

Full Length Research Paper

## Effect of lipid peroxidation and related parameters on the storability of soybean (*Glycine max*) seeds

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On the basis of reported storability and seed germination thirteen soybean [*Glycine max* (L.) Merrill] genotypes (grouped as six “good storers” and seven “poor storers”) were selected to understand the relationship between lipid peroxidation, antioxidant activity and seed storability. Good storers possessed significantly high activity ( $p < 0.05$ ) of LOX-1 and lower activity of LOX-2 as compared to poor storers. Significant increase ( $p < 0.05$ ) in HPL activity was observed in all poor storer genotypes and correlated with higher accumulation of lipid peroxides, total MDA and carbonyl content. Further our results indicated that, the good storers possessed high antioxidant activities when analyzed through DPPH and CUPRAC method than the poor storers, however the activity of antioxidant enzymes viz., SOD and catalase remained unchanged in both the good and poor storers with an exception of a good storer genotype - M1090. Although no significant difference in the antioxidant compounds like tocopherols and isoflavones were found between the good and poor storers, an increased ascorbic acid content was however observed in the good storers (8.5 mg/100g to 14.74 mg/100g) as compared to the poor storers (4.82mg/100g to 6.84 mg/100g). Thus our study reflected the possible role of ascorbic acid, LOX-1, LOX-2 and HPL enzymes as potential indicators to determine the storability of soybean seeds and also can be used as the parameters to improve their nutritional quality of the soybean seeds.

**Key words:** Soybean, storability, antioxidant enzymes, tocopherols, lipid peroxides.

### INTRODUCTION

Soybean (*Glycine max* L. Merrill) is one of the major crops grown in the world with 250 million tonnes of global production and possesses a unique combination of very high protein (40%) and oil (20%) content. Soybean has been regarded as significant ‘functional food’ as it contains several phytochemicals such as vitamin E, phenolics including phenolic acids, flavonoids and isoflavonoids with putative health benefits (Faraj and

Vasanthan, 2004). However, soybean seed quality deteriorates faster particularly under warm and humid climatic conditions, as prevalent in India. Seed deterioration also takes place when seeds are subjected to prolonged storage, but the exact cause of seed deterioration or loss of seed viability is still unknown. In the presence of oxygen, aging of seed can lead to peroxidative changes in the polyunsaturated fatty acids (Stewart and Bewley, 1980; Wilson and McDonald, 1986) and this free radical-induced, non-enzymatic peroxidation has the potential to damage membranes thereby causing the deterioration of the stored seeds (Sung and Jeng,

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1994). Alternatively, lipoxygenase (LOX) mediated pathway operating in many un-imbibed seeds, in which LOX is capable of catalyzing lipid peroxidation by acting on membrane phospholipid components such as linolenic and linoleic acid as its substrates (Priestley, 1986; Wang et al. 1990) also leads to the formation of hydroperoxides by addition of molecular oxygen to these fatty acids having cis, cis, 1,4 pentadiene motif (Feussner and Wasterneck, 2002) and these hydroperoxides produced act as a substrate for hydroperoxidelyase (HPL) -the second most important enzyme in LOX pathway, catalyzing the formation of aldehydes such as 3-2-nonenal and 3-2,6-2- nonadienal (Fauconnier et al., 1997) and  $\omega$ -oxo acids [9-oxo-nonanoic acid]. These medium chain aldehydes have been reported to be mainly responsible for the poor storability as well as poor seed germination potential of soybean (Gardner et al., 1990). Normal soybean seeds contain 3 lipoxygenase isozymes, LOX-1, LOX-2 and LOX-3 which differ in substrate specificity, optimum pH for catalytic activity, isoelectric point and thermal stability (Gardner et al., 1990; Matsui, 2006). In soybean seeds, LOXs are abundant proteins that constitute 1-2% of the total protein content (Loiseau et al. 2001). The enzymes are involved in the production of volatile compounds (such as n-hexanal) associated with grassy- beany and rancid off-flavors in soybean and soy foods (Robinson et al. 1995). Foods made from soybean lacking LOXs generate less hexanal than do those with normal LOX activities (King et al. 2001).

Many researchers (Wilson and McDonald, 1986; Kumar, 2005; Sung and Chiu, 1995; Sung, 1996) have established a well-known correlation between lipid peroxidation and seed deterioration & seed storage by quantifying LOX enzymes, lipid peroxide accumulation and various other parameters. Sung and Jeng (1994) have shown increased lipid peroxides in the form MDA (malondehyde) formation in peanut seed subjected to accelerated aging process, in addition to increased MDA (malondehyde) content, high activity of LOX-I and LOX-2 have also been reported in the bold and yellow-seeded poor-storer varieties than the black and small-seeded good-storers (Kumar, 2005).

Lipid peroxidation process in the cell can be minimized by antioxidant systems (Halliwell and Gutteridge, 1999) such as isoflavones, tocopherols and ascorbic acid by involving in different redox reactions. The antioxidant property of the isoflavones (genistein and daidzein) in soybean plays a major role in inhibiting the lipid peroxidation by scavenging both hydrogen peroxide and superoxide anions and protects the seeds against the action of free radicals, thus diminishing the deterioration effects and consequently aiding the maintenance of seed physiological quality (Avila et al., 2012; Lee et al., 2005).

Tocopherols are the important antioxidant molecules present in the PUFA (polyunsaturated fatty acid) enriched membranes of many organelles such as chloroplasts,

leucoplasts and oil bodies of seeds, which plays an important role in protection of photosynthetic thylakoids membranes and Seed triacylglycerols (TAGs) which are stored as major carbon reserves (Smirnoff, 2010). Tocopherols scavenge the lipid peroxy radicals and yield a tocopheroxyl radical that can be recycled back to the corresponding tocopherol by reacting with ascorbate or other antioxidants (Liebler, 1993). The protective mechanism within the seeds also involves several free radical scavenging enzymes such as superoxide dismutase (SOD), catalase and peroxidase (Sung, 1995). The decrease of antioxidant enzymes is linked to increase on peroxidation of lipids as well as to accelerated aging process, with a positive correlation between antioxidant capacity of the enzyme and the vigor of seeds (Bailey et al., 2002). The coordination between catalase and SOD activities are detrimental for the growth of the radicle as well as in the maintenance of the seed viability (Gidrol et al., 1994). Storage of seeds for a long time decreases the germination percentage drastically in case of peanuts resulting in their poor viability (Swain et al., 2001). Previous studies have reported that, the black-seeded tropical landraces of soybean such as T-49, Birsa-1 and Kalitur have been found to have better storability than the yellow-seeded temperate varieties such as Clark-63, Lee and Bragg (Kueneman and Wein, 1981; Kuchlan 2006).

