TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2020) 44: 577-588

doi:10.3906/tar-1909-48

Vigor difference during storage and germination in Indian mustard explained by reactive oxygen species and antioxidant enzymes

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Received: 12.09.2019 • **Accepted/Published Online:** 08.06.2020 • **Final Version:** 08.12.2020

Abstract: Canola-type genotypes in Indian mustard (*Brassica juncea*) are a new kind of quality resource developed for their low levels of erucic acid (<2%) and glucosinolate (<30 µmole/g defatted meal) contents. Single-zero (low erucic acid) and double-zero (low erucic acid and glucosinolate content) genotypes of Indian mustard have less vigor. Conventional genotypes (high erucic acid and glucosinolate contents) havea significantly higher seedling vigor index-II (SVI-II) and single-zero genotypes have a significantly higher SVI-I, whereas double-zero genotypes have been observed to have a significantly lower SVI-I and SVI-II. To know the possible reasons for the differences in vigor, the seed quality parameters, reactive oxygen species (ROS) contents (superoxide radicals ($O_2^{-}\cdot$) and hydrogen peroxide), lipid peroxidation, and antioxidant enzyme activity were examined. In the dry seeds, the conventional genotypes revealed lower ROS contents and higher catalase and peroxidase enzyme activity. This trend was reversed in the double-zero genotypes, which could be the reason why they were more susceptible to oxidative damage. During seed germination, an increase in the ROS contents, and corresponding increase in antioxidant enzyme activity, was noticed, which was highest in the conventional genotypes, followed by the single-zero genotypes. Double-zero genotypes showed the lowest increase in ROS contents and antioxidant enzyme activity during this period. This meant that the required attributes were met for maintaining oxidative balance within the cells and triggering physiological activities to reach high vigor. This study proposed 2 causes for the poor vigor of the double-zero genotypes; first, in the dry seeds, the ROS remained high due to low antioxidant activity (ROS scavengers) and the second was less generation of $O_2^{-}\cdot$ during germination.

Key words: Canola, *B. juncea*, seed quality, germination, antioxidant activity

1. Introduction

Successful seedling establishment is most critical in crop production,which determines the possibility of the potential harvest in the future. A universal rule is that the high germination ability of seeds is coupled with high vigor, and thus the seedling establishment. Hence, it is imperative to select vigorous seeds of high quality to attain maximum yields. A vigorous seed lot is potentially able to perform well, even under suboptimal environmental conditions. Poor seed quality, with low viability and vigor, results in uneven or erratic emergence and consequently, reduces plant stand and crop yields. Farmers often fail to recover from the hazardous effects of such substandard seeds (Finch-Savage and Bassel, 2015).

Reactive oxygen species (ROS) are continuously produced as metabolic by-products in the different cellular compartments of plants. In dry seeds, ROS are produced by Amadori and/or Maillard reactions, and associated lipid peroxidation mechanisms. These are generated as a result of the partial reduction of oxygen, which results in the formation of superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). Imbalances in intracellular ROS status-driven processes have been reported as the reason for loss of seed viability. According to the free radical theory of aging, the loss of seed viability during senescence is caused by the excessive production of ROS ($O_2^{-\bullet}$, H_2O_2, and $^{\bullet}OH$) combined with reduced antioxidant potential in cells and the gradually accumulated oxidative damage of seed cells (Ratajczak et al., 2015). The major source of ROS in cells is oxidative phosphorylation occurring in the mitochondria; thus, the free radical theory of aging may essentially be a mitochondrial theory of aging in seed-bearing plants (Kurek et al., 2019). As a consequence of the above processes, during storage, seeds undergo deteriorative processes that limit their viability and ultimately cause the loss of their inherent property, i.e. the ability to germinate.

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Due to these reasons, the uncontrolled production of ROS has long being considered as deleterious material. In particular, the accumulation of ROS has often been indicated as the major cause of seed deterioration (Lehner et al., 2008).

On the other hand, seed germination is the most important event that ensures the very existence of plants over generations. The progression of germination is a complex process that starts with the imbibition of water, resulting in several morphogenetic, physiological, and biochemical changes. It is associated with the transition of a dry quiescent seed into a metabolically active state, resulting in elongation of the embryonic axis and emergence of the radicle after rupturing the testa (Singh et al., 2014). It has also been reported that ROS plays a dual role, where they function as cellular messengers, and regulators of growth and development, signaling molecules, and responses to biotic and abiotic stresses,mainly when seeds become hydrated, and act as the main modulators in programmed cell death in aged seeds (Karkonen et al., 2015; Dunand et al., 2007). For a seed to germinate, it has to imbibe water and in the hydrated state, mitochondria, glyoxysomes, and plasma membrane-embedded nicotinamide adenine dinucleotide phosphate (NADPH) oxidases act as major sources for ROS production (Jeevan Kumar et al., 2015). ROS produced after imbibition plays a role in seed germination. The localized accumulation of ROS has been correlated with the endosperm loosening process in *Lepidium sativum* (Muller et al., 2009).

