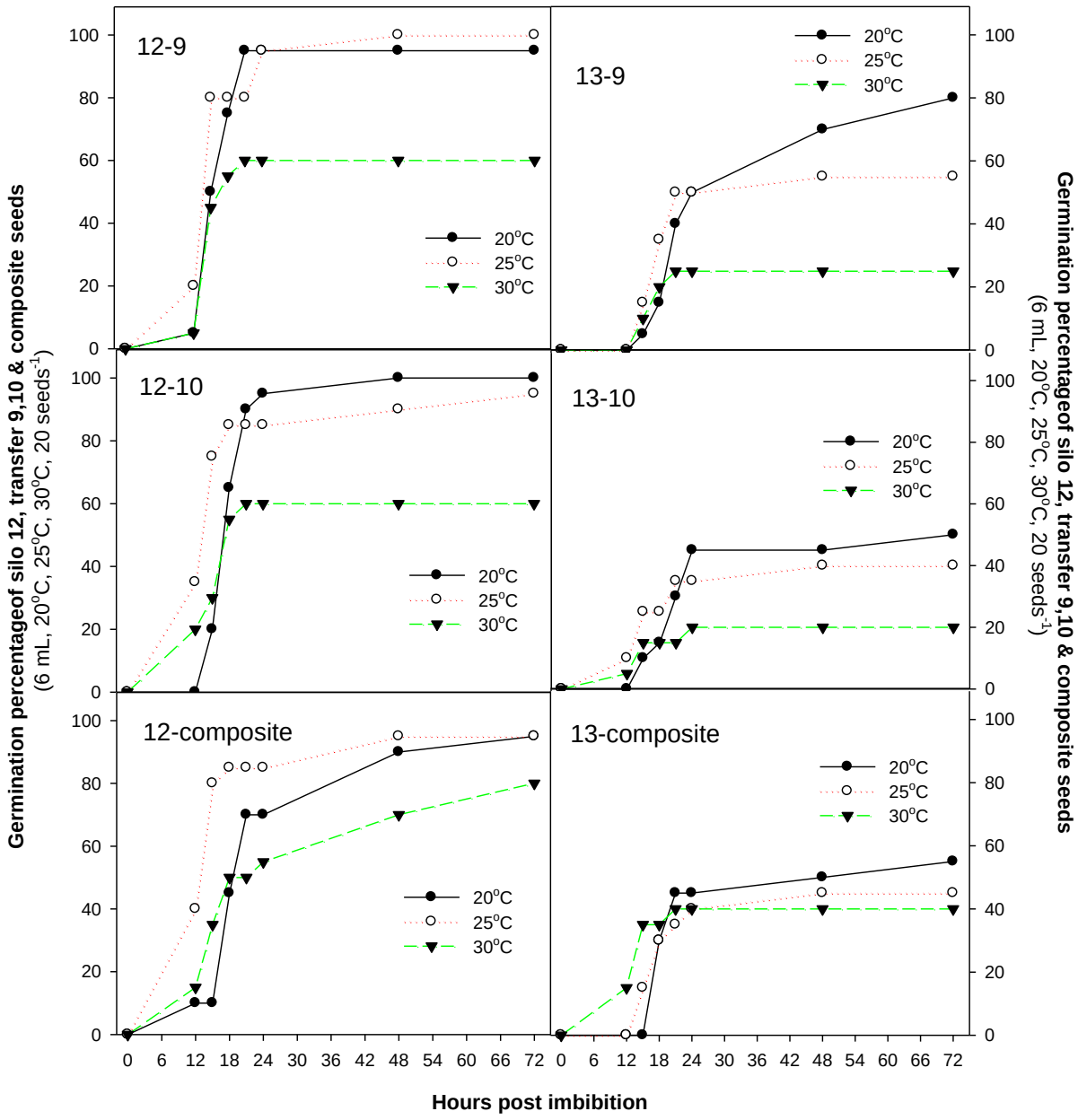

Figure 3.1.3. The effect of varying temperature on the germination ability of barley (cv., Erica) stored in silo 12 and silo 13 (2012), transfers 9,10 and composite.

The silo 13 stored seeds transfer 10 and composite had the lowest germination percentage compared to other transfers (Fig. 3.1.3).

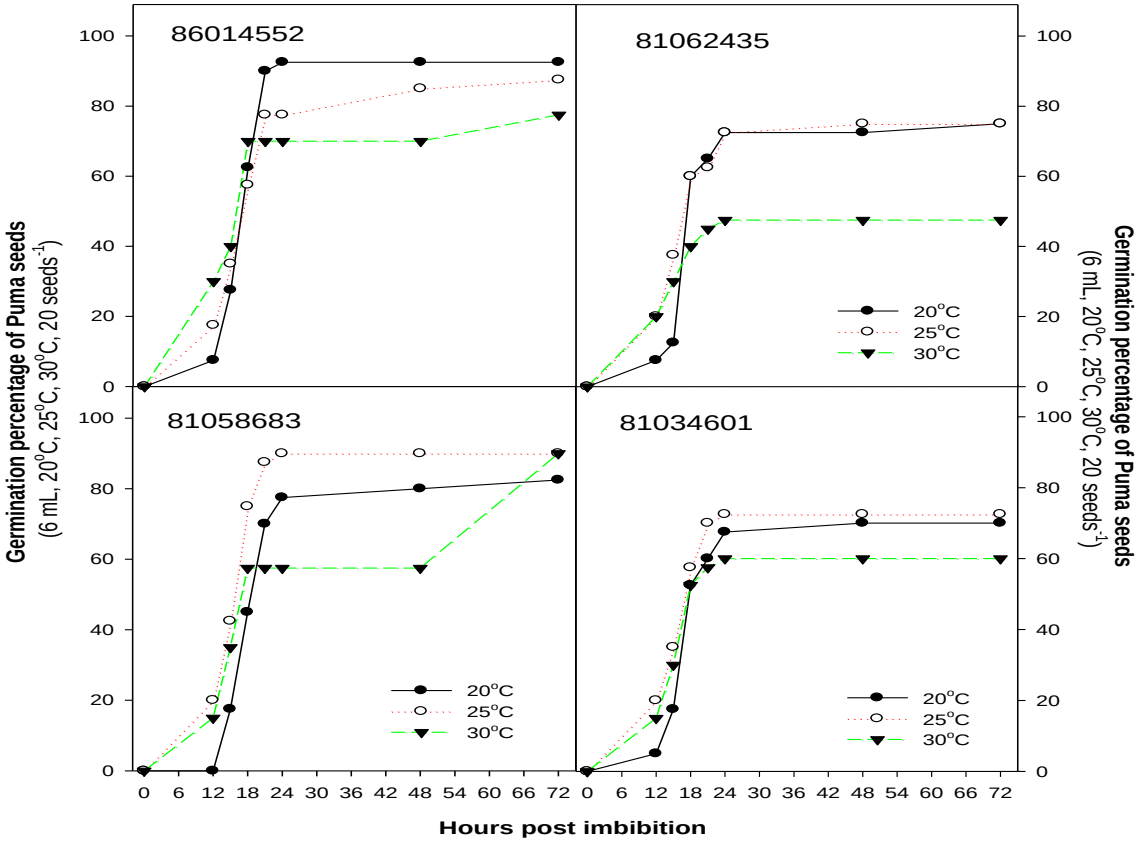

Figure 3.1.4. The effect of varying temperatures on the germination ability of barley (cv., Puma) seeds. Experiments were done in duplicate.

