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Effect of Ageing on Oxidative Stress and Ascorbate-Glutathione Cycle in various RILs of Soybean

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ABSTRACT: Effect of ageing on the antioxidant system was studied in three years old ambient-stored seeds of cultivated soybean variety DS 9712 (*G.max.*), wild type genotype DC 008-1(*G.soja*) and 3 recombinant inbred lines (RILs) developed from inter-crossing of DS 9712 and DC 008-1. Germination in the seeds of DS 9712 was 13% as against 85% and above in the seeds of DC 008-1 and the 3 RILs. The concentration of the membrane-damageing substances like thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) in the seeds of DS 9712, DC 008-1 and the recombinant inbred lines (RILs) was high, low and intermediate, respectively, which corresponded inversely with their level of germination. Among the RILs, RIL-2 (2_6_2) had the least concentration of TBRAS and CDs and the highest level of germination. TBRAS equivalent was found to be negatively correlated with the germination percent (r= -0.9966). The antioxidant enzymes involved in ascorbate-glutathione (AsA-GSH) cycle *viz.*; ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) were significantly low in DS 9712, while their concentration was medium to high among the RILs and DC008-1. The AsA-GSH system possessed weaker reduced: oxidized forms in DS 9712 as compared to DC 008-1 and the RILs. Efficient AsA-GSH cycle in the seeds of DC 008-1 and the 3 RILs might have prevented accumulation of reactive oxygen species (ROS) saving the membrane from damage leading to increased germination.

Keywords: Soybean, Storage, Oxidative Stress, Ascorbate-Glutathione Cycle, RILs

The soybean {*Glycine max* (L.) Merr.} is a legume crop native to East Asia, is one of the major agricultural commodities in the world. It is pre-eminent for its high protein (38–45%) as well as high oil content (approximately 20%). Soybean is also rich in vitamins, minerals, and other antioxidants. The health benefit of soybean includes improved digestive and bone health improved blood circulation and heart health, reduced risk of cancer, insomnia, and diabetes [1]. Soybeans occur in various sizes, and in many hull or seed coat colors. The seeds of *G. max* are yellow, bold, with a low number of non-shattering pods, early maturity duration, and exhibit permeable seed coat. It is believed that *G. max* was domesticated about 5,000 years ago from its wild relative (*G. soja* Sieb. & Zucc.) found in China [2]. The seeds of *G. soja* are black, small, having more number of shattering pods with delayed maturity duration and have hard non-permeable seed coat.

Seed deterioration is a complex physiological and biochemical process. Seed deterioration refers to the decrease in the quality of seed and may lead to a reduction to complete loss of seed viability, delayed germination followed by germination suppression, seed damage and lower tolerance to unfavorable or stressed conditions etc [3, 4]. Losses in seed quality occur during field weathering, harvesting, and storage and are exacerbated if seeds are exposed to high temperature and/or high humidity. Soybean is one of the most sensitive agronomic seeds where significant deterioration can occur after just one year of storage [5] and has been grouped as least storable among all the grain crops [6].To manage the problem of rapid seed deterioration in soybean, it is imperative to understand the basic mechanisms involved. Several mechanisms such as impairment of membrane function, inhibition of protein synthesis, the decline in sugar content, damage to enzyme systems, damage to nuclear material including RNA, DNA and chromosomes and biochemical changes resulting in lower levels of ATP have been suggested for loss of seed vigour [7]. In recent years, growing evidence

point to the toxicity of by-products of catabolic reactions such as lipid peroxidation, modification of proteins and sugars thorough Amadori and Millard reaction resulting in deterioration of seeds [8, 9].

To guarantee future global food security and sustainable crop production, there exists a strong need for broadening the genetic base and looking for new resources to develop soybean cultivars. The closest variety of soybean to *G. max* is the wild, undomesticated ancestor *G. soja*. Wild soybean is cross-compatible with the cultivated soybean [10] and has the potential to improve soybean yield [11]. The population was derived from a cross between *Glycine max* and *Glycine soja*. The present work was undertaken to study the germination, the degree of oxidative stress and the anti-oxidative enzymatic defense system of 3-year aged seeds of the parents and RILs of these crosses.