For the regulation of excess ROS, the cells are equipped with enzymatic and nonenzymatic antioxidant systems. The performance of these antioxidant defense systems is associated with seed vigor. Superoxide dismutase (SOD) activity directly modulates the amount of ROS, where it catalyzes the dismutation of superoxide radical ($O_2^{-}\cdot$) with great efficiency, resulting in the production of H_2O_2 (Lin and Kao, 2000). Catalase (CAT) and peroxidase (POD) have been demonstrated to scavenge the H_2O_2 produced by interacting under oxidative stress. CAT reduces H_2O_2 into water and dioxygen, and POD requires a reductant, since it reduces H_2O_2 into H_2O (Noctor and Foyer, 1998). Bailly (2004) proposed a positive relationship between antioxidant enzyme capacity and seed vigor. The detoxification of oxidative damage is an essential aspect for seeds to maintain their quality. Hence, a higher level of antioxidant activity is positively correlated with seed vigor, resulting in germination and stand establishment. It has also been reported that a reduction in SOD, CAT, and glutathione reductase activity yielded higher lipid peroxidation in sunflower seeds, as indicated by increased MDA contents, and was responsible for seed deterioration (Bailly et al., 2008).

Rapeseed-mustard is the third important oilseed crop in the world after soybean (*Glycine max*) and palm (*Elaeis guineensis* Jacq.) oil. Among the 7 edible oilseeds cultivated in India, rapeseed-mustard (Brassica spp.) constitutes 28.6% of the total production of oilseeds. Indian mustard [*Brassica juncea* (L.) Czern & Coss] is one of the most important oilseed crops, which accounts for nearly one-third of the oil produced in India. Although the crop constitutes about 24.02% of the total production of oilseeds in the country, there is the presence of undesirable substances like erucic acid (35.7%–51.4%) in the oil and glucosinolates (49.9–120.3 μmole/g) in the defatted seed meal. Therefore, Indian breeders have developed quality mustard genotypes with low erucic acid (<2%) contents, known as single-zero mustard, and double-zero mustard genotypes with low erucic acid contents in the oil and glucosinolates (<30 μmole/g) in the defatted oilcake. Although these quality mustard genotypes are nutritionally enriched, they have been reported to suffer from low vigor (Swami et al., 2016).

In the present study, it was aimed to determine the possible reasons for differences in the seedling vigor of various types of Indian mustard genotypes. Herein, the ROS contents, antioxidant enzyme activity, and seed quality traits in dry, as well as during the process of germination, in conventional, as well as quality Indian mustard genotypes, were examined.

2. Materials and methods

2.1. Experimental materials

The seeds of Indian mustard were collected from the Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi. These included 3 conventional genotypes: Pusa Bahar, PM 28, and BEC 144; and 6 quality genotypes. Among the quality genotypes, 3 of each were of single-zero: PusaKarishma, PM 24, and PM 30; and double-zero: PDZ 1, PDZ 4, and PDZ 5.

2.2. Germination percent and mean germination time (days)

The germination study of the seeds under different treatments was performed in replicates of 4 sets, each containing 100 seeds, following standard procedures (ISTA, 2015). The first count was taken on day 5 and the final count was taken on day 7. During the period of germination, it was ensured that the paper in the petri dish did not dry out and distilled water was added to the petri dish as required. The evaluation was performed by categorizing them into normal seedling, abnormal seedling, and hard and dead seeds. The average number of normal seedlings was used to calculate the standard germination and expressed as a percentage.

The mean germination time (MGT) was calculated following the method of Nicholas and Heydecker (1968).

The number of seeds germinated each day was noted upto the final count date. Results were calculated with the mean of replicates using the following formula:

MGT=∑ (N*d)/ ∑ (N),

where N is the number of seeds germinated on'd' days and d is the day number.

2.3. Seedling length

On the day of the final count, 10 normal seedlings were randomly selected from the germination test. The length between the collar region and the tip of the primary shoot was measured as the shoot length, and the length between the collar region and tip of the primary root was measured as the root length. The total seedling length (cm) was calculated by adding the shoot and root lengths together.

2.4. Seedling fresh weight

Leaves and cotyledons of 10 normal seedlings, which were used to measure the length, were removed and their weights (g) were recorded.

2.5. Seedling dry weight

After recording the fresh weights (FWs), the seedlings were placed onwax paper and allowed to dry in a hot air oven at 70 ± 1 °C for 48 h. The seedling dry weights (g) were measured after cooling for 30 min in a desiccator with silica gel.

2.6. Vigor indices

Seedling vigor indices were calculated using the formula below, following the method of Abdul-baki and Anderson (1973):

Seedling vigor index-I (SVI-I) = germination (%) × total seedling length (cm).

Seedling vigor index-II (SVI-II) = germination (%) × seedling dry weight (g).

2.7. Estimation of the $O_2^{-}\cdot$ contents

The spectrophotometric assay of the total $O_2^{-}\cdot$ contents was performed following the method of Chaitanya and Naithani (1994) with modifications. First, 1 g of seeds was homogenized in precooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM of diethyl dithiocarbamate. The homogenate was centrifuged at 10,000 g and the supernatant was used immediately for the estimation of $O_2^{-}\cdot$. The superoxide content was measured spectrophotometrically at 540 nm and the result was calculated as DA_{540}/min/gFW.