According to Figure 3.1.4, Puma 86014552 and 81058683 had a higher germination ability than the other two (Puma 810624435 and 81034601). However, the germination percentage difference between the ''bad'' seeds and ''good'' seeds was minimal. Similar to silo 12 and 13 stored seeds, the 20^{o}C and 25^{o}C germination temperatures were better than the 30^{o}C germination temperature, with 25^{o}C performing better in the good seeds and 20^{o}C better in bad seeds (Fig. 3.1.4). In agreement with our results, germination at 25^{o}C was also found to be optimal in barley (Mei and Song 2010).

Since similar conditions were maintained in both silos, the difference in germination could not be related to the temperature inside the silo. As a result, it was more applicable to investigate the involvement of antioxidative enzymes (SOD, POD, GR

and APX) in seed germination because ROS is the primary cause of seed deterioration during storage (McDonald 1999).

3.2. Germination tests for the seeds used in the enzyme activity assays

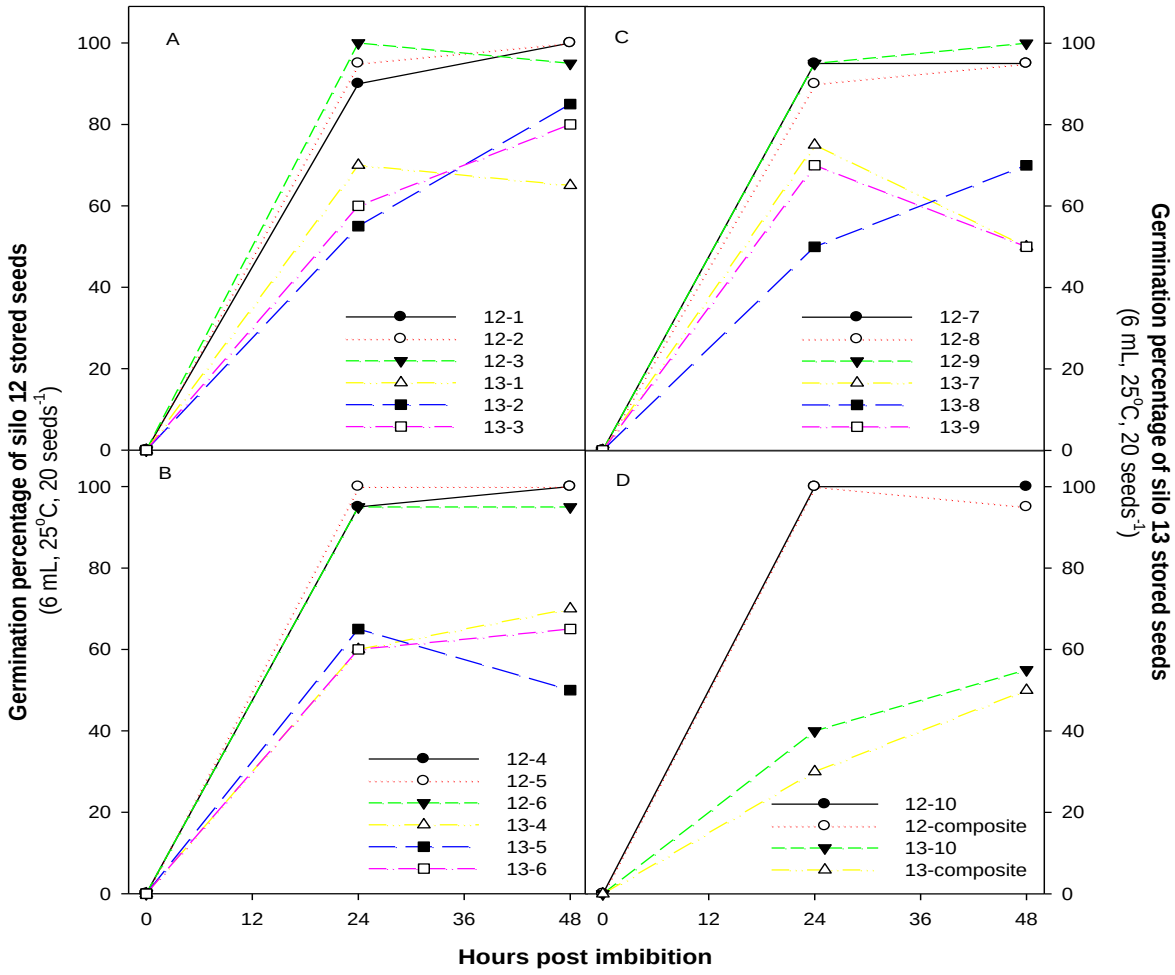

Figure 3.2.1. Germination ability of the silo 12 and 13 stored barley seeds (2012) used for the antioxidative enzyme assays. The 6 mL and 25°C germination conditions were used.

The results presented in Figure 3.2.1 confirmed that the silo 12 stored seeds were indeed better seeds with good germination ability in contrast to the silo 13 stored

seeds. The germination percentage of all silo 12 stored seeds ranged between 95-100 %, whereas that of silo 13 stored seeds ranged between 45-80 %.

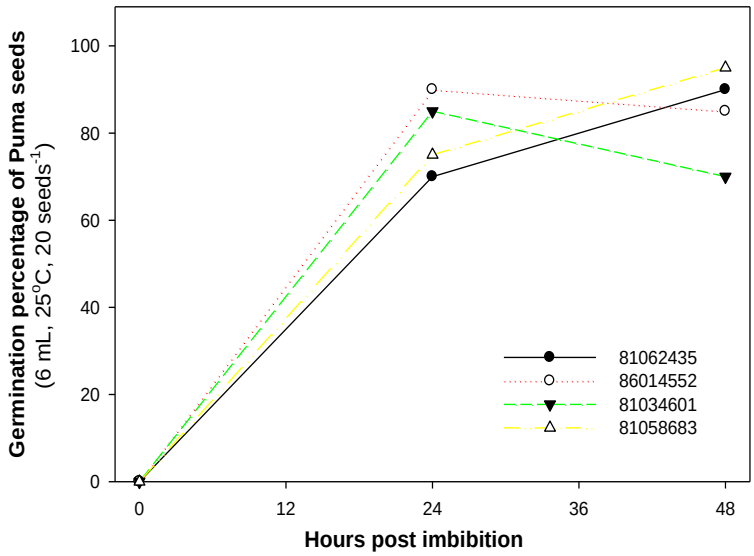

Figure 3.2.2. Germination ability of the barley seeds (cv., Puma) used for the antioxidative enzyme assays. The 6 mL and 25^{o}C germination conditions were used.

According to Figure 3.2.2, there was no significant difference in the germination percentage of Puma seeds. Also, the results show no consistency. For example at 48 h.p.i , the germination percentage pattern indicates that 81058683 and 81062435 had higher germination than the other two. In contrast in Figure 3.1.4 however, 81058683 and 86014552 (instead of 81062435) had the highest germination percentage.

With a longer period of storage (± 1 year later), the same seeds tested in 2012 i.e. (silo 12 and 13 stored seeds) had a slight decrease in the germination percentage (i.e. ± 20 % loss). However, the germination percentage of the silo 12 seeds was still found to be significantly higher than that of the silo 13 seeds (P = 4.03 x 10^{-9}). The germination percentage of the silo 12 stored seeds was found to be between 87 and 70% and that of the silo 13 stored seeds between 48 and 25% (Fig. 3.2.3).