MATERIALS AND METHODS

Cultivated soybean varieties are known for their poor storability, contrary to that the wild types are reported to have better longevity. Transfer of desirable genes from wild types to cultivated varieties is feasible using modern tools. Nowadays scientists are developing recombinant inbred line (RIL) populations to map quantitative trait loci. For the development and phenotypic evaluation of RIL populations, parent strains are selected based on phenotype, marker availability and compatibility. A construction design scheme is determined, including the target population size, if and how advanced inter-crossing will be done, and the number of generations of inbreeding. Parent crosses and F1 crosses are performed to create an F2 population. Depending on the design, advanced inter-crossing may be implemented to increase mapping resolution through the accumulation of additional meiotic crossover events. Finally, lines are inbred to create genetically stable recombinant lines. Genetically stable RILs; 2_6_2, 4_8_1 and 4_32_1 developed from diverse parents (*G. max* and *G. soja*) by Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi were taken for this study (Plate 1). Three years old seeds of soybean, the harvest of the year 2013, were supplied by the Pulse Laboratory, ICAR-IARI, New Delhi. The seeds sealed in envelopes were kept in desiccators under laboratory conditions to naturally age for 3 years. After the ageing of three years, the seeds were used to study the Ascorbate-Glutathione cycle and oxidative stress caused due to ageing.

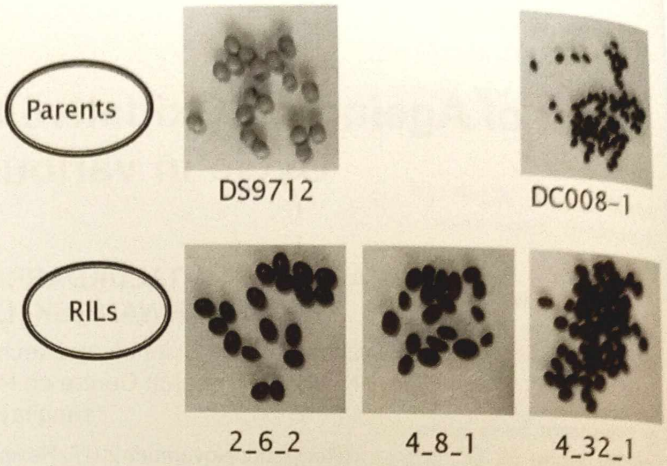

Plate 1. Diverse parents and genetically stable RILs used in the study

Germination (%)

Four replications of 50 seeds each were put in rolled towel paper for germination in an incubator set at 25°C. The count of normal seedlings was recorded [12] after incubation for 10 days and expressed in germination percentage.

Oxidative Damage

The oxidative damage to membrane lipids caused stress due to ageing was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS) with thiobarbituric acid (TBA) test as described by Heath and Packer [13]. The specific and non-specific absorbances were measured at 532 and 600nm, respectively. The TBARS content was expressed in μmol g^{-1} FW. The lipid conjugated dienes (CD) were estimated by the method proposed by Gidrol *et al.* [14]. The lipid conjugated dienes were expressed in μmol g^{-1} FW.

Antioxidant Enzymes Activity

Ascorbate peroxidase (APX)

The APX activity was measured according to Nakano and Asada [15]. The seeds (0.15 g) were homogenized in 1.5 mL extraction buffer containing 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM EDTA, 2% (w/v) PVP and 1 mM ascorbic acid. The homogenized samples were centrifuged at 12000 x g for 20 min at 4°C. The reaction mix for monitoring enzyme activity consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2, 0.1 mM EDTA, 0.5 mM ascorbic acid and enzyme extract. The kinetic changes were observed at 290nm for 180 seconds. The enzyme activity was expressed in μmol Vit min^{-1} mg^{-1} protein.

Dehydroascorbate reductase (DHAR)

The DHAR activity was measured according to the method described by Nakano and Asada [15]. The enzyme was extracted in extraction buffer containing 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM EDTA and 2% (w/v) PVP. The reaction mix consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA and enzyme extract. The enzyme kinetics was monitored at 265 nm for 180 seconds. The specific enzyme activity expressed as µmol AsA min^{-1} mg^{-1} protein.