2.8. Estimation of the H_2O_2 contents

The H_2O_2 content was estimated by following the method of Teranishi et al. (1974) with modifications. First, 1 g of seeds was homogenized in prechilled acetone and filtered through Whatman No. 1 filter paper. The filtrate was mixed with 4 mL of titanium reagent and 5 mL of ammonia solution to precipitate the titanium-hydro peroxide complex. The total reaction mixture was centrifuged at 10,000 g for 15 min and the pellet was further dissolved in 2 M of H_2SO_4 and then recentrifuged. The absorbance of the supernatant was read at 415 nm against a blank and the H_2O_2 content was expressed as μmol g^{-1} FW.

2.9. Lipid peroxidation (TBA reactive substances content)

The lipid peroxidation assay was performed following the method of Heath and Packer, (1968). First, 0.1g of the seed sample was homogenized in 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,600 g for 15 min. To a 1-mL of aliquot of the supernatant, 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated to 95 °C for 30 min in anelectric oven at the laboratory and cooled in an ice bath. After cooling, the aliquot was centrifuged at 10,000 g for 15 min. The absorbance of the clear supernatant was recorded at 532 nm. Values of nonspecific absorption, recorded at 600 nm, were subtracted from the values recorded at 532 nm. The TBA reactive substances content was calculated according to its extinction coefficient at 155 $mM^{-1}cm^{-1}$.

2.10. Histochemical assay for the in vivo detection of $O_2^{\bullet-}$ production

In vivo production of $O_2^{\bullet-}$in the germinated seeds was visualized by incubating them in K-phosphate buffer (10 mM, pH 7.0) containing 1.0 mM of nitroblue tetrazolium for 15 min (Frahry and Schopfer, 2001). The sections were photographed using a Nikon D3500 camera (Tokyo, Japan).

2.11. CAT assay

CAT activity was measured spectrophotometrically at room temperature following the method of Braber (1980). First, 1 g of mustard seeds was homogenized in phosphate buffer (100 mM, pH 7). The homogenate was centrifuged at 15,000 g for 30 min and the supernatant served as the enzyme extract. The reaction was started after adding H_2O_2 into the reaction mixture. The change in absorbance was measured at 240 nm at 15-s intervals for 3 min.

2.12. POD assay

POD activity was determined using guaiacol as the substrate for the assay following the method of Castillo et al. (1984). First, 1 g of mustard seeds was homogenized in phosphate buffer (50 mM, pH 7.0). The homogenate was centrifuged at 15,000 g for 30 min and the obtained supernatant was used as the enzyme extract. In a 3-mL cuvette, the reaction was started after adding guaiacol and H_2O_2,and the change in absorbance was measured at 470 nm at 15-s intervals for 3 min.

2.13. SOD assay

SOD activity was determined by measuring the inhibition in the photo-reduction of nitroblue tetrazolium (NBT) by the SOD enzymes (Kumar et al., 2012). A control reaction was performed without a crude extract and a nonirradiated complete reaction mixture served as the blank. The SOD reaction was performed by exposing the reaction mixture to light from a 15-W fluorescent

bulb for 15 min at room temperature. After 15 min of incubation, the absorbance was recorded at 560 nm using a spectrophotometer. One unit (U) of SOD activity was defined as the amount of enzyme that resulted in 50% inhibition of the photochemical reduction of NBT.

2.14. Statistical analysis

All data were taken in replications of 3. The data were laid out in the Latin square of order m, in an arrangement of m Latin letters, in a square of m rows and m columns, such that every Latin letter occurred once in each row and once in each column, using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Germination, MGT, and seed vigor indices

Seeds carry the full genetic complement of a crop and are therefore the delivery system for agriculture and crop improvement. Therefore, the real value comes with the germination of seeds. The germination of Indian mustard seeds occurred in 24 to 30 h inside of a germinator at 20±2 °C. The percentages of germination of the conventional, single-zero, and double-zero Indian mustard seeds were 96%, 96.44%, and 94.89%, respectively. No significant differences in the germination percentages were observed (Table) among these 3 different types of Indian mustard genotypes. This could have been because of the fact that the seeds had been freshly harvested and negligible deterioration might have occurred. However, under similar germination conditions, the average MGT of double-zero Indian mustard seeds (1.74 days) was significantly higher than those of the conventional and single-zero seeds. The conventional and single-zero mustard was statistically similar in terms of the average MGT (Table). The average MGT for the single-zero mustard seeds was 1.24 days and for the conventional mustard seeds, it was 1.41 days.

Although there were no significant differences among the various types of Indian mustard in terms of germination, they showed significant differences in terms of inherent seed vigor, which actually reflected the real potential of the performance of seeds under varied environmental conditions. The seed vigor indices provided a better prediction of field emergence than the germination percentage of the seeds under laboratory conditions alone. The single-zero mustard genotypes exhibited a significantly higher SVI-I (1401.70),which was statistically similar tothat of the conventional mustard genotypes (1318.16). Similarly, in terms of the SVI-II, both the conventional (1.36) and single-zero (1.29) mustard genotypes were statistically similar (Table), whereas the double-zero Indian mustard genotypes were significantly lower than the conventional and single-zero genotypes in terms of both the SVI-I (1067.96) and SVI-II (1.06).