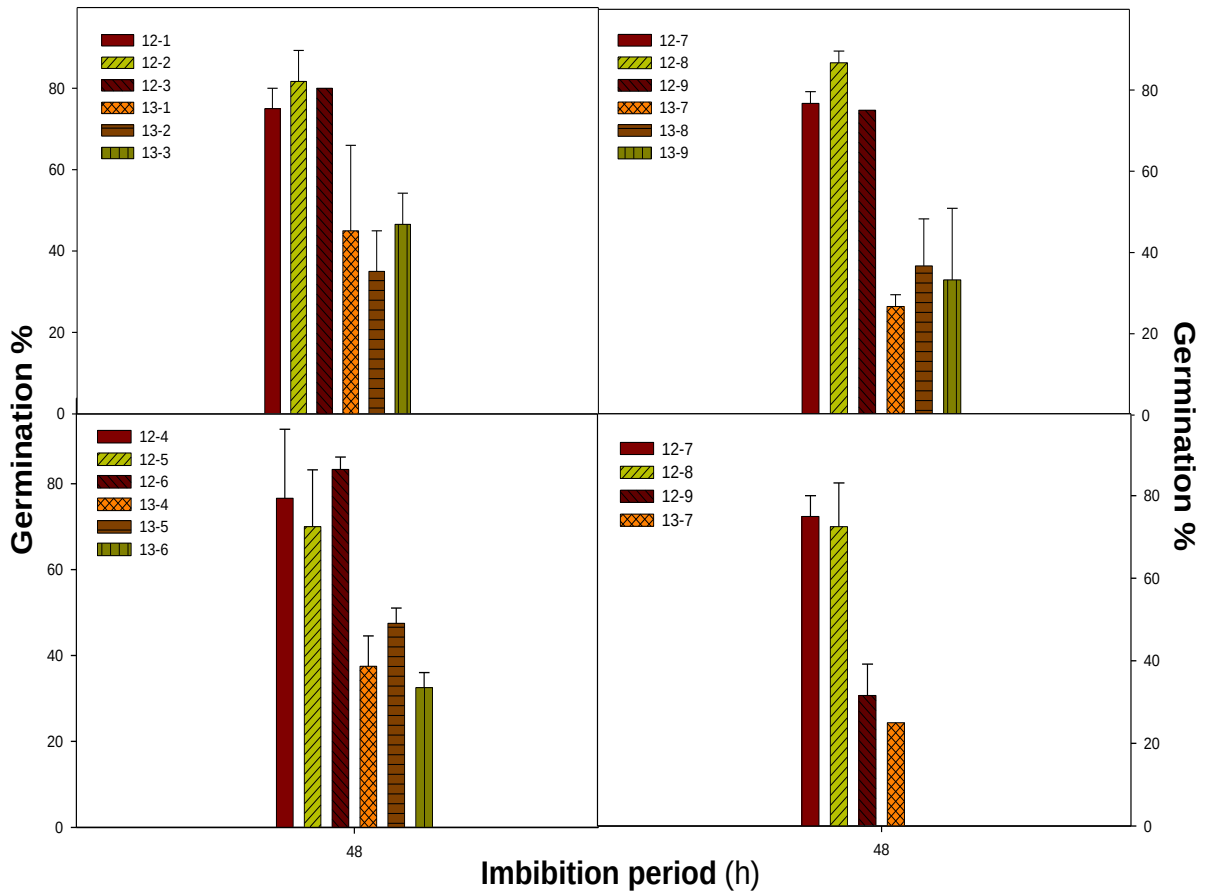

Figure 3.2.3. Germination percentage of silo 12 and 13 stored seeds at different sampling intervals (i.e. per 50 tons), a year later (i.e. 2013). Values are means ± SD (n=3).

3.3. Antioxidative enzyme activities

Germinating seeds have high antioxidant enzyme activities (Kiran et al. 2012). In our study, however, not all antioxidative enzymes tested were found to be involved in germination. The SOD activity of the silo 12 and 13 seeds was inconsistent (Fig.3.3.1). No link between SOD activity and germination (Fig. 3.2.1) was established. These findings were also confirmed in another study using barley seeds (cv., Puma), where the difference in SOD activity was not evident (Figure 3.3.2). In disagreement, a positive correlation between SOD activity of dry *Picea omorika* seeds and germination was established in another study (Pronanovic et al. 2007). Similarly, when antioxidative enzymes including SOD were low, ROS accumulation

was high leading to a 50% decrease in germination (Lehner et al. 2008; Tian et al. 2008). Interestingly, Pukacka and Ratajczak (2005) found that seeds *of Beech fagus sylvatica* still lost their viability despite the increase in antioxidative enzymes, meaning that the level of antioxidative enzymes could be important in the ROS detoxification process only.

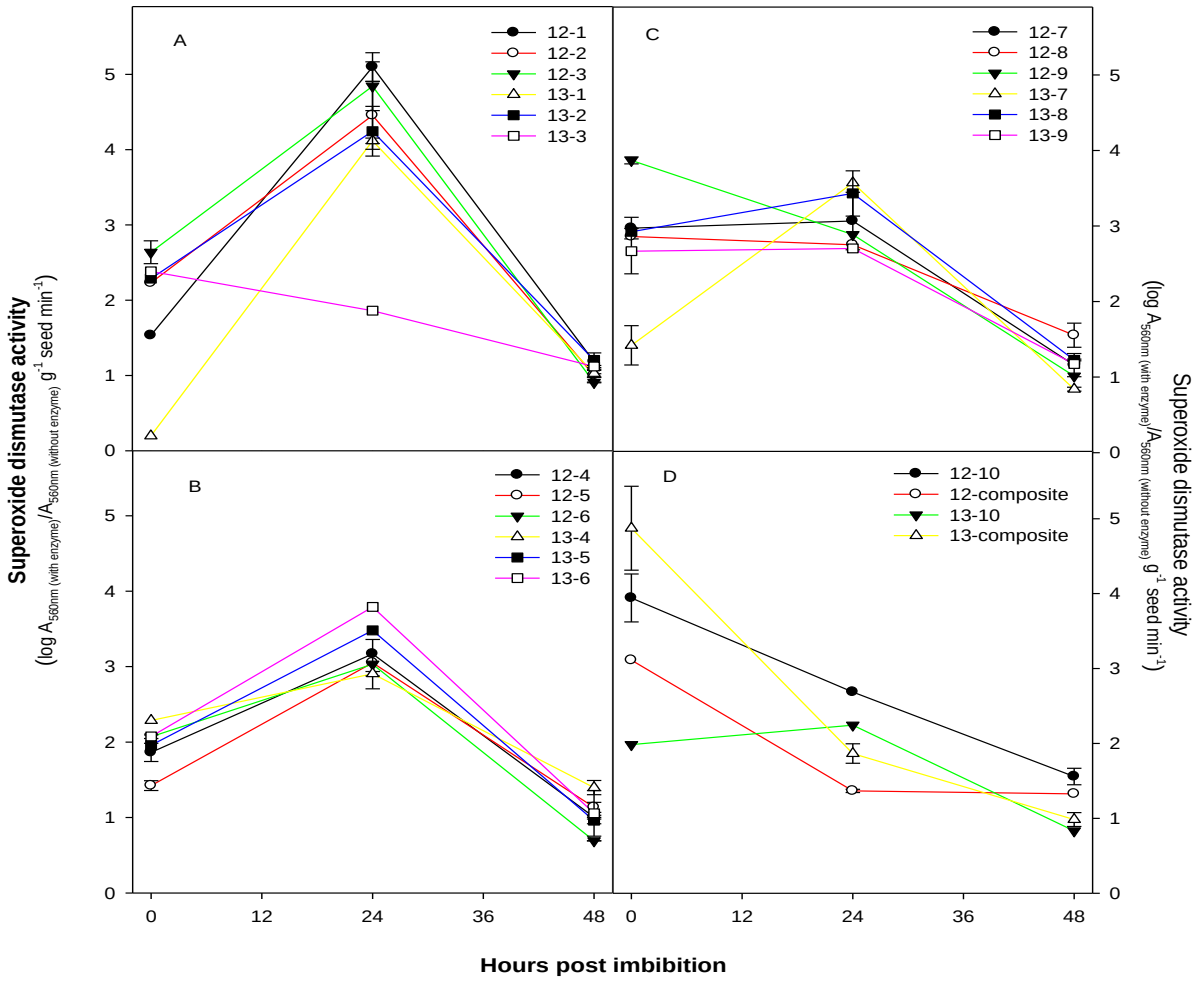

Figure 3.3.1. Superoxide dismutase activity of barley (cv. Erica) seeds stored in silo 12 and silo 13. Values are means SD ± (n=3).