To address the correlation between lipid peroxidation and seed storability, we have determined LOX and HPL enzyme activities, radical scavenging enzyme activities, level of antioxidants and other lipid peroxidation parameters in the selected thirteen soybean genotypes which were grouped as "good storers" and "poor storers" with aim to identify the parameters influencing the seed storability and viability.

## MATERIALS AND METHODS

### Plant materials and seed storage

Thirteen soybean germplasm lines (7 yellow seeded and 6 black seeded) were obtained from Division of Genetics, Indian Agricultural Research Institute, New Delhi. The genotypes were selected based on the preliminary work conducted by earlier workers (Hosamani et al., 2013; Kumar, 2005; Dr. S.K. Lal, unpublished) wherein, soybean genotypes were categorized into good, medium and poor storers. In order to validate the given soybean genotypes as poor and good storers, 100 g of soybean seeds were packed in brown coloured paper bags (after drying the seeds to an average moisture content of 8%) and stored under uncontrolled conditions in the laboratory with an average relative humidity of  $60 \pm 10$  % (max 90 % and min 14 %) and temperature of  $25 \pm 2$  °C (max 38°C-40 °C and min 8°C-13 °C) upto 12 months; samples were analyzed for germination test at the start of experiment

and 1 year after storage.

### Germination test

Germination was tested at 25°C in three replications of 50 seeds each following the between-paper method given by ISTA 2008 and seeds were evaluated for germination percentage corresponds to the mean percentage of normal seedlings obtained after 10th day of germination.

### Lipoxygenase (LOX) assay

LOX activity was assayed by a modified method shown by Axelord, 1981 and the time course of the peroxide formation was measured by increase in the absorbance at 234 nm. One gram of overnight-soaked (distilled water) soybean seeds were homogenized in 6 ml ice-cooled 0.1M Phosphate buffer (pH 6.8) using pestle and mortar. The homogenate was allowed to stand for 10 min at 4°C and centrifuged at 15000 g for 30 min at 4°C; the supernatant was kept at 4°C for investigations. Substrate for LOX-1 was prepared by adding 35µl of linoleic acid to 5 ml of double distilled water containing 50 µl Tween-20, then the linoleic acid was dissolved by adding 600µl of 0.5 M NaOH to the above solution and the final volume made up to 100 ml using 0.2M Boric acid borax buffer (pH 9.0). To 2.99 ml of substrate, 10µl of supernatant was added and the increase in OD at 234 nm was measured for 6 min. at the room temperature. For LOX-2 activity, substrate was prepared in the same way except that 0.2M phosphate buffer pH6.8 was used in place of borate buffer. For LOX-3 activity, 0.1M Phosphate buffer (pH 7.1) was used for assay and the activity was measured at 280 nm for 6 min at the room temperature.

### Hydroperoxidelyase (HPL) assay

HPL was assayed as method shown by Vick , (1991). The enzyme extract was prepared by stirring 1g of soya flour with 50mM phosphate buffer, pH 7.0, for 2hrs followed by centrifugation at 20000 g for 30 min. at 4°C; the resultant supernatant was filtered by passing through two layers of muslin cloth. Enzyme activity was determined by adding 2600µl of assay mixture (prepared by mixing 50ml of 0.1mM potassium phosphate buffer (pH 6.0) 3.5mg of NADH and 6.6mg of Yeast alcohol dehydrogenase), 200µl of substrate - fatty acid hydroperoxide and 200µl of enzyme extract. The change in absorbance was followed at 340nm at 30 sec. intervals for 6 min. HPL activity was expressed as  $\Delta OD/min/mg$  of protein; protein concentrations were determined by the Lowry's, (1951) method using bovine serum albumin as a standard extract.

### Lipid peroxidation

Soybean seeds were powdered and lipid peroxidation was determined by estimating malondehyde (MDA) content based on the methods developed by Ohkawa, (1979) and modified by Hodges,(1999). Powdered tissue sample (0.2g) was macerated in 1.5ml. of 10% trichloro acetic acid, the homogenate centrifuged at 16000 rpm for 15 min. and the supernatant was used as extract. 200µl of the extract received 200µl of SDS (8.1%) and 1.5ml of 20% acetic acid and after adjusting pH to 3.5, the volume was made up to 4ml. with distilled water. The mixture was heated at 95°C for 60 min. and cooled rapidly on ice; after adding 1ml of distilled water, the mixture was filtered through whatman No.1 filter paper and 5ml of n-butanol and pyridine (15:1 v/v) was added into the filtrate and vortexed; the mixture was then centrifuged at 10000 rpm for 10 min. The absorbance was measured at 532nm and lipid peroxides were expressed as nmoles MDA/g fresh weight.

### Carbonyl value

The carbonyls were determined as 2, 4-dinitrophenylhydrazone derivatives, as described by Henick's method (1954). Five ml of 2, 4-nitrophenylhydrazine (in 25% HCl) and 5 ml. of soybean extract were added in a volumetric flask and incubated for 30 min. at 60°C. After cooling, 10 ml. of 4% KOH solution was added; resulting black colour was rapidly cleared to the characteristic wine red. The absorbance was measured at 480 nm, after 10 min., against a blank prepared in the same manner, using 5 ml. of double distilled water in place of the sample; calculations were made using the average value of  $E_{max}$  2.72  $\times 10^4$  at 480 nm.