3.2. $O_2^{-}\cdot$ content and its localization

The seed germination period was accompanied by an increase in superoxide content. The mean superoxide content, in both the dry seeds and the germinated seeds, varied among the different types of Indian mustard (Figure 1). In the dry seeds, the double-zero mustard was observed to have significantly higher mean superoxide content (0.154 OD/min/gFW) than the conventional mustard (0.067 OD/min/gFW). The $O_2^{-}\cdot$ content in the conventional genotypes was similar tothat of the single-zero genotypes. In the germinated seeds, the superoxide content was significantly increased in the conventional mustard (0.320 OD/min/gFW), followed by the single-zero (0.239 OD/min/gFW), and it was the lowest in the double-zero mustard (0.204 OD/min/gFW). Thus, with germination, a burst of superoxide generation was observed and the trend was reversed in the dry seeds. The conventional mustard showed a 375.85% increase in superoxide content from the dry to germination state, followed by the single-zero mustard (159.12% increase), which was significantly higher than in the double-zero mustard (32.17% increase).

Table. Seed quality parameters of the different types of Indian mustard.

Types of mustard	Germination (%)	MGT (days)	SVI-I	SVI-II
Conventional	96.00 (78.76)*[a]	1.41[a]	1318.16[b]	1.36[b]
Single-zero	96.44 (79.35)[a]	1.24[a]	1401.70[b]	1.29[b]
Double-zero	94.89 (77.16)[a]	1.74[b]	1067.96[a]	1.06[a]
Mean	95.78 (78.42)	1.46	1262.61	1.24
CD type	NS	0.18	97.43	0.09

NS: Nonsignificant.

*Figures in parenthesis are the arcsine transformed values of the percent data. Means followed by the same letter in different rows of various parameters did not differ statistically from each other according to the Duncan homogeneous subsets test(statistical significance was accepted as $P \leq 0.05$).

The deposition of $O_2^{-}\cdot$ was visualized with NBT, which formed a dark blue formazan precipitate upon contact with superoxide (Bielski et al., 1980). NBT staining could not be detected on the testa, but it strongly accumulated after radicle protrusion in the growing tip of the radical,where superoxide was probably necessary for cell division and root elongation (Figure 2) (Dunand et al., 2007).

3.3. H_2O_2 content

The H_2O_2 content, just like the superoxide content, was higher in the dry seeds of the double-zero genotypes than in those of the conventional and single-zero genotypes . This showed that the cumulative increase of ROS in the dry seeds during storage enhanced deteriorative changes due to oxidative damage. As a result, there was early loss

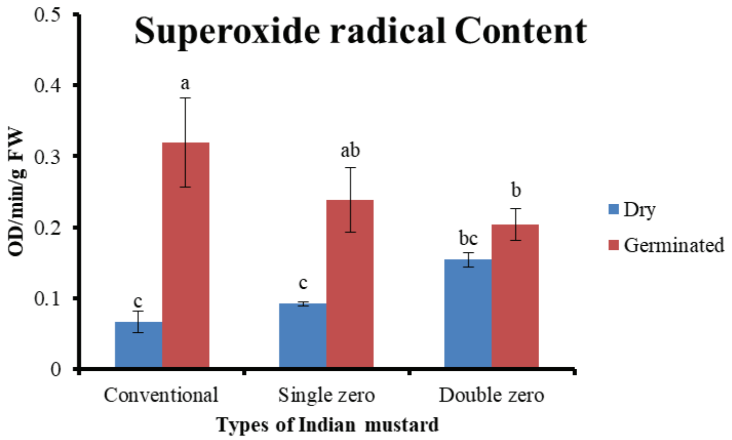

Figure 1. $O_2^{-}\cdot$ contents (OD/min/gFW) of the dry and germinated seeds of the different types of Indian mustard seeds (values are given as the mean±SEM of 3 genotypes in each group).

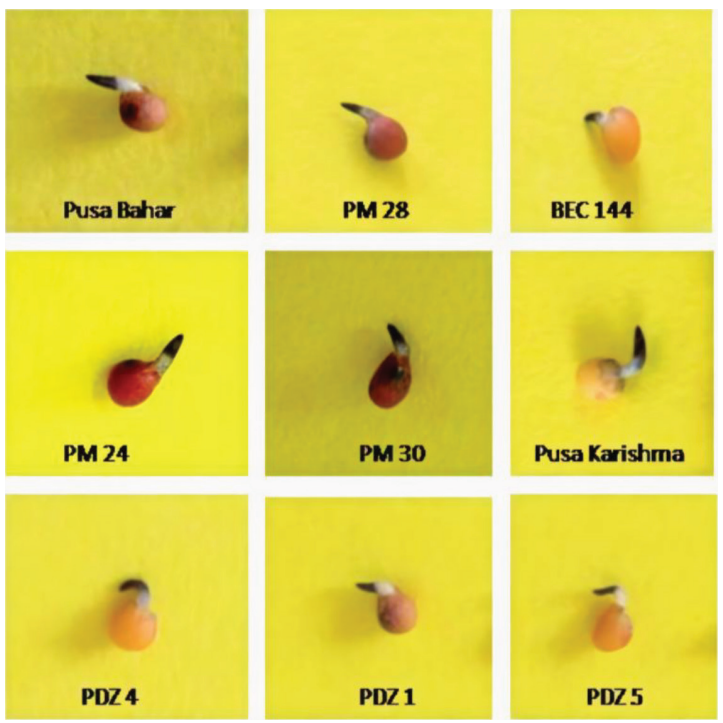

Figure 2. Production of $O_2^{-}\cdot$ in the different types of Indian mustard seeds visualized by NBT staining (the darker and more pronounced formazan accumulation in: Pusa Bahar, PM 28, and BEC 144; Pusa Karishma, PM 24, and PM 30. Lighter and less pronounced formazan accumulation in double-zero; PDZ 1, PDZ 4, and PDZ 5).