Table 3.3.1. *P* values for superoxide dismutase activity of silo 12 and silo 13 barley (cv., Erica) stored seeds at transfers 1-10 and composite.

Samples	*P* value (0 h.p.g)	*P* value (24 h.p.g)	*P* value (48 h.p.i)
Silo-12 stored seeds (cv., Erica) vs	0.45998	0.56970	0.6045
Silo-13 stored seeds (cv., Erica)			

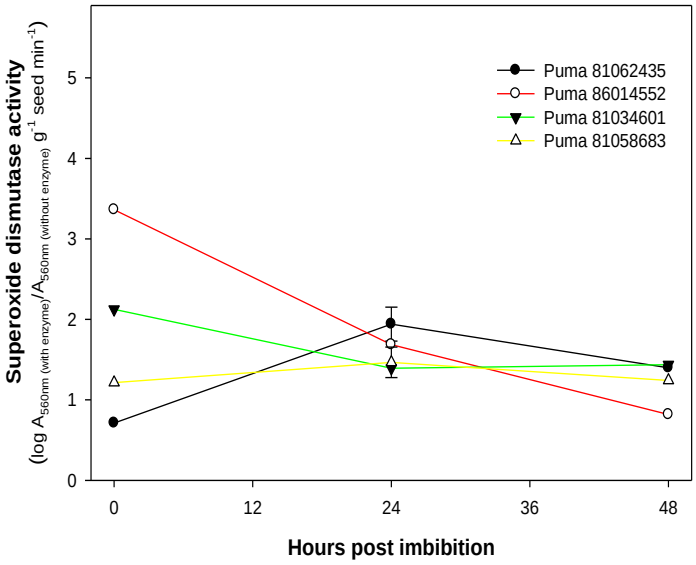

Figure 3.3.2. Superoxide dismutase activity of barley seeds (cv., Puma). Values are means SD ± (n=3).

The results indicated that POD is not involved in preservation of barley because POD activity of dry seeds was not significantly different between silo 12 and 13 seeds (P > 0.05). A significant induction of POD activity to higher levels in silo 12 than 13 seeds (48 h.p.i; Fig. 3.3.3) shows that this increase was a result of an increase in germination (Kiran et al. 2012). Similarly, POD was found to be involved during germination and not in protection against ROS during storage (Prodanovic et al. 2007; Demirkaya et al. 2010). Figure 3.3.4 further confirmed an increase in POD activity using a different cultivar (cv., Puma), with the highest induction in the 'good' seeds, 48 h.p.i.

Significantly higher (P = 0.03) levels of APX activity in dry silo 12 seeds (Fig. 3.3.5), indicates that these seeds have more ROS scavenging ability than silo 13 seeds. This increase may be followed by an increase in germination because the success of germination depends on the ROS detoxification efficiency of dry seeds (Bailly 2004; Prodanovic et al. 2007). In agreement, it was found that a decrease in APX activity of stored dry *Ginkgo biloba* seeds results to a decrease in germination (Tommasi et al. 2006). Furthermore, high APX activity was also associated with high germination of maize (Wattanakulpakin et al. 2012). Although these studies suggest a strong relation between APX activity of non-germinating seeds and germination, our results contradicted these statements. A moderate correlation (0.51) between APX activity (0 h.p.i) and germination indicates that APX activity of dry seeds cannot always be a prediction for good germination (see Table 3.3.2). Higher APX activity of silo 12 seeds may suggest that this enzyme is only involved in the prevention of lipid peroxidation and consequently membrane leakage (Pukacka 1991; Bailly 2004) during storage. A strong positive correlation (0.8) between germination and APX activity (48 h.p.i), is in agreement with findings that germinating seeds have high antioxidative enzyme activities (Kiran et al. 2012).

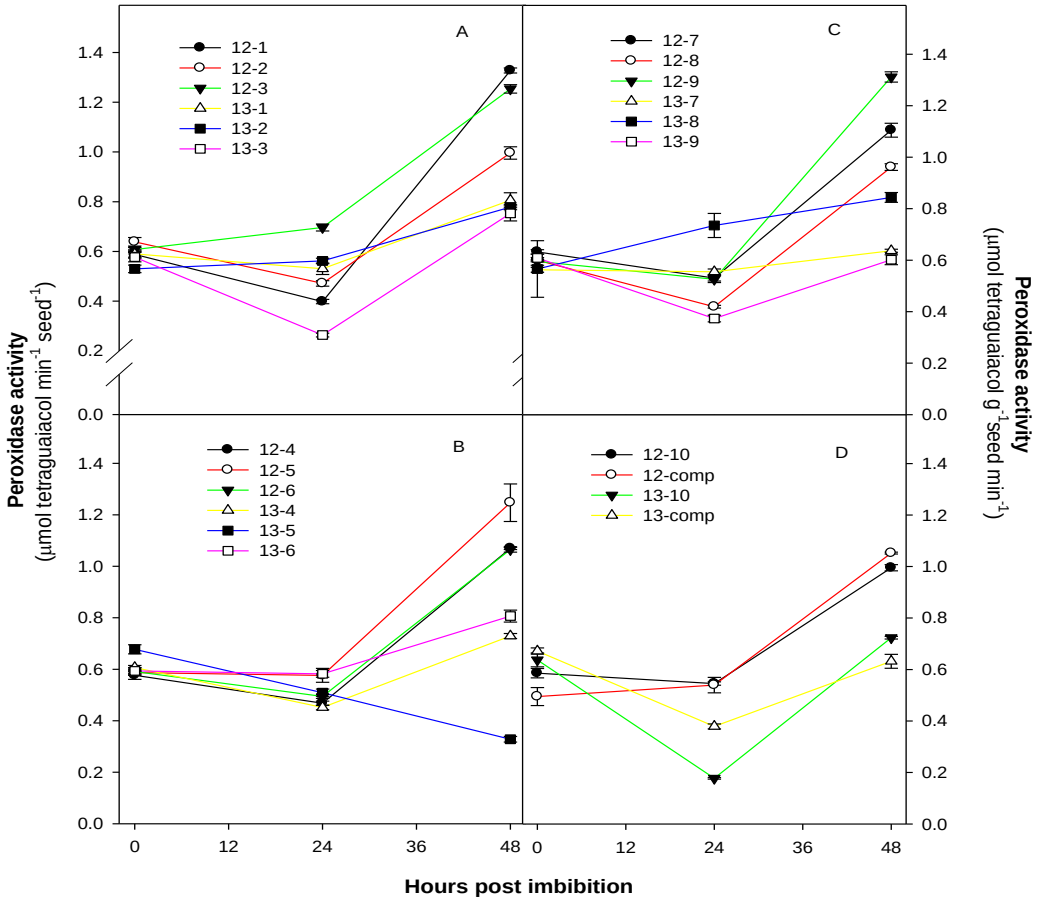

Figure 3.3.3. Peroxidase activity of barley (cv. Erica) seeds stored in silo 12 and silo 13. Values are means ± SD (n=3).