### Ferrous oxidation-xyleneol (FOX) assay

FOX version2 assay is used to quantify lipohydroperoxides (LOOHs) in various tissue extracts. LOOHs oxidize ferrous (Fe<sup>2+</sup>) ions to ferric (Fe<sup>3+</sup>) ions which bind to ferric-sensitive dye, xyleneol orange, yielding an orange purple complex (colour depends on the abundance of -OOH) that can be measured at 560nm. The FOX assay for determining lipid peroxides was performed as described by Griffith et al., (1997). FOX-2 reagent was prepared according to Delong et al., (2002) by mixing 1mM xyleneol orange and 2.5mM ammonium ferrous sulphate in 250mM H<sub>2</sub>SO<sub>4</sub>. One volume of this concentrated reagent was added to 9 volumes of HPLC grade methanol containing 4.4mM BHT to make a working reagent (250µM ammonium ferrous sulphate, 100µM xyleneol orange, 25mM H<sub>2</sub>SO<sub>4</sub> and 4mM BHT in 90% (v/v) methanol). Soybean seed extract was prepared

by homogenizing 0.2g. of seed powder with pestle and mortar containing 1ml. of acetic acid (0.15M) and 100ml chloroform/methanol (1:2 v/v); the mixture was shaken for 5min and then centrifuged at 3000 rpm for 10 min. The residue was re-suspended in either 100 $\mu$ l of HPLC grade methanol or 100 $\mu$ l of 2.5mM TPP in methanol and incubated at room temperature for 30 min. in dark. FOX-2 reagent was added and the absorbance at 560nm was recorded exactly 10 min. after reagent addition. Hydro peroxide values were determined using a molar absorption coefficient derived for standard linoleate – hydroperoxide ( $\epsilon= 6.0 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ ) as given by Gay et al., (1999).

### Total antioxidant capacity - CUPRAC method

Cupric reducing-antioxidant capacity (CUPRAC) method is based on the ability of antioxidants (polyphenols, Vit-C and Vit-E) utilizing the copper (II)-neocuproine (CU (II)-NC) reagent as chromogenic oxidizing agent (Apak et al., 2008). The oxidizing agent was prepared by mixing 0.01M of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  and 0.075M neocuproine stock solution with 2.5mM ammonium acetate buffer. The reaction mixture was diluted with ethanol. Sample extract was prepared by centrifuging overnight-soaked seed powder (with acetone) at 3000rpm for 10min; the resultant supernatant (100 $\mu$ l) was mixed with 1 ml. of each of Cu(II), alcoholic neocuproine and ammonium acetate buffer (pH-7) and final volume made up to 4.1 ml. with ethanol. The mixture was incubated for 30 min. and the absorbance read at 450nm. The antioxidant capacity is expressed as Trolox equivalent ( $\mu\text{mol}/\text{TE/g}$ ) using the formula,

Where,  $V_{\text{initial}}$  = initial volume ;  $m$  = weight of sample;  $r$  = dilution factor;  $V_f$  = final volume;  $V_s$  = volume of aliquot;  $A_f$  = absorbance;  $\epsilon_{\text{TR}} = 1.67 \times 10^4 \text{Lmol}^{-1}\text{cm}^{-1}$ .

### DPPH radical scavenging activity

The antioxidant activity was determined as the scavenging activity of stable DPPH free radicals by the method of Mellors and Tappel, (1996) with few modifications. Sample extract was prepared as described for CUPRAC assay. 0.1ml. of extract solution was added to 3ml. of 0.004% (w/v) DPPH solution in ethanol (95%). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517nm until reaction reached a steady state. The DPPH radical scavenging activity (S %) was calculated using the following equation.

$S\% = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$ , where  $A_{\text{control}}$  is the absorbance of the blank control (containing all reagents except the extract solution) and  $A_{\text{sample}}$  is the absorbance of the test sample.

### Assay of superoxide dismutase (SOD)

The activity of SOD was assayed by measuring the SOD inhibition of photochemical reduction of NBT (Machly and Chance, 1959). Each 7ml. reaction mixture contained 50mM phosphate buffer (pH 7.0) 200mM methionine,

1.125mM NBT, 1.5mM EDTA, 75 $\mu$ M riboflavin was added at the end, after the samples were incubated for 10 min. under 15W fluorescent lamp to reach a constant temperature of 30 $^{\circ}\text{C}$ . The reaction was run for 15 min. and stopped by switching the light off and absorbance was recorded at 560nm. A mixture without extract was used as a control and dark control mixture served as blank. The activity was calculated as described by Giannopolitis and Ries, (1977),  $\text{SOD units/ml} = (V/v-1)$  (dilution factor), where 'V' is the rate of reaction without enzyme and 'v' is the rate of reaction in the presence of enzyme. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of the initial rate of the reaction.

### Assay of peroxidase

Guaiacol peroxidase activity was measured as described by Machly and Chance, (1959). In 3ml. of reaction mixture containing 100mM phosphate buffer pH 6.1, 96mM guaiacol and 100 $\mu$ l of enzyme extract; 0.5ml of  $\text{H}_2\text{O}_2$  (12mM) was added into the reaction mixtures and change in the absorbance was measured at 470nm at 15 second intervals for 3 min. Blank consisted of reaction mixture without enzyme extract. Peroxidase activity was expressed as  $\mu\text{mol tetraguaiacol formed /min/g fresh weight}$ .

### Catalase activity

Catalase activity was determined by titrimetric method of Beers and Sizer, (1952) with minor modifications; the activity was assayed at 25 $^{\circ}\text{C}$  in a reaction mixture containing 3ml. of 0.1M phosphate buffer (pH 7.0), 2ml. 5mM  $\text{H}_2\text{O}_2$  (freshly prepared). The reaction was initiated by the addition of 1ml of enzyme extract and 10 ml of 0.35M  $\text{H}_2\text{SO}_4$  was added exactly one minute after adding of enzyme extract which was then titrated against 0.01M  $\text{KMnO}_4$ .  $\text{H}_2\text{O}_2$  was used as reaction substrate. Residual  $\text{H}_2\text{O}_2$  was oxidized by  $\text{KMnO}_4$  (0.1M) standard solution. The activity of catalase was expressed by the amount of enzyme that decomposes  $\text{H}_2\text{O}_2$  per gram fresh weight.