of seed viability in the double-zero genotypes. During germination, the H_2O_2 content was highest in the single-zero genotypes, which was statistically similar to that of the conventional genotypes, while it was lowest in the double-zero genotypes (Figure 3) The increase in H_2O_2 content from the dry to germinated stage was 47.61% in the conventional genotypes and 37.05% in the single-zero genotypes. However, the lowest increase, of 6.37%, in the H_2O_2 content was observed in the double-zero genotypes.

3.4. Lipid peroxidation

Lipid peroxidation reflects the level of oxidative damage in seeds. The dry seeds of the double-zero genotypes had the highest lipid peroxidation, which was statically similar to that of the single-zero genotypes, whereas the seeds of the conventional genotypes had a low level of lipid peroxidation (Figure 4). There was an increase in lipid peroxidation with germination. This increase was highest in single-zero genotypes, followed by the double-zero genotypes. Conventional genotypes had the highest increase in lipid peroxidation. This increase was highest in the single-zero genotypes (41.14%), followed by the conventional genotypes (37.90%), which had an increase of 1.41- and 1.38-fold, respectively.

3.5. SOD activity

There was no significant difference observed in the SOD activity of the dry seeds among the 3 types of Indian mustard; however, during germination, the SOD activity increased in all of the Indian mustard seeds (Figure 5). In the germinated seeds, the SOD activity was highest in the conventional mustard (6.122 U/min/gFW). It increased by 1.35 times more than the activity in the dry seeds. The SOD activity in the single-zero mustard was 5.792 U/min/gFW, which was 1.13 times more than that in the dry seeds. The lowest SOD activity was recorded in the germinated seeds

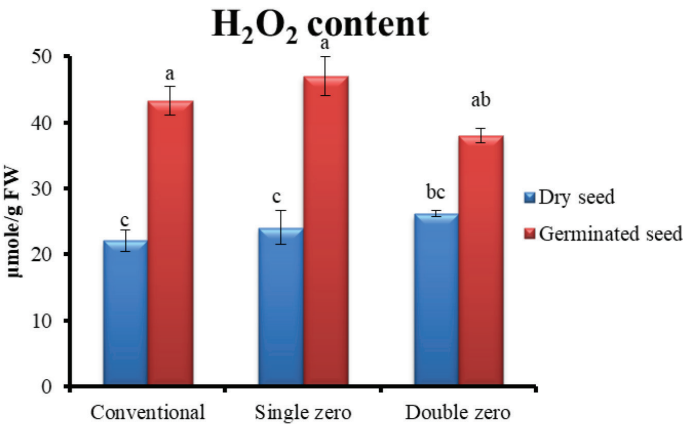

Figure 3. H_2O_2 contents of the dry and germinated seeds of the different types of Indian mustard (values are given as the mean±SEM of 3 genotypes in each group).

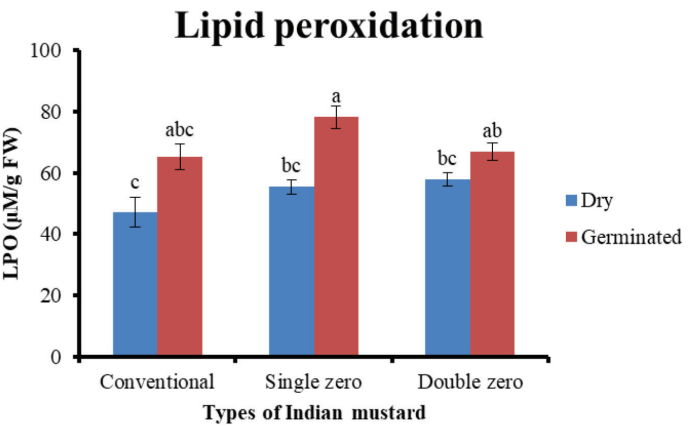

Figure 4. Lipid peroxidation of the dry and germinated seeds of the different types of Indian mustard (values are given as the mean±SEM of the 3 genotypes in each group).

of the double-zero mustard (5.218 U/min/gFW). However, the increase in SOD activity was found in germinated seeds over dry seeds. This SOD activity increase in the double-zero genotype was 4.03 times lower than that in the conventional Indian mustard during germination.

3.6. CAT activity

Differences in the CAT activity were observed in the germinated seeds of the 3 types of Indian mustard (Figure 6). In the dry seeds, no significantly different higher value was found in the conventional mustard (0.109 µM/min/gFW). The CAT activity in the single-zero mustard was between that of the conventional and double-zero mustard. A sharp increase in CAT activity was found in the germinated seedsofall of the Indian mustards. However, during germination, the highest CAT activity was recorded in the conventional mustard (0.389 µM/min/g FW), which was 256.9%higher than that in the dry seeds. These increases were 1.01- and 1.08-fold higher than the increase in the dry seeds of the single-zero and double-zero genotypes, respectively. In the single-zero mustard, the CAT activity (0.335 µM/min/gFW) was similar to that of the conventional mustard. The lowest enzyme activity was observed in the germinated seeds of the double-zero mustard.