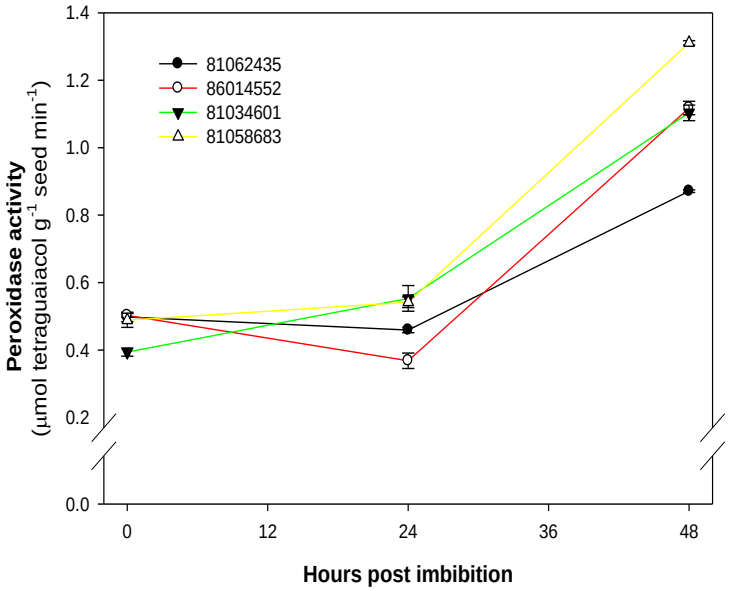

Figure 3.3.4. Peroxidase activity of barley (cv., Puma) seeds. Values are means ± SD (n=3).

There was no consistency in glutathione reductase (GR) activity of the silo 12 and silo 13 stored seeds (before and after germination). The GR activity of barley stored in silo 13, transfers 4,5,6,10 and composite, was higher than that of silo 12 stored seeds. In contrast, the GR activity of the silo 12, transfers 1,2,3,7,8,9, was higher than that of silo 13 stored seeds (Fig.3.3.7). In overall, there was no significant difference in the GR activity of the silo 12 and silo 13 stored seeds for all hours tested. Figure 3.3.8 further confirmed that there is no big difference in the GR activity of seeds with good or bad germination ability.

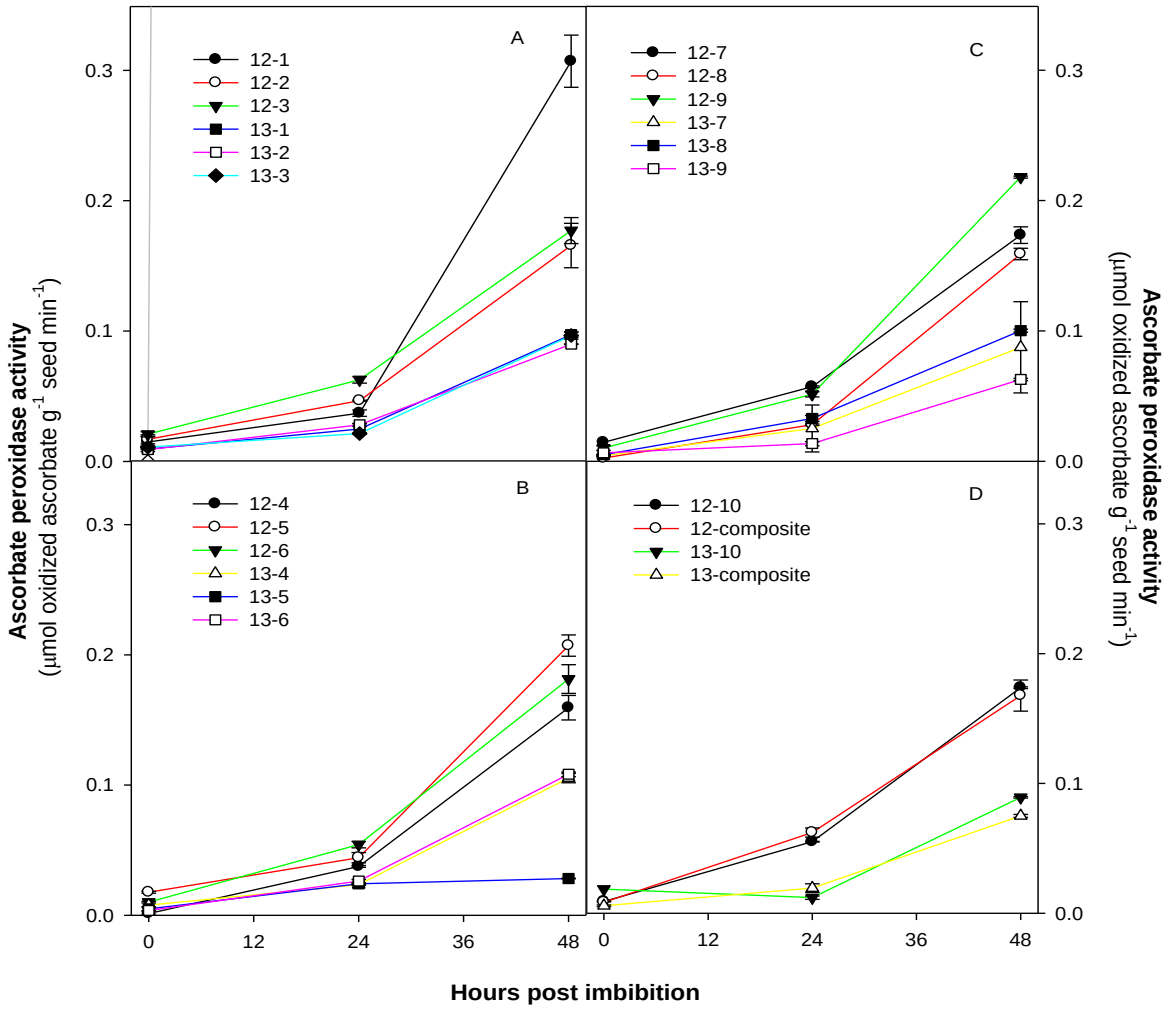

Figure 3.3.5. Ascorbate peroxidase activity of barley (cv., Erica) seeds stored in silo 12 and silo 13. Values are means ± SD (n=3).

Table 3.3.2. *P* values for ascorbate peroxidase activity of silo 12 and silo 13 barley (cv., Erica) stored seeds.

Sample	***P* value (0 h.p.g)**	***P* value (24 h.p.g)**	***P* value (48 h.p.g)**
Silo-12 stored seeds (cv., Erica)	0.02918	0.00001377	0.0001659
Silo-13 stored seeds (cv., Erica)			

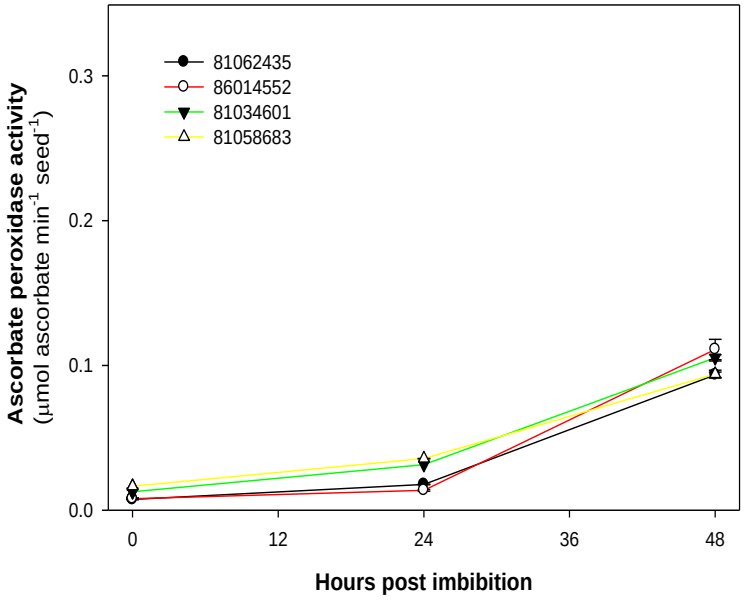

Figure 3.3.6. Ascorbate peroxidase activity of *Puma* seeds. Values are means ± SD (n=3).