### Total tocopherol content

Total tocopherol content was estimated by the method of Rani et al., (2007) with few modifications. Soybean seeds were ground in single clamp Cyclo-Tech sample miller; 0.3g of ground soybeans were extracted in 6ml of n-hexane after overnight soaking. Extracted sample was separated with a phase separation filter paper; the filtrate was subjected to evaporation; the resultant residual oil was weighed, dissolved in 5 ml of methanol and filtered through 4.5 $\mu\text{m}$  syringe filter; 20 $\mu$ l of clear filtrate was analysed by HPLC method. Separation was carried out

using Shimadzu HPLC equipped with C18 silica column (250 X 4.6mm, 5 $\mu$ m); mobile phase of methanol (100%) was used at the flow rate of 1ml/min. 20  $\mu$ l of sample extract was injected into a HPLC column. The fluorescence detector was set at 290nm. The tocopherols were identified by comparison of retention time of  $\alpha$ ,  $\beta$ + $\gamma$ ,  $\delta$  tocopherol standards. Standard curves (concentration versus peak area) were calculated by linear regression analysis. Injection in triplicate was made at each concentration for both standards and samples. The amount of each compound was expressed as  $\mu$ g/g of oil present in the sample.

#### Estimation of isoflavones

Isoflavones (diadzein and genistein) were estimated by using HPLC method followed by Kumar et al., (2010). The soybean seeds (125mg) were powdered and 1ml. 80% ethanol and 1 ml conc. HCl were added. The mixture was sonicated for 2hr. at room temperature and centrifuged at 16000 rpm for 10 min; supernatant was filtered through 0.45 $\mu$ m syringe filter and transferred into HPLC vials. Analysis was performed on Shimadzu HPLC as described earlier for tocopherol estimation; mobile phase A was 10% acetonitrile and mobile phase B was 38% acetonitrile. The injection volume was 20 $\mu$ l and elution was kept at 0.8ml/min for 25 min. with gradient programme for % solventA/solventB (0 min-0/100, 5 min-10/90, 20 min – 0/100 and 25 min – 0/100); Detection was done at 260nm. The amounts of daidzein and genistein present in the sample were estimated by plotting the value of peak area of sample into the equation derived from the standard graph and were expressed as  $\mu$ g/g of sample.

#### Ascorbic acid content

The ascorbic acid was measured by titrimetric method described by Dahuja and Madaan, (2004). Two gram of overnight soaked seeds were homogenised in 10ml of stabilizing media (3% w/v metaphosphoric acid) and centrifuged at 5000 rpm for 20 min; the supernatant was filtered through Whatman no.1 filter paper and 2ml. of clear filtrate was titrated with 2, 6dichloro phenol indophenol dye solution to a faint pink colour. Amount of ascorbic acid was expressed as mg per /100g fresh weight.

#### Statistical analysis

Data were analyzed using SPSS (SPSS for Windows, version rel. 10.0.5., 1999, SPSS Inc., Chicago, IL). One-way ANOVA was conducted using data obtained in triplicate. Tukey's test was used to determine differences between means after ANOVA.

## RESULTS AND DISCUSSION

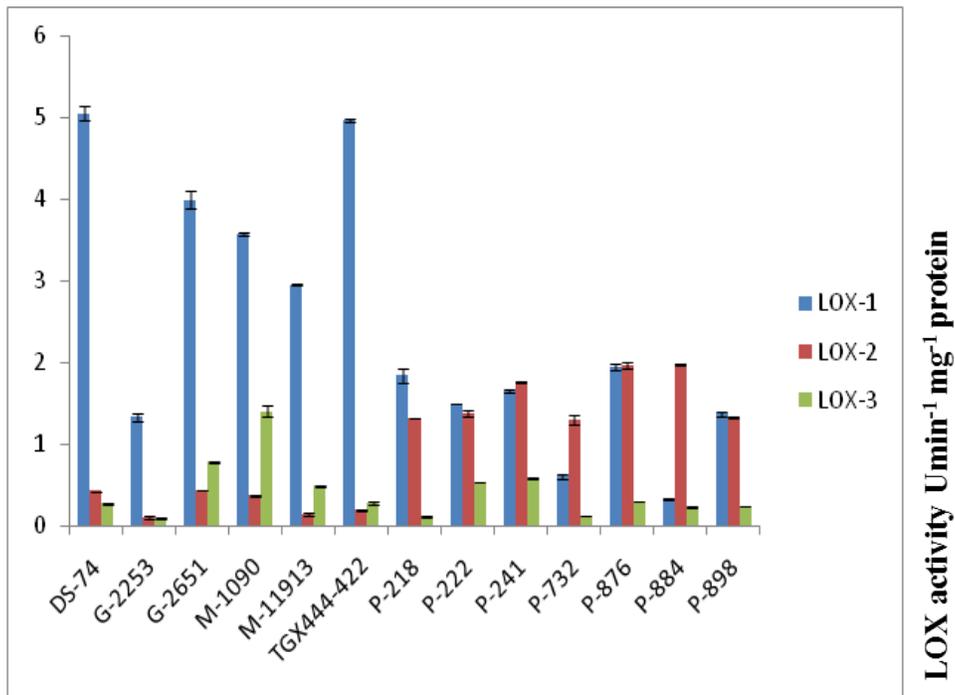
To understand the relationship between lipid peroxidation and seed storability, the impact of both lipid peroxidising enzymes and their compounds as well as the influence of antioxidant enzymes and antioxidant molecules was studied in the selected good and poor storer genotypes of soybean.