Monodehydroascorbate reductase (MDHAR)

The MDHAR activity was measured according to Hossain *et al.* [16]. The seeds were homogenized in the extraction buffer consisting of 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM EDTA and 2% (w/v) PVP. The reaction mixture for enzyme activity ingredient 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH, 2.5 mM AsA, 1U ascorbate oxidase and enzyme extract. The oxidation of NADH was determined by observing the kinetic changes of reaction mix at 340 nm for 180 seconds and enzyme activity was expressed in µmol NADH mg^{-1} protein.

Glutathione reductase (GR)

The GR activity was measured according to the method suggested by Foyer *et al.* [17]. The enzyme was extracted using extraction buffer consisted of 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM EDTA and 2% (w/v) PVP. The reaction mix for GR contains 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.2 mM NADPH, 1 mM GSSG and enzyme extract. The enzyme kinetics was observed at 340nm for 180 seconds. The specific enzyme activity was expressed as µmol NADPH min^{-1} mg^{-1} protein.

Non-enzymatic Antioxidants

Ascorbate pool

The ascorbate pool (t-AsA, ASH, and DHA) was estimated according to Wu *et al.* [18]. The seeds were homogenized in 5% (w/v) TCA and centrifuged at 12000X g for 30 min at 4°C. The supernatant obtained was used for estimation of Ascorbate pool. The reaction mix for ASH contains the supernatant containing sample, 50 mM potassium phosphate buffer (pH 7.7), 2% (w/v) TCA, 8.8% (v/v) H_3PO_4, 1.25% (w/v) 2,2'- bipyridyl, 0.3% (w/v) $FeCl_3$. The mix was incubated at 37°C for 60 min followed by cooling at room temperature. For estimation of t-AsA, the sample was initially incubated with 50 mM potassium phosphate buffer (pH 7.7) and 0.0125 mM DTT for 10 min, followed by a procedure similar to that ASH estimation. The absorbance of both the reaction mixes was recorded at 525nm. The DHA content was calculated from the formula, DHA= t-AsA – ASH. The contents of the ascorbate pool were expressed as µg g^{-1} FW.

Statistical Analysis

The correlation analysis was performed with SPSS correlate (Bivariate, two-tailed) (ver.21.0).

RESULTS AND DISCUSSION

Germination (%)

In practice, seeds are stored at low temperature and low moisture content. However, there are reports which showed that if seeds are stored for a long duration even at ambient conditions, seed deterioration may occur [3]. The seeds of the three RILs obtained from a cross between *G. max* (DS 9712) (yellow and bold seeded having permeable seed coats) and *G. soja* (DC 008-1) (black and small seeds having non-permeable seed coats) were brown colored and medium-sized having permeable seed coats. The seeds of both the parents and their RILs were stored in sealed bags for 3 years at ambient laboratory conditions. The germination percentage of seeds stored for 3 years (Table-1) was highest (85.66%) in case of DC 008-1 (*G. soja)* and lowest (15%) in DS 9712 (*G. max)*, among the RILs, 2_6_2 show highest (81.66%) germination percentage followed by 4_32_1 and 4_8_1 with 70% and 69% germination, respectively. The results indicate that even after 3 years of ageing the seeds of *G. soja* and RILs retained the potential to germinate, while it almost declined in *G. max* [11].

Oxidative Damage and Decline in Germination

The CDs and TBARS contents were highest in *G. max* and lowest in *G. soja*, with in-between ranges in the three RILs. It was found that the lipid-conjugated dienes (CDs) were highest in DS 9712 (1.17±0.056 mmol g^{-1} FW) and lowest in DC008-1 (0.59±0.030 mmol g^{-1} FW) with in-between ranges (0.76 mmol g^{-1} FW - 0.99 mmol g^{-1} FW) in the three RILs. Among the RILS, the lowest CD was obtained in RIL 2_6_2. Similar results were obtained in TBARS content (Table-1). The results indicate that

Table 1. Mean germination percentage, lipid conjugated dienes (CDs) and thiobarbituric acid reactive substances (TBARS) of three year old seed lots of the parents and RILs of soybean