Table 3.3.3. Correlation between APX activity and germination of silo stored seeds.

Imbibition period	**Correlation (APX vs germination)**
0 h (based on the 2013 germination % values)	0.54
0 h (based on the 2012 germination % values)	0.51
48 h (based on the 2013 germination% values)	0.83
48 h (based on the 2012 germination % values)	0.78

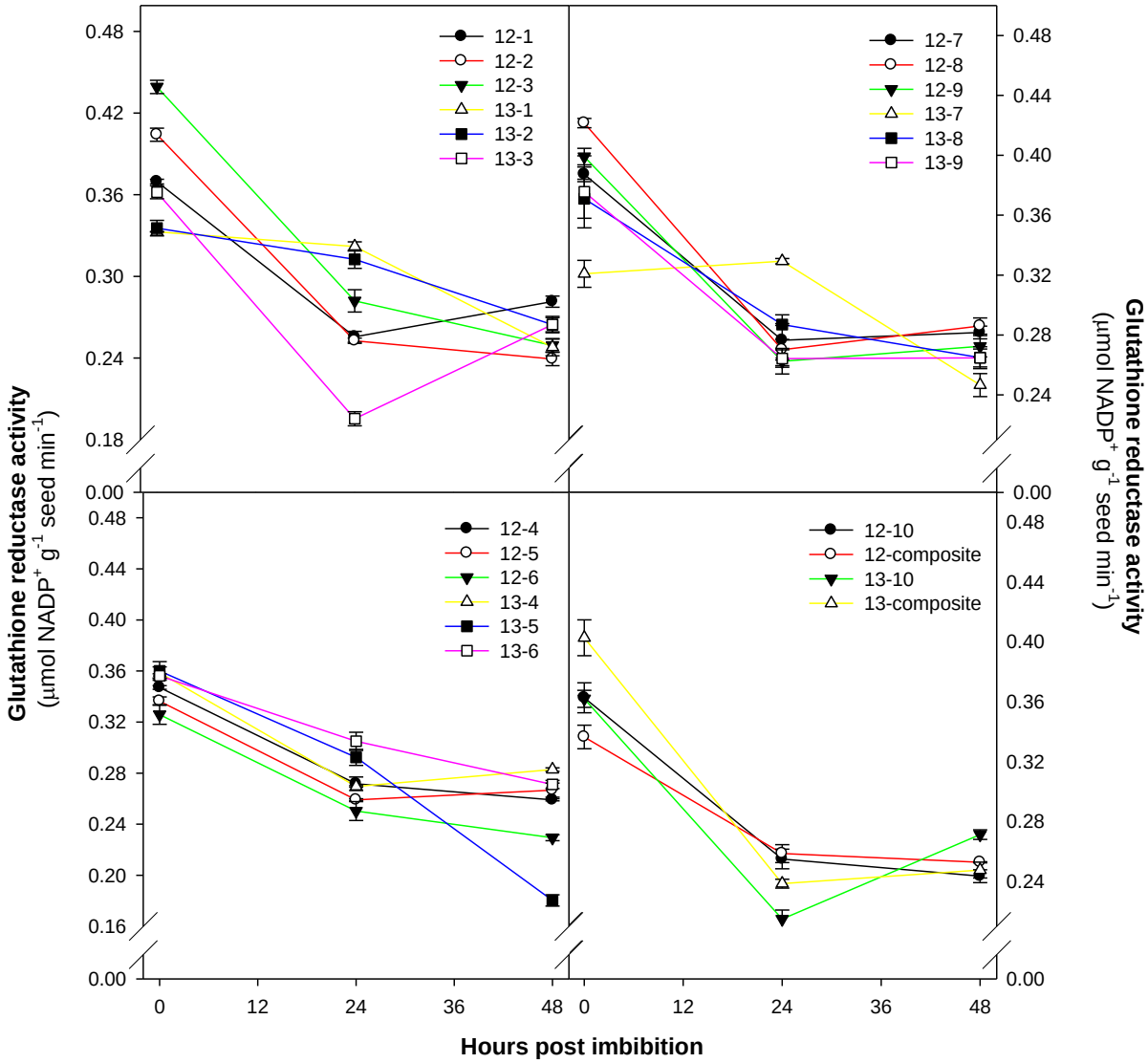

Figure 3.3.7. Glutathione reductase activity of barley (cv., Erica) seeds stored in silo 12 and silo 13. Values are means ± SD (n=3).

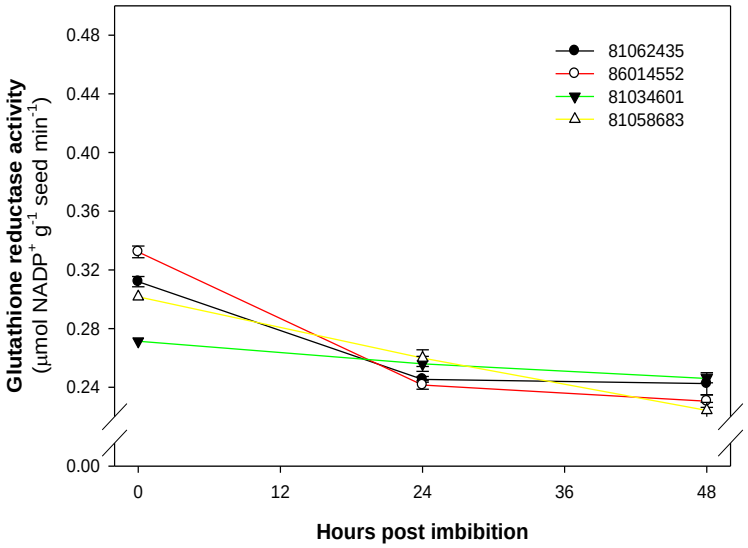

Figure 3.3.8. Glutathione reductase (GR) activity of good and bad barley (cv., Puma) seeds. Values are means ± SD (n=3).

3.4. Electrolyte leakage

The above discovery led to the investigation of the intactness of the seed membranes during storage. To initiate this, total electrolyte measurements was done on the silo 12 and 13 seeds imbibed for 0 h and 48 h period. Ion leakage was already observed as early as 0 h of imbibition with de-ionised water. This leakage was significantly less in the silo 12 stored than silo 13 stored seeds (P = 1.677 x 10^{-8}). As germination progressed (48 h.p.i), more leakage was observed. However, leakage in the silo 12 seeds was still less than that of silo 13 seeds (P = 1.07 x 10^{-6}) (Fig. 3.4.1). The leakage found in the silo 12 seeds could be because germination was no longer 100% (compared to 2012 results), meaning that those seeds that did not germinate could be the ones responsible for the observed leakage.