#### Lipoxygenase activity

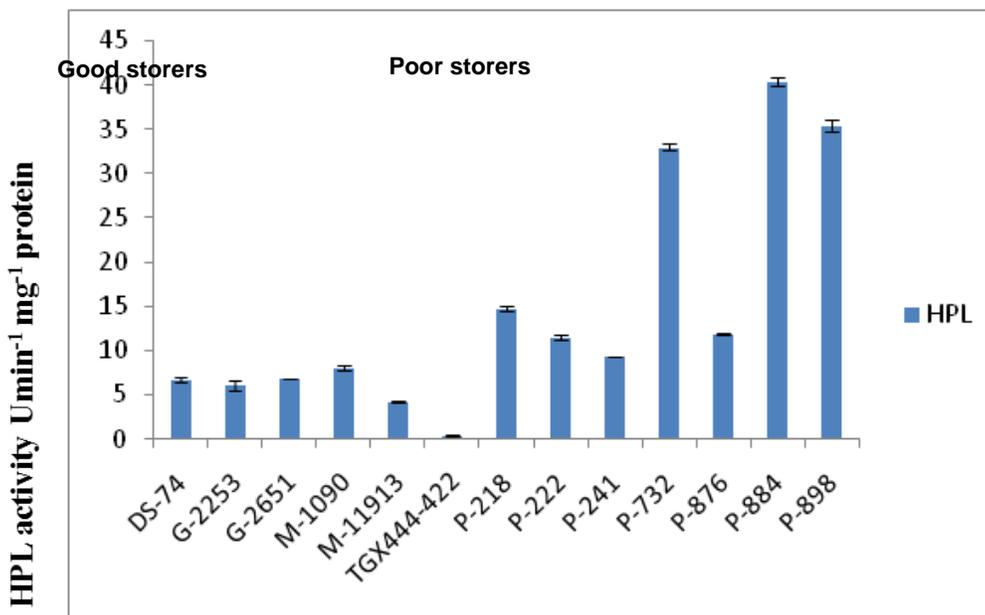
In order to determine the extent of lipid peroxidation in good and poor storers of soybean genotypes, lipoxygenase isozymes namely LOX-1, LOX-2 and LOX-3 activities were evaluated. The results revealed a significant variation in LOX-1 and LOX-2 activities among two contrast group of genotypes. Good storers possessed significantly high activity ( $p < 0.05$ ) of LOX-1 and low activity of LOX-2 ranging between 1.33 to 5.05 Umin-1mg-1 and 0.09 to 0.43 U min-1mg-1 protein respectively compared to poor storers, whereas no significant changes in LOX-3 activities were observed between two contrasting genotypes (Figure 1). The high activity of LOX-2 in poor storers could attribute to the loss of seed viability and storability through accumulation of LOX-2 catalyzed hydroperoxide products. Trawantha et al., (1995) investigated the role of LOXs in soybean seed deterioration by employing century isolines (lacking either of the two LOX isoenzymes) under natural ageing conditions and reported that century lines lacking LOX-2, LOX-3 did not show rapid loss in seed vigour and germination, thereby implicating LOX1 as one of the indices for better storability for seeds. Whent et al., (2011) observed a negative correlation between LOX-1 and linolenic acid (18:3) and a positive correlation between LOX-1 and oleic acid (18:1) in some of the soybean genotypes thereby suggesting that combination of low 18:3 and elevated 18:1 is a desirable trait in oil seeds to achieve oil stability. These findings indirectly support our results showing higher LOX-1 activity in good storers, which in turn implicates that LOX -1 might be playing an important role in maintaining the keeping quality of the seeds. Davies et al., (1987); Warner and Knowlton (1997); Shen et al., (1996) reported that, soymilk or oil prepared from soybean seeds lacking LOX-2 had lower beany rancid and oily flavors than the soybean lacking LOX-1, LOX-3, both LOX-2 and LOX-3 or both LOX-1 and LOX-3, thereby implying that, LOX-2 might be directly affecting the deterioration of soybean seeds or its products. Frankel et al., (1988) showed higher peroxide value of the oil from soybean lacking LOX-1 as compared to normal soybean oil. The evaluation of the activities of lipoxygenase isozymes in our study thus suggests that high LOX-1 activity in the good storers might contribute to their high oxidative stability and thus help in improving the shelf life of the seeds. These results are also supported by the previous findings from our lab where we have reported a significant positive correlation of LOX-2 with the off-flavor measuring parameters (Mandal et al., 2013).

#### Hydroperoxidelyase activity

A high HPL activity in the range of 9.34 to 40.18 Umin-1 mg-1 protein was observed in all the poor storers as compared to good storers where the activity in the range of 0.39 to 8.05 Umin-1 mg-1 protein (Figure 2) was observed. This observation was consistent with the previous finding of higher LOX-2 activity in the poor storers, leading to a higher production of hydroperoxide fatty acids which acts as a potential substrate for the HPL enzyme. Hosamani et al., 2013 reported higher activity of hydroperoxide lyase during storage of soybean seeds resulted in poor storability.



**Figure 1.** Variation in lipooxygenase (LOX) iso viz., LOX-1, LOX-2, LOX-3 activities between good and poor storers after 12 months of storage. Each value is expressed as mean± standard deviation (n = 3). Significant difference between 'poor' and 'good' storers are analyzed by ANOVA with Tukey's HSD test (P < 0.05).



**Figure 2.** Variation in hydroperoxidelyase (HPL) activities between good and poor storers after 12 months of storage. Each value is expressed as mean± standard deviation (n = 3). Significant difference between 'poor' and 'good' storers are analyzed by ANOVA with Tukey's HSD test (P < 0.05).

lity of seeds. Matoba et al., (1985) has also reported that soybean seed homogenates with LOX-2 isozymes showed increased n-hexanal formation (one of the HPL

catalysed product) as compared to LOX-2 null seeds and further showed the inhibition of soybean germination in the presence of n-hexanal compound with short time exp-

osure in a petridish bioassay. Poor germination % in the poor storers was also observed in the present study could be correlated with the high activity of HPL observed in the poor storer genotypes. It has also been reported that, the product of HPL catalysed reaction, such as hexanol, contributes to the undesired aroma and poor germination of soybean seeds (Bradow and Connick, 1990). The evidences related to the direct relationship between hydroperoxide lyase and lipid peroxidation process during seed storage are scarce, however this result might help in deciphering the role of hydroperoxide lyase in determining the storage life of seed.