3.7. POD activity

POD activity was also found to be significantly different in the various types of Indian mustard. The dry seeds of the conventional mustard exhibited the highest POD activity (0.272 µM/min/gFW), followed by the single-zero (1.288 µM/min/gFW), and double-zero (0.511 µM/min/gFW) Indian mustard (Figure 7). Like the CAT activity, increased POD activity was observed during germination of the various types of Indian mustard genotype seeds. However, in the germinated seeds, the highest POD activity was

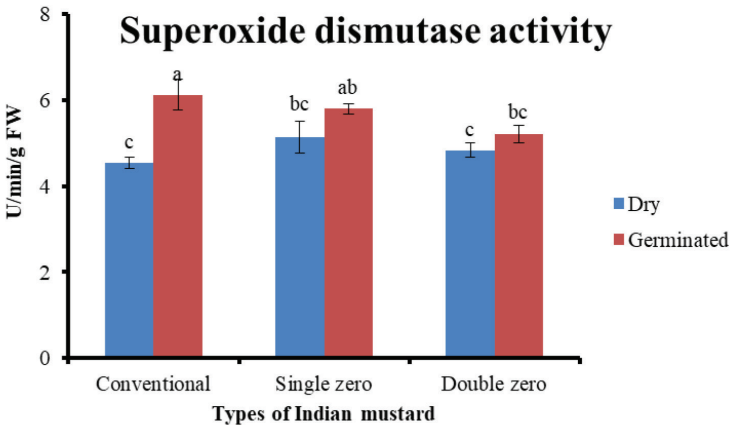

Figure 5. SOD activity (U/min/gFW) of the dry and germinated seeds of the different types of Indian mustard seeds (values are given as the mean ± SEM of the 3 genotypes in each group).

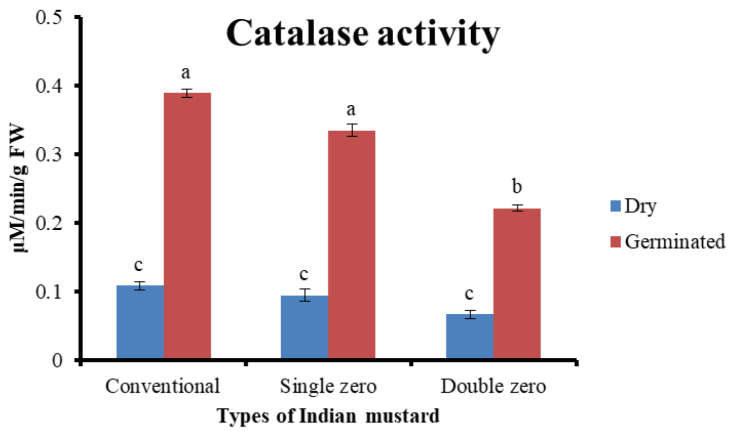

Figure 6. CAT activity (µM/min/gFW) of the dry and germinated seeds of the different types of Indian mustard seeds (values are given as the mean±SEM of the 3 genotypes in each group).

observed in the conventional mustard (5.297 µM/min/gFW), which was 0.8- and 1.23-fold higherthan that ofthe single-zero and double-zero mustard, respectively.

4. Discussion

Seed quality is affected by the different constituents of the seed. In general, seeds are considered to be of high quality when they exhibit fast and homogeneous germination. Seed germination is the most tangible manifestation of seed quality; however, is not closely related to field emergence, particularly under unfavorable environmental conditions. Thus, the development of the seed vigor concept is a more promising seed quality character to reflect potential seed germination, field emergence, and seed storage ability under different conditions than standard germination. There were no significant differences in germination among the types of Indian mustard under laboratory conditions. This may have been because of the freshly harvested seed lot of all of the genotypes that were used in study;hence, no or negligible deterioration might have occurred. One of the early manifestations of seed aging is an increase in the MGT. The MGT is the time taken by the seeds to germinate. It can be said that it is a measure of the rate and time-spread of germination, and it is widely used by seed scientists (Soltani et al., 2016). Rapid and uniform seed germination is a crucial prerequisite for crop establishment and high yield levels in crop production. A lower MGT indicates the high and uniform speed of germination of the seeds. The MGT has a strong negative correlation with germination (r=–0.952**) as well as the SVI-I (r=–0.680*). The MGTs of the conventional and single-zero genotypes were significantly lower, which may have been attributed by their higher SVI-I and SVI-II. The double-zero Indian mustard was observed to have a higher MGT (1.74 days) and low SVI-I and SVI-II, which required a longer preradical protrusion period that may have been due to it having a less equipped repairing mechanism in place. Asimilar relationship of the SVI and MGT was also reported by Grahn (2015) in lettuce.