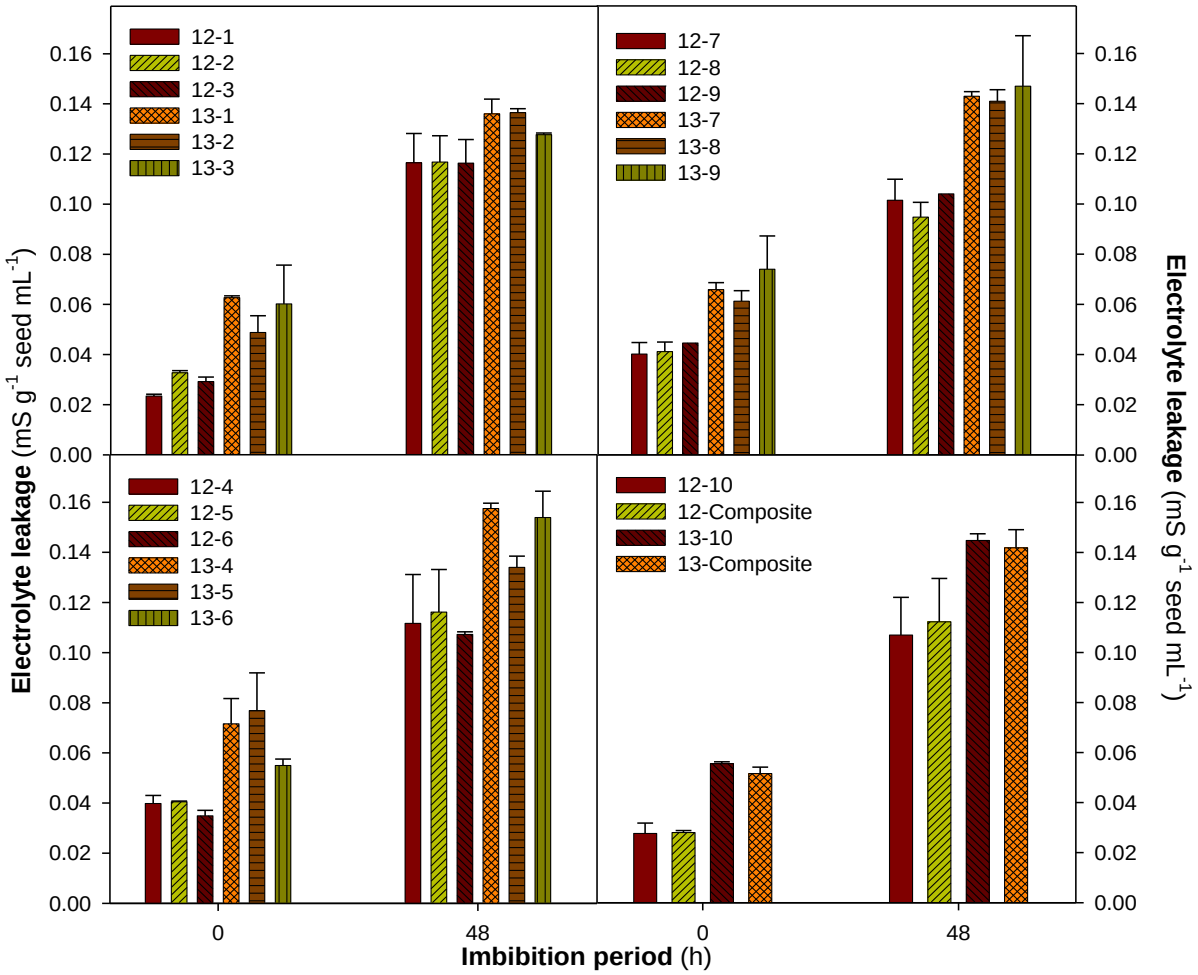

Figure 3.4.1. Total electrolyte leakage of silo 12 and 13 stored seeds at different sampling intervals (i.e. per 50 tons). Values are means ± SD (n=3).

To investigate this suggestion, correlation between germination and electrolyte leakage was conducted. The results suggested a strong negative relationship between these variables (both at 0 h and 48 h imbibition periods) (Table 2). According to these results, germination may be predicted on the dry seeds by measuring the electrolyte leakage on the seed testa. This is based on the finding that the correlation coefficient between germination and electrolyte leakage at 0 h of imbibition was - 0.80 (Table 3.4.1).

Table 3.4.1. Correlation between electrolyte leakage and germination of silo stored seeds.

Imbibition period	**Correlation (electrolyte leakage vs germination)**
0 h (based on the 2013 germination % values)	-0.79
0 h (based on the 2012 germination % values)	-0.82
48 h (based on the 2013 germination% values)	-0.93
48 h (based on the 2012 germination % values)	-0.82

Table 3.4.2. Correlation between APX activity and electrolyte leakage of silo stored seeds.

Imbibition period	**Correlation (APX vs electrolyte leakage)**
0 h	-0.60
48 h	-0.70

According to Table 3.4.2, the relationship between APX activity and ion leakage at 0 h of imbibition was found to be moderate. At 48 h.p.i, the relationship was much stronger. This could be a result of the metabolic processes involved during the germination stage, which may involve more leakage on the membranes.

Based on the above results, it can be concluded that main problem is somehow related to the membrane damage.

3.5. Malondialdehyde

Malondialdehyde (MDA) is a final product of lipid peroxidation and its concentration can be used as a measure of lipid peroxidation (McDonald 1999). The MDA content was measured to investigate if observed electrolyte leakage was somehow associated with lipid peroxidation. The results below (Table 3.5.1) represent the MDA content of the silo 12 and 13 stored seeds. Although there were significant differences in the germination percentages of silo 12 and 13 seeds, no correlation (r at 0 h.p.i = 0.146 and 0.1902 at 48 h.p.i) between seed germination and MDA content was observed. Furthermore, MDA content of the silo 12 and 13 seeds was not significantly different (P = 0.485 and 0.59 for 0 h.p.i and 48 h.p.i respectively). These results clearly indicate that lipid peroxidation may not be the cause of observed electrolyte leakage. There could be other contributing factors.

Table 3.5.1: The MDA content of silo 12 and 13 stored barley. Values are means ± SD (n= 3).

SAMPLE	MDA (0h)	MDA (48h)	GERMINATION %
12-1	0.942±0.03	0.2646±0.025	90
12-2	0.4533±.045	0.2542±0.101	95
12-3	0.596±0.062	0.2647±0.052	95
12-4	0.9883±0.025	0.247±0.07	100
12-5	0.5756±0.124	0.2281±0.034	85
12-6	0.6327±0.07	0.3926±0.027	85
12-7	0.7667±0.061	0.4033±0.121	95
12-8	0.7802±0.116	0.2997±0.011	90
12-9	0.8284±0.148	0.226±0.038	95
12-10	0.974±0.228	0.1814±0.025	95
12-Composite	0.8587±0.076	0.2899±0.034	95
13-1	0.7054±0.084	0.212±0.077	60
13-2	0.6682±0.065	0.2931±0.019	80
13-3	0.5742±0.182	0.2532±0.119	70
13-4	0.8376±0.106	0.4548±0.145	85
13-5	0.9783±0.195	0.2558±0.048	60
13-6	1.3189±0.191	0.2037±0.091	40
13-7	1.027±0.0097	0.2778±0.044	50
13-8	0.8126±0.111	0.2793±0.069	50
13-9	0.6097±0.2134	0.1934±0.041	50
13-10	0.6983±0.048	0.287±0.064	40
13-Composite	0.6527±0.043	0.3219±0.119	45

3.6 Ascorbate peroxidase activity and germination of cultivars grown and stored at different places.

Another batch of seeds was received in February 2013 for further investigations. These consisted of different barley cultivars (cv., Nemesia, SSG564, S5,S6, S9, Puma, Marthe, Cocktail and Kristalia) stored in different silos, at different locations/ places (Bredasdorp, Caledon, Harts water, H/ Berg,Rietpoel, Napier, K/Dale, K/Melk, OAB Protem). The aim was to further investigate the involvement of APX in the germination loss of silo stored seeds, whether it is cultivar related or place (i.e. a place where barley was stored) related. According to the literature, storage environment plays a secondary role in seed deterioration. The rate of seed viability and vigour loss is mainly a function of temperature and moisture content (MC) (Pukacka and Rajatszac 2005). However, seed viability and vigour differs with species and cultivars (Demirkaya et al. 2010).