S.No.	Seed lots	Germination percentage (%)	CDs (mmol g^{-1} FW)	TBARS (µmol g^{-1} FW)
	Parents			
1.	DS9712 *(G. max)*	15.00±2.645	1.17±0.056	17.54±2.854
2.	DC008_1*(G. soja)*	85.66±1.527	0.59±0.030	7.67±1.373
	RILS			
3.	2_6_2	81.66±8.736	0.76±0.044	9.03±0.170
4.	4_8_1	69.00±6.082	0.99±0.037	10.19±0.654
5.	4_32_1	70.00±8.740	0.81±0.016	10.45±0.465

*Each value in table is the mean of three biological replicates with ± representing standard deviation.

ageing induced the exaggerated generation of ROS especially in case of *G.max* which initiates oxidative stress as indicated by high CDs and TBARS contents. However, low levels of CDs and TBARS in *G. soja* and 2_6_2 explains the high germination percentage even after 3 years. The adverse impact of higher levels of free oxygen radicals on lipid peroxidation and membrane integrity of seeds was evident in our studies. The TBARS and CD content, which is an indicator of the extent of lipid peroxidation, which is an indicator of membrane perturbation, was very high for the *G.max* compared to *G. soja* seed. The results showed that ageing seeds for long duration affect the integrity of cell membrane which may lead to a decrease in germination especially in *G. max* and not in RILs. The CDs and TBARS were found to be negatively correlated to seed germination as the Pearson correlation between CDs and germination was -0.884 and that between TBARS and germination was -0.997, suggesting that decrease in germination ability is accompanied with an increase in CDs and TBARS. Lipid peroxidation and oxidative stress have been widely indicated as the major causes of deterioration of oilseeds during ageing [4, 22].

Ascorbate-Glutathione Cycle

To access the effects of ageing on the anti-oxidative system we explore the Ascorbate-Glutathione cycle- an active mechanism operated in plants meant for quenching ROS. The comparative analysis of the enzymes (APX, DHAR, MDHAR, and GR) among both the parents and the RILs (Figure 1-5). The ageing significantly affected ROS scavenging enzymes in *G.max* when compared to *G. soja*. APX activity was found to be highest in all the RILs in comparisons to the parents (Figure 1). Among the parents, minimum activity was found in DS 9712 (0.15 µmol Vit min^{-1} mg^{-1} protein)

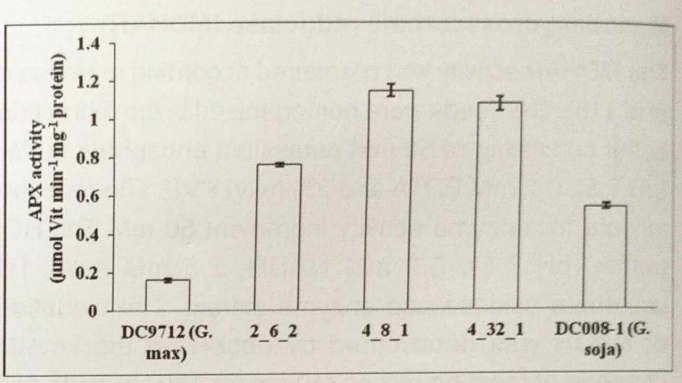

Figure 1. Mean ascorbate peroxidase (APX) activity (µmol Vit min^{-1} mg^{-1} protein) in three year old seed lots of the parents (*G.max* and *G. soja*) and the RILs (2_6_2, 4_8_1 and 4_32_1) of Soybean (error bars representing standard deviation)

compared to DC008-1(0.540 µmol Vit min^{-1} mg^{-1} protein). Thus, *G.max* exhibited about 70.55% low activity of APX when compared to *G. soja*. Data on DHAR showed that DHAR activity was found to be highest in DC008-1 (0.95 µmol AsA min^{-1} mg^{-1} protein) followed by RILs and least activity was found in DS 9712 (0.10 µmol AsA min^{-1} mg^{-1} protein) (Figure 2). Among the RILs, the highest activity was observed in 2-6-2 (0.21 µmol AsA min^{-1} mg^{-1} protein).