### Lipid peroxidation

Determination of malondehyde (MDA) content, carbonyl and hydroperoxide molecules are the convenient methods for quantifying the extent of lipid peroxidation. Our previous results clearly stated that, poor storers exhibited high activity of LOX-2 and HPL have correlated with significantly high accumulation of lipid peroxidation compounds viz., lipid hydroperoxides, total MDA and carbonyl content (Figure 3). In the poor storers, MDA content varied in the range of 122nmol/g to 177nmol/g as compared to the good storers which showed a variation range of 74 nmol/g to 112nmol/g. Hydroperoxide content was also higher (568nmol/g to 1084 nmol/g) in the poor storers than in good storers (219nmol/g to 375 nmol/g). Parkhey et.al., (2012) showed remarkably increased amounts of lipid hydroperoxides and MDA levels during the long term storage of Sal seed (*Shorearobusta*) and also observed a loss of seed viability in terms of reduced seed germination. Similar kind of results were shown by Sung and Chiu, (1995) and Zacheo et al., 1998 that, higher accumulation of lipid peroxides such as hydroperoxides, carbonyls and MDA content were correlated with reduced germination percentage of soybean and almond seeds and similar observations were also found in both naturally occurring and artificially aged soybean seeds which resulted in reduced germinability and germination time due to the higher accumulation of MDA content (Sung, 1996). Our results thus indicate that, the enhanced lipid peroxidation in the poor storers might result in reduced germination and poor storability of seeds through increased MDA, carbonyl and hydrogen peroxide accumulation (figure 3).

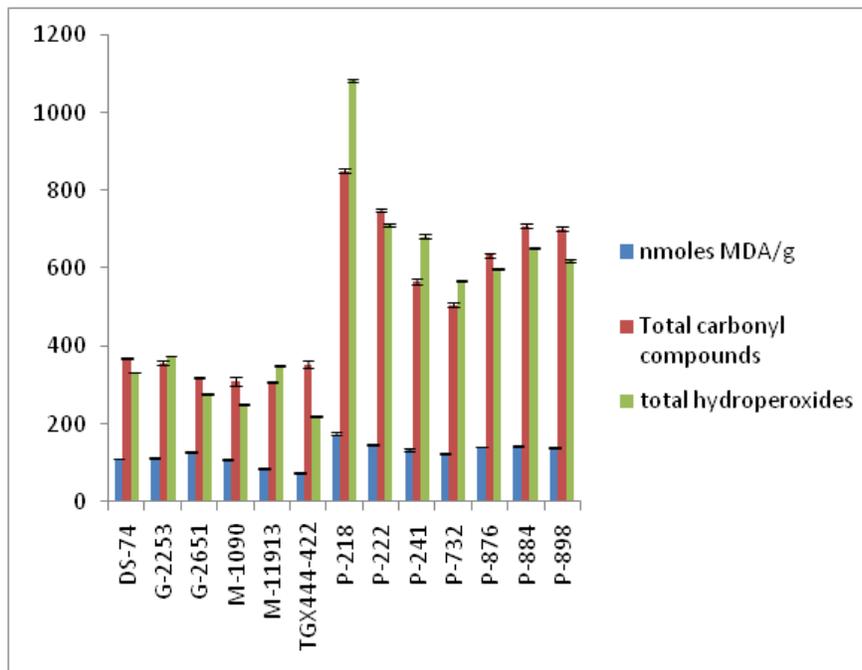
### Antioxidant enzyme activity

In view of significant differences observed in the MDA, carbonyl and hydroperoxide levels between the good and poor storers, several radical scavenging enzymes were assayed. Some of the good storer genotypes viz., M1090 and DS-74 showed significantly high ( $P < 0.05$ ) activity of

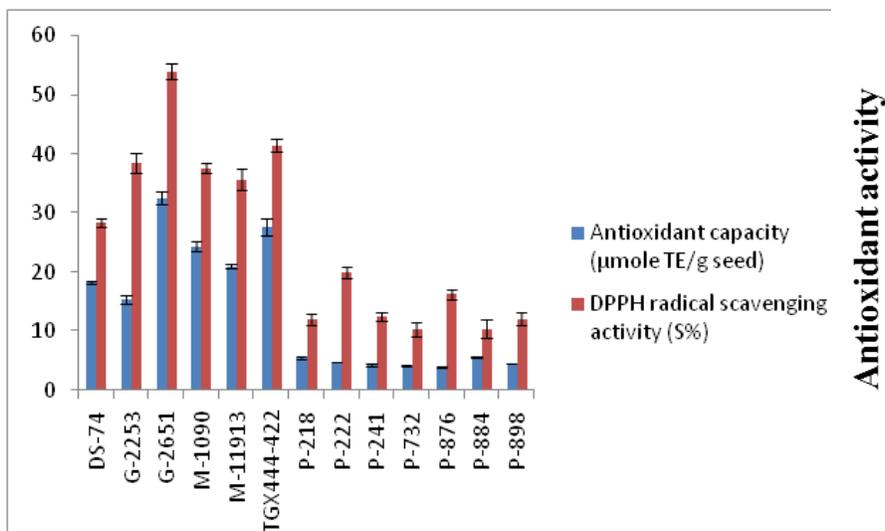
SOD in the range of 0.35 to 0.39 Umin<sup>-1</sup> mg<sup>-1</sup> protein, whereas the rest of the good storer genotypes showed the activity less than 0.3 Umin<sup>-1</sup> mg<sup>-1</sup> protein. Although catalase activity was higher in the good storers (3.63 Umin<sup>-1</sup> mg<sup>-1</sup> protein to 6.92 Umin<sup>-1</sup> mg<sup>-1</sup> protein), the extent of increase was not significant in comparison with the poor storers (Table 2). Our results indicated that, the activity of antioxidant enzymes SOD and catalase remained unchanged in both good and poor storers with an exception of M1090 - a good storer genotype. Similar kind of results were found by Sung and Jeng, (1994) that, aging effect was not evident on catalase activities in peanut seeds, further observations made by Stewart and Bewley, (1980); Sung and Jeng, (1994) indicated no significant detectable SOD activity in soybean and peanut seeds during aging process. Cakmak et al., (2010) showed a decline in the germination speed of alfalfa seeds kept for long time storage was correlated with the decreased activity of peroxidase (Cakmak et al., 2010) thereby supporting the results observed in our present study where decreased peroxidase activity in the poor storers correlated with lower germination percentage and enhanced accumulation of lipid peroxidation compounds which might result in poor storability of soybean seeds.