Ascorbate peroxidase activity was measured in these seeds at a dry state (i.e. 0 h of imbibition) and the results are represented below (Table 3.6.1). There was no relationship between APX and germination across all the silos, cultivars and locations ($r = -0.0254$), indicating that APX values were not a good indication of germination rate. When an analysis of variance was done on APX values of one cultivar (Erica) for all the silo's, it was found that the effect of the silo from which the samples were taken was highly significant ($p=0.0000$), indicating that there were large differences between the different silos (data not shown).

For Erica the germination rate between the different silo's varied from as high as 100% to as low as 5%. For Nemesia, the germination rate varied from 62.5% to as low as 5% (Table 3.6.1). SSG564 had very low germination values (5-27%), as had S6 (5-20%) and S5 (5-50%) at the different silo's. Puma, Cocktail and Marthe did not have germination rates of less than 45% at any of the silo's, but it should also be noted that these cultivars were produced and stored in the Northern Cape region (Jan Kempdorp, Hartswater and Magogong), contrary to the other cultivars. But this still indicated germination far under the optimal value. It was therefore clear that most of these seeds were from problem batches, as germination rates were very low

on average. It was clear that for this set of seeds the APX values had not predictive value in terms of germination rates, and that this test will not have use in the case where a large percentage of the seed has already died.

SABM would have sent more batches of seed for us to test in simulated silo conditions, but after dr. Meijering left for the UK there was no further communication in terms of the seed, and we received no further seed.

Table 3.6.1. Ascorbate activity and germination of various cultivars stored at different locations.

Cultivar	*Place*	*Silo #*	*Germination %*	*Average per location*	*APX activity(dry seeds)*
Erica	**R/poel**	R 5	12.5		0.159
		R 9	10		0.152
		R 19	22.5		0.218
		R 23	25		0.16
		R 25	20		0.161
		R 28	42.5		0.168
		R 6	5		0.165
		R 29	5	17.81	0.141
	K/Melk	B9	60		0.103
		B10	40	50.00	0.117
	H/Berg	B2	40		0.18
		B6	100		0.15
		B7	67.5		0.129
		B11	62.5		0.156
		B16	65	67.00	0.168
	S/Dam				
		B6	60		0.128
		B10	67.5		0.157
		B17	80		0.131
		B13	90	74.38	0.13
	K/Dale	K14	62.5		0.123
		K5	72.5	67.50	0.171
	Krige	K9	30		0.156
		K10	35		0.17
		K6	32.5		0.141
		K11	32.5	32.50	0.136
	Caledon	C2	45		0.089
		C22	35		0.094
		C27	5		0.149
		C24	27.5		0.133
		C25	10		0.106
		C9	7.5		0.129
		C26	32.5	23.21	0.131
	Napier	N16	62.5		0.106
		B11	22.5	42.5	0.15
	OA Protem	B13	52.5		0.168
	B/Dorp	B25	40		0.117
		B7	52.5		0.108
		B12	57.5		0.106
		B8	32.5	45.63	0.162
Nemesia	**R/poel**	R 1	5		0.083
		R 21	10		0.085
		R 8	22.5	12.50	0.174

	K/Dale	K18	52.5		0.202
		K17	25	38.75	0.165
	Krige	K5	37.5		0.146
		K13-K2	17.5		0.09
		K13-K7	12.5	22.5	0.11
	Caledon	C13	25		0.104
		C7	10		0.118
		C3	25	20.00	0.085
	Napier	N9	7.5		0.13
		N10	5	6.25	0.175
	OA Protem	B12	27.5	27.50	0.11
	B/Dorp	B35	30		0.197
		B6	42.5		0.138
		B27	27.5		0.137
		B33	10		0.11
		B5	10		0.115
		B36	22.5		0.119
		B13	37.5		0.11
		B1	62.5		0.083
		B30	20		0.117
		B34	27.5		0.106
		B32	35		0.115
		B31	25	29.17	0.102
S9	**R/poel**	R 22	27.5	27.50	0.16
	H/Berg	B22	55	55.00	0.16
	B/Dorp	B81	77.5	77.50	0.07
SSG564	**R/poel**	R 20	5	5.00	0.113
	K/Melk	B4	15	15.00	0.079
	H/Berg	B1	27.5	27.5	0.116
	S/Dam	B9	10		0.102
		S9	20	15.00	0.105
	K/Dale	K11	5	5.00	0.096
	Napier	N6	27.5	27.50	0.083
	Caledon	C21	17.5	17.50	0.096
	B/Dorp	B28	15	15.00	0.087
		B29	15	15.00	0.089
S6	**R/poel**	R 10	20	20.00	0.069
	K/Dale	K9	5	5.00	0.092
	Krige	K4	10	10.00	0.133
	Caledon	C19	7.5	7.50	0.112
	OA Protem	B1	10	10.00	0.143
	B/Dorp	B10	5	5.00	0.065
S5	**K/Dale**	K10	15	15.00	0.186
	Caledon	C12	5	5.00	0.203
		C20	15	15.00	0.21
		C10	10	10.00	0.181
		C23	40	17.50	0.184

	OA Protem	B4	50	50.00	0.162
	B/Dorp	B4	27.5		0.074
		B16	37.5		0.119
		B24	30		0.099
		B11	17.5		0.081
		B15	7.5	24.00	0.078
Puma	**J/Kemp**	L7	70		0.121
		L2	52.5		0.068
		L5	75	65.83	0.068
Cocktail	**H/water**	R 10	45	45.00	0.11
	Magogong	L1	55		0.108
		L2	70	62.50	0.121
Marthe	**H/water**	R 11	55		0.066
		R 1	52.5		0.051
		R 2	60	55.83	0.045

4. CONCLUSIONS

4.1. This study suggests that germination in 6 mL fluid at 25^{o}C is optimal for sustained root and coleoptile development.

4.2. Germination studies confirm that the silo 12 stored seeds which were the “good seeds”, had, as expected, higher germination percentage than the silo 13 stored seeds which were the “bad seeds”.

4.3. There were no significant differences in the germination percentages of the supplied Puma seeds. In addition, the germination pattern was not consistent.

4.4. Low activities of the antioxidative enzymes glutathione reductase (GR), guaiacol peroxidase (POX) and superoxide dismutase (SOD) in “dry” seeds strongly indicate that they are not involved in the preservation of silo stored seeds.

4.5. Higher ascorbate (APX) activity of silo 12 (good) compared to silo 13 (bad) stored seeds could suggest the involvement of this enzyme in

enhancing germination of the seeds during storage. However, this enzyme is known not to be involved in seed preservation during storage. The total lack of correlation between APX activity and germination rate in the batch of seed received in 2013 from silo's in different regions indicates that when seed has already died or are in the process of dying, APX does not give any indication of potential germination rates.

4.6. High electrolyte leakage in the silo 13 compared to silo 12 stored seeds indicates that membranes of the silo 13 seeds were damaged during storage, how or why this happens is not known. This is strongly linked to APX activity in that high APX activity enables the seeds to scavenge toxic radicals thereby preventing membrane damage, hence high germination. Therefore electrical conductivity of the membranes may be a quick and reliable method to predict germination of the silo stored seeds.

4.7. In this study, malondialdehyde content assay was not found to be a reliable method for predicting membrane damage of the dry seeds.

5. RECOMMENDATION

Of all the tests done in this study it seems that electrical conductivity of the membranes may be a quick and reliable method to predict germination of the silo stored seeds. Death of seeds seems to be due to membrane damage during storage. More research will be needed to determine why these membranes are damaged, and how this can be prevented.