When seeds are stored, they undergo different degrees of damage that have detrimental effects on their quality. Therefore, accurate prediction of the quality and viability levels of a seed lot is of high importance in the seed-producing industry.The production and accumulation of ROS depend on the metabolic and physiological state of the seeds. In dry seeds, ROS are synthesized in a nonenzymatic reaction. Uncontrolled accumulation of ROS is highly toxic for the cell, as ROS are capable of reacting with the majority of biomolecules, which results in oxidative stress that can become irreversible and cause cellular damage. This affects seed germination and vigor. The double-zero mustardexhibited a higher superoxide and H_2O_2content in the dry seeds. This higher $O_2^{-}\cdot$ content was possibly due to the conversion of oleic acid into linoleic acid,since the erucic acid content was less than 2%. At the same time, the activity of the ROS scavenging enzyme, POD, was also low. They also had a higher lipid peroxidation value, resulting in the accumulation of MDA. High lipid peroxidation occurs as a result of the attack of ROS on polyunsaturated fatty acids, leading to seed deterioration and reduced viability. The accumulation of MDA during aging has been reported in several species, including sunflower (Kibinza et al., 2006), wheat (Lehner et al., 2008), cotton (Goel et al., 2003), and soybean (Sharma et al., 2013), during artificial and natural aging. However, this trend was reversed in the conventional and single-zero genotypes (Figure 8). The POD activity showed a positive correlation with the SVI-II and the $O_2^{-}\cdot$ content was observed to have a negative

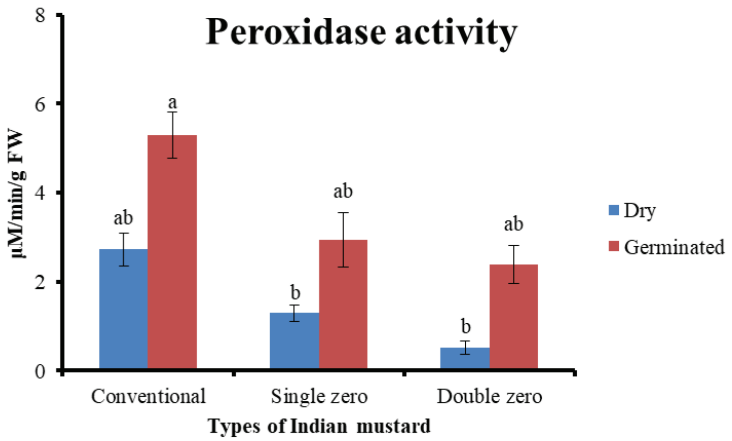

Figure 7. POD activity (µM/min/gFW) of the dry and germinated seeds of the different types of Indian mustard seeds (values are given as the mean±SEM of the 3 genotypes in each group).

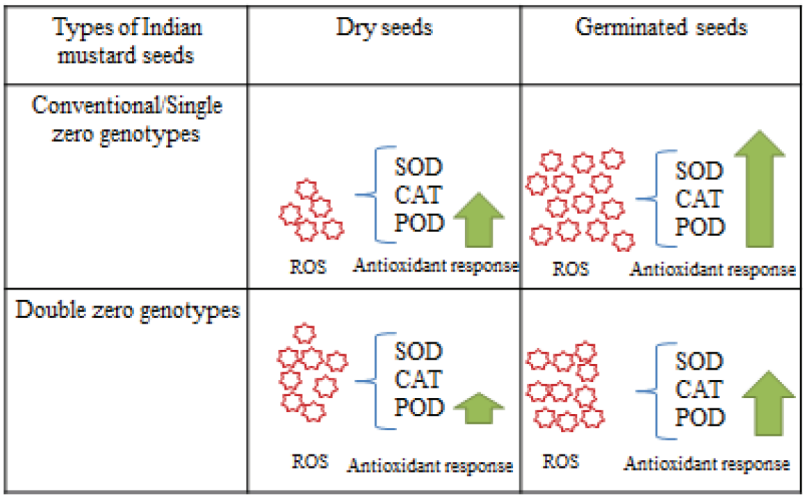

Figure 8. Proposed model for explaining differences in the seed vigor of the double-zero genotypes when compared to the conventional and single-zero genotypes based on the $O_2^{-}\cdot$ contents and antioxidant enzymes.

correlation with the SVI-I. Similar results were obtained by Balesevic-Tubic et al. (2007), Oracz et al. (2007), Varghese and Naithani (2008), and Parkhey et al. (2012), wherein ROS induced the oxidative damage of proteins and lipids. Membrane damage and the generation of toxic byproducts are common features of lipid peroxidation. The increase in ROS production and accumulation in the mitochondria of aging seeds reduces antioxidant system activity. Other researchers have also observed a decrease in the ascorbic-glutathione cycle activity and enzymatic antioxidant activity (Kurek et al.,2019). Such oxidative damage led to severe cellular damage that eventually resulted in the loss of viability and vigor in several types of seeds (Halliwell and Gutteridge, 2007; Varghese and Naithani, 2008; Sahu et al., 2017). Hence, the higher ROS content and lower antioxidant defense system activity in the double-zero genotypes when compared to the conventional and single-zero genotypes was possibly the reason for their lower seed vigor, and, in the long run, it may lead to a reduction in the storability of the double-zero genotypes, as also reported by Swami et al. (2016).