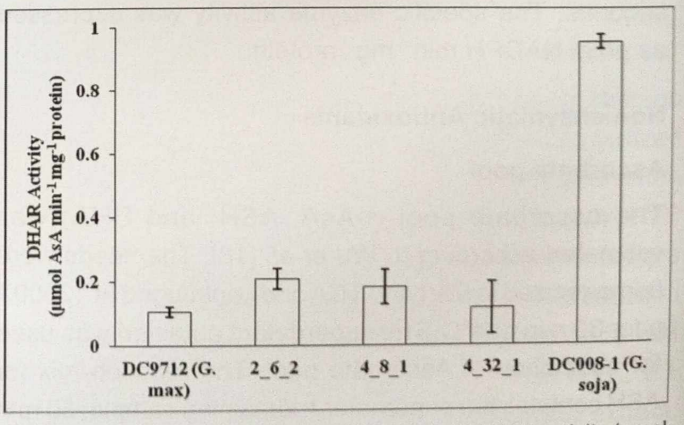

Figure 2. Mean dehydroascorbate reductase (DHAR) activity (µmol AsA min^{-1} mg^{-1} protein) in three year old seed lots of the parents (*G.max* and *G. soja*) and the RILs (2_6_2, 4_8_1 and 4_32_1) of Soybean (error bars representing standard deviation)

Here also about 80%, less activity of DHAR was found in *G. max* as compared to *G. soja*. The plant contains ROS scavenging systems including enzymatic and non-enzymatic components These anti-oxidative system works efficiently to maintain the redox homeostasis in the plant to perform it better under stressed conditions [3].

A similar trend was observed in MDHAR and GR activities of parents and RILS. Highest activities of MDHAR (Figure 3) and GR (Figure 4) was observed in DC008-1 (5.9 µmol NADH min^{-1} mg^{-1} protein) and 1.23 µmol NADPH min^{-1} mg^{-1} protein), respectively. The activities of MDHAR and GR were intermediate in RILs and lowest in DS 9712. Among the RILs, 2-6-2 showed the highest activity of both the enzymes. The *G. max* exhibited about 65.76% and 71.89% low activity of MDHAR and GR, respectively, when compared to *G. soja*. Activities of all the enzymes of Halliwal Asada pathway were found to be highest in

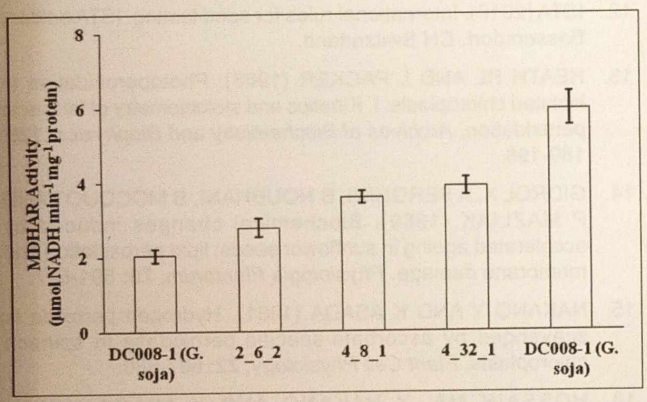

Figure 3. Mean monodehydroascorbate reductase (MDHAR) activity (µmol NADH min^{-1} mg^{-1} protein) in three year old seed lots of the parents (*G.max* and *G. soja*) and the RILs (2_6_2, 4_8_1 and 4_32_1) of soybean (error bars representing standard deviation)

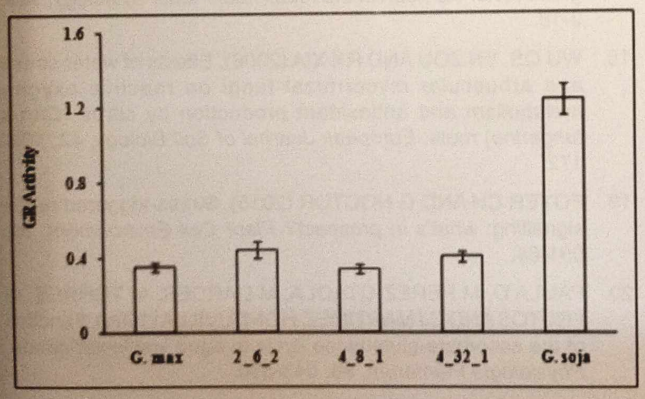

Figure 4. Mean glutathione reductase (GR) activity (µmol NADPH min^{-1} mg^{-1} protein) in three year old seed lots of the parents (*G. max* and *G. soja*) and the RILs (2_6_2, 4_8_1 and 4_32_1) of soybean (error bars representing standard deviation)

G. soja followed by RILS and least in *G.max*. Among the RILS 2_6_2, had the highest antioxidant activity except for APX and MDHAR activity which was higher in RIL 4_32_1. The results imply that ageing results in compromised ROS scavenging system in *G.max* as indicated by low enzyme activities. Similar changes were also reported in sunflower [23]. The enzyme activities in all the RILs were within the ranges of the two parents except for the APX activity in 4_32_1 and 4_8_1 which was much higher than the two parents.