### Total antioxidant molecules

In addition to examining the antioxidant enzyme activity, the DPPH radical scavenging assay and total antioxidant activity by CUPRAC method were employed to evaluate the anti-oxidative properties of the soybean genotypes under study. The results revealed that good storers possessed high antioxidant activities in the range of 28% to 54% than the poor storers (10% to 19%) when analysed through DPPH radical scavenging activity (Figure 4). The total antioxidant activity by CUPRAC method also followed a similar trend. Significantly high ( $p < 0.05$ ) activity was observed in the good storers (15  $\mu$ mole TE/g seed to 32  $\mu$ mole TE/g seed), whereas the poor storers showed significantly low antioxidant activity ranging from 3.9  $\mu$ mole TE/g seed to 5.5  $\mu$ mole TE/g seed (Figure 4). Higher antioxidant activity in good storers could be attributed to better scavenging activity of the antioxidant molecules which could be correlated to lower accumulation of lipid peroxides thereby leading to improved storability in good storers as evident through higher germination % (88% to 93%) (Table 1). Avila et al. (2012) observed high vigor and viability of some soybean genotypes during aging process (both by natural and artificial process) by retaining high antioxidant activity. Kaewnaee et al., 2011 Showed increased antioxidant activity in accelerated aging (0-10 days) peanut seeds with high seed vigour and observed severe deterioration of sweet pepper seeds (15-25 days of accelerated aging) with lower antioxidant activity. Thus higher level of antioxidant activity helps to restore the seed viability and vigor



**Figure 3.** Modulation in the level of lipid peroxides in good and poor storers after 12 months of storage. Each value is expressed as mean± standard deviation (n = 3).Significant difference between 'poor' and 'good' storers are analyzed by ANOVA with Tukey's HSD test (P < 0.05).



**Figure 4.** DPPH radical scavenging activity and antioxidant capacity of soybean genotypes. Each value is expressed as mean± standard deviation (n = 3).Significant difference between 'poor' and 'good' storers are analyzed by ANOVA with Tukey's HSD test (P < 0.05).

which might lead to better storability of the seeds.

**Relative contents of natural antioxidant**

To understand whether the natural antioxidants play a role in seed longevity during seed storage or natural

aging process, isoflavones, tocopherols and ascorbic acid levels were measured. Results revealed no significant difference in tocopherol components such as α, β, γ, δ tocopherols and isoflavone components viz. genestein and diadzein between good and poor storers, except in one of the good storer M-1090 which showed a significant increase in total tocopherol

**Table 1.** Seed coat color and germination percentage before and after 12 months of storage.

SI no.	Genotypes	Seed coat color	germination % before storage	germination % after storage
<b>Good storers</b>				
1.	DS-74	Black	99(87.46) <sup>a</sup>	88.32(70.68) <sup>bdc</sup>
2.	G-2253	Black	98(85.32) <sup>ba</sup>	90(73.46) <sup>ba</sup>
3.	G-2651	Black	98(83.84) <sup>ba</sup>	93(80.14) <sup>a</sup>
4.	M-1090	Black	98(87.92) <sup>ba</sup>	88(68.46) <sup>bdc</sup>
5.	M-11913	Black	99(86.90) <sup>a</sup>	90(72.08) <sup>ba</sup>
6.	TG X 444-422	Black	97(85.23) <sup>bc</sup>	89(61.83) <sup>bac</sup>
	Mean		98.16(86.11)	89.66(71.10)
<b>Poor storers</b>				
7.	P-218	Yellow	93(80.31) <sup>c</sup>	72(56.87) <sup>gh</sup>
8.	P-222	Yellow	95(79.42) <sup>bc</sup>	74(60.16) <sup>gf</sup>
9.	P-241	Yellow	98(87.45) <sup>bac</sup>	72(54.11) <sup>gh</sup>
10.	P-732	Yellow	96(82.13) <sup>bc</sup>	69(52.35) <sup>gh</sup>
11.	P-876	Yellow	99(74.11) <sup>ba</sup>	66(55.04) <sup>gh</sup>
12.	P-884	Yellow	96(80.62) <sup>bc</sup>	77(60.41) <sup>ef</sup>
13.	P-898	Yellow	98(82.67) <sup>bac</sup>	77(63.56) <sup>ef</sup>
	Mean		96.42(80.96)	72.42(57.5)
	MSD at 5%		9.483	5.641
	SE (d)		1.514	1.286

Value in *parentheses* is arc sin transformed. Similar alphabets indicate non significant differences between the values following Tukey's studentized range (HSD) test.

**Table 2.** Variation in antioxidant enzymes activity between good and poor storers of soybean genotypes after 12 months of storage<sup>a</sup>.