The process of seedling emergence from the seed embryonic axis is known as germination (Bewley and Black, 1994). The major sources of ROS production during germination are due to the resumption of respiration by the mitochondria (Noctor et al., 2007), and this can also lead to electron leakage and the increased production of ROS (El-Maarouf-Bouteau and Bailly, 2008). There have been several reports describing the involvement of H_2O_2 and $O_2^{-}\cdot$ in the early imbibition period, which have indicated their signaling roles during seed germination (Puntarulo et al., 1991; Caro and Puntarulo, 1999; Schopfer et al., 2001). The early increase in the superoxide content indicated its physiological role in contributing to the germination of the seed and seed vigor (Biswas et al., 2017). Dunand et al. (2007)reported that the burst of ROS generation during germination may possibly act as a factor to loosen the cell wall and other barriers to the embryo, and aid in the protrusion of radicals, thereby helping in the early stages of germination, and embryo and seedling growth. The higher $O_2^{-}\cdot$ content in the germinated seeds of the conventional and single-zero mustard might have triggered a set of sequential cellular events in various subcellular compartments, whose completion was associated with the realization of germination, as also reported by Leymarie et al. (2011). Whereas, in the germinated seeds of the double-zero mustard, the burst of superoxide generation was the lowest, which may not have been sufficient for the early breaking of the seed coat and protrusion of radicals. This could possibly have resulted in the longer MGT in the seeds and thus, more time was taken for recovery from any damage during germination of the double-zero in comparison with other types of Indian mustard. Moreover, the lower $O_2^{-}\cdot$ content in the double-zero mustard was insufficient to stimulate other physiological events required for germination. The conventional and single-zero genotypes had low MGTs with high H_2O_2 contents during germination. This increase in the H_2O_2content was perhaps associated with the reserve mobilization to support embryo growth (Diaz-Vivancos et al., 2013). It has been reported that H_2O_2 would not only decrease the abcisic acid (ABA) content in amitogen-activated protein kinases-dependent manner, but also increase the carbonylation of seed storage proteins, favoring their mobilization. Moreover, it could also stimulate the phosphate pentose pathway by activating some glycolytic enzymes that, in

turn, could provide NADPH for the thioredoxin system, which is involved in seed germination and seedling development. Additionally, H_2O_2 could, either directly or indirectly, impair ABA transport from the cotyledon to the embryo, causing a decrease in ABA and thus, stimulate the germination process (Barba-Espín et al., 2012). The increase in lipid peroxidation with germination can be correlated with the accumulation of ROS production with germination (Jaleel et al., 2007).

ROS are kept in check by antioxidant enzymes. The increased SOD, CAT, and POD activities in the germinated seeds in all of the Indian mustards reflected that increased antioxidant enzyme activity was required for the seeds to counter effect or buffer the deleterious activities of ROS (Verma et al., 2015). The SOD content was found to be significantly different during seed germination, and similar results were observed by Bogdanović et al. (2008), who reported that SOD activity was significant at the final stages of germination and early seedling development of *Chenopodium murale*. The increased CAT and POD activity in the conventional and single-zero mustard during germination conferred the superiority in seed vigor and other seed quality attributes. Alternatively, CAT also plays a key role in H_2O_2 removal during fatty acid oxidation in glyoxysomes (Olsen and Harada, 1995). Therefore, high CAT activity could be associated with better mobilization of lipid reserves and faster seedling development (Bailley et al., 2007). The resultswere thus in accordance with the earlier reported results of involvement of CAT in the germination and early growth of sunflower seedlings; thus, suggesting that the control of H_2O_2 homeostasis is an important event in the expression of seed vigor. Hence, the ability to increase SOD, CAT, and POD activity in the conventional and single-zero genotypes of Indian mustard is essential for maintaining the balance between the formation and degradation of free radicals that are potentially harmful to seed homeostasis.

However, in the double-zero mustard, the SOD, CAT, and POD activities were lower in the germinated seeds, which could not prevent deleterious events, such as lipid peroxidation caused by the increased ROS content. As a result, the seeds gradually accumulated oxidative damage. ROS oxidize lipids and inactivated enzymes damage the structure and function of proteins and carbohydrates, and modify or disrupt the DNA structure. Lipid peroxidation also modifies membrane permeability, which affects the decrease in seed viability,which could have been the reason for the poor seed vigor in the double-zero genotypes (Figure 8).

5. Conclusion

The present study on seed quality parameters, ROS, lipid peroxidation, and antioxidant enzymatic activity revealed that both ROS and antioxidant enzyme activity were important for different physiological activities, and their balance was especially required for maintaining the planting value of seed, i.e. seed vigor indices. The conventional and single-zero Indian mustard had higher seed vigor indices, whereas the double-zero mustard had a lower SVI-I and SVI-II. This study proposed that a higher accumulation of ROS, leading to more oxidative damage and lower antioxidant activity, in the dry seeds, could have been the cause of low vigor during storage of the double-zero genotypes. On the other hand, the lower burst of ROS and insufficient increase in antioxidantscould have been the possible reasons for the lower vigor in the double-zero mustard seeds during germination. Thus, the ROS content, lipid peroxidation, and antioxidant enzymes can explain the differences in seed vigor of the various types of Indian mustard.

Acknowledgments

The authors are thankful to the Indian Council of Agricultural Research, New Delhi, for providing the required funds for smooth conduct of the research work and the Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi, for providing the necessary facilities for the studies.

Conflict of interest

The authors declare that they have no conflicts of interest.

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