Ascorbate Pool

To further investigate the effect of ageing in these soybean varieties, we quantify the ASH, DHA, and their corresponding redox ratios. The ASH/DHA ratio was 1.66, 5.98, 1.03, 1.81, and 7.40 in *G. max*, 2_6_2, 4_8_1, 4_32_1 and *G. soja*, respectively (Figure 5). The redox ratios are the indicators of a plant's efficiency to combat the oxidative stress aroused due to ageing. The comparative studies in the parents and RILs revealed that the Ascorbate-Glutathione system possessed weaker reduced/oxidized forms in aged seeds of *G. max* followed by 4_32_1 and 4_8_1, as indicated by low ASA/DHA ratios which might lead to decrease in efficiency of the seed to quench ROS and hence results in seed deterioration during storage. On the other hand, *G. soja*

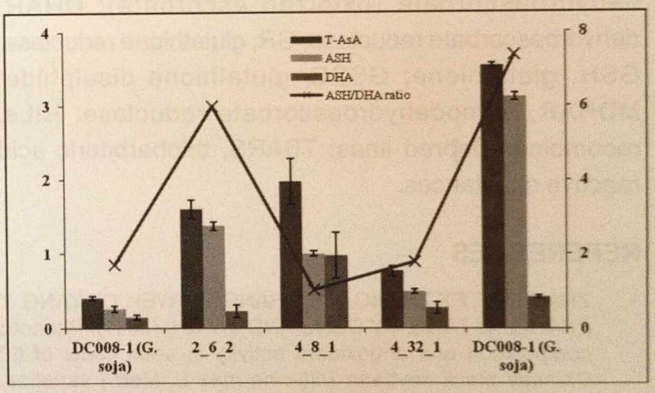

Figure 5. Mean total ascorbate (T- AsA), reduced ascorbate (ASH) and oxidized ascorbate (dehydroascorbic acid, DHA) content expressed as µg g^{-1}FW on primary Y-axis and ASH/DHA ratio represented on secondary Y-axis, in three year old seed lots of the parents (*G. max* and *G. soja*) and the RILs (2_6_2, 4_8_1 and 4_32_1) of soybean (error bars representing standard deviation)

and 2_6_2 shows better reduced/oxidized forms, maintained even after ageing. The exploration of the Ascorbate-Glutathione cycle revealed that the better germination percentage in DC 008-1 (*G. soja*) and RILS especially 2_6_2 even after ageing the seeds for 3 years is might be due to the proficient functioning of the

enzymes involved in the Ascorbate-Glutathione cycle. The enzymes result in the generation of non- enzymatic antioxidants which provide the balanced redox status in the cells and quench ROS efficiently, while the redox status in the other three members of soybean is quite low in comparison to these two which might be the reason of low germination percentage after 3 years of ageing. On the other hand, loss of germination in *G. max* was associated with accumulation of oxyradicals, which in turn leads to rapid lipid peroxidation, and loss of membrane integrity of seeds. In this case, these antioxidant enzymes are not able to quench high levels of the free radicals as well as other peroxides generated, which lead to faster and severe damages to seed tissues. The level/amount of antioxidant was either not sufficient or not available at the proper time so the balance between generation and quenching get disturbed. Therefore, the imbalance of the highly coordinated defense mechanism against reactive oxygen species leads to the damages and cell death of seed tissues and finally resulted in the loss of germinability and vigour of seed [24].

Abbreviations

APX, ascorbate peroxidase; AsA, ascorbic acid; ASH, reduced ascorbate; CDs, lipid conjugated dienes; DHA, dehydroascorbate (oxidized ascorbate); DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; MDHAR, monodehydroascorbate reductase; RILs, recombinant inbred lines; TBARS, thiobarbituric acid reactive substances.

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