SI no.	Soybean genotypes	Superoxide dismutase (units/min/mg protein)	Catalase ( $\mu$ moles of H <sub>2</sub> O <sub>2</sub> /min./g of fresh wt.)	Peroxidase (O.D./min./mg protein)	( $\Delta$ of of
<b>Good storers</b>					
1.	DS-74	0.35 $\pm$ 0.01	6.92 $\pm$ 0.13	167.35 $\pm$ 2.06	
2.	G-2253	0.29 $\pm$ 0.01	6.62 $\pm$ 0.43	158.02 $\pm$ 0.80	
3.	G-2651	0.27 $\pm$ 0.01	3.63 $\pm$ 0.16	143.35 $\pm$ 2.56	
4.	M-1090	0.39 $\pm$ 0.02	5.13 $\pm$ 0.35	178.46 $\pm$ 1.03	
5.	M-11913	0.23 $\pm$ 0.01	5.21 $\pm$ 0.44	78.81 $\pm$ 1.26	
6.	TG X 444-422	0.23 $\pm$ 0.02	3.73 $\pm$ 0.47	99.89 $\pm$ 0.45	
<b>Poor storers</b>					
7.	P-218	0.23 $\pm$ 0.01	5.00 $\pm$ 0.50	217.17 $\pm$ 0.98	
8.	P-222	0.28 $\pm$ 0.01	3.35 $\pm$ 0.37	112.41 $\pm$ 1.99	
9.	P-241	0.30 $\pm$ 0.01	5.53 $\pm$ 0.07	71.02 $\pm$ 0.91	
10.	P-732	0.28 $\pm$ 0.01	5.22 $\pm$ 0.22	104.90 $\pm$ 1.81	
11.	P-876	0.19 $\pm$ 0.01	3.22 $\pm$ 0.10	74.04 $\pm$ 2.01	
12.	P-884	0.12 $\pm$ 0.00	2.68 $\pm$ 0.12	146.63 $\pm$ 1.99	
13.	P-898	0.19 $\pm$ 0.01	3.75 $\pm$ 0.09	120.72 $\pm$ 2.19	

Note: <sup>a</sup> Data represent the mean  $\pm$ SD (n = 3). Significant difference between 'poor' and 'good' storers were analyzed by ANOVA with Tukey's HSD test (P > 0.05).

**Table 3.** Variation of individual forms of isoflavones, tocopherols, total isoflavones, total tocopherols and ascorbic acid content between poor and good storers of soybean genotypes after 12 months of storage.

Sl no.	Genotypes	Genistein <sup>1</sup>	Daidzein <sup>2</sup>	Isoflavone <sup>3</sup> (Genistein + Diadzein)	α-tocopherol	(γ+β)-tocopherol <sup>4</sup>	δ-tocopherol <sup>5</sup>	Tocopherol[(γ+β) + δ] <sup>6</sup>	Ascorbic acid <sup>7</sup> (mg/100g)
1.	Good storers								
2.	DS-74	600.36±2.49	1344.37±3.13	1944.73	1.32±1.40	669.99±1.92	95.46±2.23	766.77	14.74±0.35
3.	G-2253	525.12±2.97	1406.35±3.61	1931.47	4.34±0.90	603.45±1.87	141.41±2.88	749.20	13.60±0.29
4.	G-2651	288.81±1.60	714.07±1.57	1002.88	1.4±2.23	736.84±3.18	88.50±2.31	826.74	8.50±0.04
5.	M-1090	993.72±2.79	926.57±2.06	1920.29	1.52±1.68	1037.73±2.52	153.14±1.94	1192.39	10.57±0.18
6.	M-11913	271.60±2.09	550.48±1.85	822.08	1.38±2.42	528.01±2.02	85.81±2.21	615.19	11.75±0.14
7.	TG X 444-422	974.58±2.09	1113.07±2.44	2087.64	0.86±0.79	1082.43±1.15	37.72±1.70	1121.00	13±0.94
	<b>Poor storers</b>								
8.	P-218	434.26±2.18	1148.58±2.86	1582.83	1.64±1.49	346.95±1.60	93.82±1.21	442.42	5.62±0.37
9.	P-222	608.31±3.92	1189.28±4.73	1797.59	1.9±1.65	1123.57±2.83	189.92±2.45	1315.39	6.65±0.07
10.	P-241	167.53±2.05	555.67±1.58	723.20	2.82±1.87	847.40±2.27	100.30±2.11	950.52	4.82±0.11
11.	P-732	121.82±4.21	1367.69±2.01	1489.51	4.86±1.24	748.68±5.94	109.56±2.03	863.10	6.84±0.07
12.	P-876	640.41±1.09	1477.50±0.90	2117.91	2.52±2.06	488.47±2.41	68.38±2.84	559.36	5.68±0.07
13.	P-884	136.41±2.10	603.33±1.82	739.74	7.2±2.80	989.61±4.22	68.15±1.02	1064.96	5.42±0.06
14.	P-898	760.82±2.65	1184.30±3.01	1945.12	2.2±0.78	538.19±2.32	61.50±2.17	601.88	5.51±0.15

Values are <sup>1-3</sup>(µg g-1 seed); <sup>4-6</sup>(mg g-1 oil); <sup>7</sup>(mg<sup>-1</sup>100g) and are mean ±SD of triplicate samples; Significant difference between 'poor' and 'good' storers were analyzed by ANOVA with Tukey's HSD test (P >0.05).

content (1192.39 mg/g oil) and isoflavone content (1920.29 mg/g seed) (Table 3). An increased ascorbic acid content was however observed in the good storers (8.5 mg/100g to 14.74 mg/100g) as compared to the poor storers (4.82mg/100g to 6.84 mg/100g). Similar observations were made by Priestley et al., (1980) and Avila et al., (2012) in soybean genotypes. Although direct evidence correlating ascorbic acid levels and seed storability are not available to the best of our knowledge, there are however reports on priming of seeds with ascorbic acid showed improved germination of cotton seeds as compared to the control (Goel et al.,2003). This indirect evidence could support our observations leading to the probability of ascorbic acid playing an important role in maintaining the better storability of the seeds.

**CONCLUSION**

The present study thus clearly demonstrated the role of LOX pathway enzymes in determining the storability of soybean seeds. The results support the possibility of use of LOX-2 and HPL as an indices in the analysis of soybean food quality and seed viability. Significant increase in antioxidant activity and ascorbic acid levels in good storer of soybean could be exploited in future to achieve longer shelf life of the seeds. Suppression of LOX-2 and HPL gene expressions and the enhancement of antioxidant molecules in the seeds of soybean may

further help in improving the seed storability and nutritional quality by reducing its off-flavour property.

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