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OXYRADICAL ACCUMULATION AND THEIR DETOXIFICATION BY ASCORBIC ACID AND α-TOCOPHEROL IN SOYBEAN SEEDS DURING FIELD WEATHERING

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SUMMARY

The role of ascorbic acid and α-tocopherol in detoxification of oxyradicals were observed in different degree of field weathering in seeds of susceptible and tolerant cultivars of soybean [*Glycine max* (L.) Merr]. Loss of viability and vigour were associated with accumulation of oxyradicals and their inadequate detoxification by ascorbic acid and α-tocopherol. The result of present study shows that level of both ascorbic acid and α-tocopherol decreases because of their utilization during detoxification. But the amount and availability of these antioxidants is not sufficient to quench all the radicals, hence unable to prevent the seeds from deterioration. The extent of seed deterioration is regulated by the accumulation of oxyradical along with the degree of field weathering and availability of these antioxidants. Field weathering inexorably weakens the antioxidant defense mechanism of seeds and leads to rapid deterioration.

***Key words*: Alpha-tocopherol, ascorbic acid, EPR, field weathering, oxyradicals, soybean**

INTRODUCTION

Seed vigour as well as viability deteriorates prior to harvest. This deterioration is usually referred to field weathering (Tekrony *et al.* 1980). Field weathering negatively affects the membrane integrity of seeds and reduces the germinability and vigour (Bhatia 1996, Yadav *et al*. 2003). The field weathering also enhances the accumulation of oxyradicals, as the accumulation of reactive oxygen species (ROS) is one of the earliest symptoms against biotic and abiotic stress (Bhaumik *et al*. 1995, Wajtaszek 1997, Jain *et al*. 2004). The influence of free radical-mediated oxidations are amplified because it proceeds by a chain mechanism, i.e. one radical can initiate chain reaction which may propagate over and over again until they scavenged by some antioxidants. Ascorbic acid and α-tocopherol act as water-soluble and lipid soluble chain breaking antioxidants, respectively and protect lipid, proteins and membranes from oxidative damages (Burton *et al.* 1982, Lee *et al*. 1984, Alscher and Hess 1993, Yu 1994). Ascorbic acid scavenges oxygen radicals from aqueous phase, whereas α-tocopherol scavenges oxygen radicals within the membranes. Ascorbic acid regenerates the α-tocopherol by reducing the α-chromanoxyl radicals formed when α-tocopherol scavenges the oxygen radicals. This interaction between ascorbic acid and α-tocopherol radical (α-chromanoxyl radical) can take place not only in homogeneous solution but also in liposomal membrane system where ascorbic acid and α-tocopherol reside separately outside and within the membranes respectively, and ascorbic acid can act as a synergist (Niki 1987).

These endogenous small molecules play an important role in the removal of toxic oxygen species because of their quenching property and they can reduce potentially,

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destructive effect of reactive oxygen species (Wise and Naylor 1987). Plants respond to the oxidative stress by changing the in vivo level of different antioxidants, which may provide protection to particular stress. The present study analyzed the role of ascorbic acid and α-tocopherol in detoxification of oxyradicals, generated in soybean seeds during different degree of field weathering. The study may also help to understand variability of cultivars in response to seed deterioration due to field weathering.

MATERIALS AND METHODS

The soybean [*Glycine max* (L.) Merr. cvs. JS 71-05 and Punjab-1] was planted in *Kharif* 2000 and 2001 at the research farm of National Research Centre for Soybean (ICAR), Indore, India, in a randomized block design (RBD) with four replications. Each block was of 3m x 4m in area; the row-to-row and plant-to-plant distance was maintained at 45 and 5 cm respectively. At the time of planting, seeds were inoculated with recommended fungicides, viz. bevistin and diathane M @ 2 g/kg seeds and also with *Rhizobium* culture. A basal dose of N, P and K @ 20, 60 and 20 kg/ha, respectively was applied. The plants were kept free of weeds and insects by hand weeding and application of recommended insecticides respectively. In order to insure the same age of pods, at seed initiation [R5-stage (Fehr *et al.* 1971)], about 2000-3000 pods were tagged in each plot. The unweathered seeds and the seeds with different degree of field weathering were obtained as described by Dassou and Kueneman (1984).Tagged pods (about 500 pods from each block) were harvested at physiological maturity (R7 stage) and were kept for natural drying in laboratory before they were hand threshed to obtain unweathered seeds. The harvested seeds were stored in paper bags at laboratory temperature (25± 2° C) till further analysis. For field weathered seeds, tagged pods (about 500 pods from each block) were harvested at weekly interval up to four weeks after physiological maturity (R7 stage) and hand threshed and stored similarly as above.

Germination test was performed on 50 seeds each per four replicate of each genotype and weathering treatments, in rolled paper towels (AOSA 1983). The paper towels were kept at 30 °C in a seed germinator (Reico seed germinator, India) at 90% RH. Seedlings were counted after five days. The seed vigour was tested as per the method described by Franca Neto *et al*. (1998). Four replicate of 50 seeds from each treatment were kept in wet paper towels for 16 hrs at 29 °C. These preconditioned seeds were incubated in 0.075% 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution for 180 min at 40 °C in dark. The seed vigor was evaluated according to their topographical staining. The differently stained seeds were grouped in different vigour classes (Franca Neto *et al*. 1998) as class I, very high vigour seeds [stained superficially with glossy faint red (pink) colour]; class II, medium vigour seeds (stained deeply with intense red colour); class III, very low vigour seeds (typical white lesions of dead tissues on embryonic axis and more than 50% of cotyledons) and class IV, non-viable seeds (completely unstained and chalky white fragile seeds). Only class I and class II seeds were considered as vigorous and represented the percent vigour of the seed lot.

Spin trapping of oxyradicals: Seeds (200 mg) were homogenized in 1.98 ml phosphate buffer saline (100 mM, pH 7.4 containing 100 μM EDTA) and 20 μl of 100 mM Di-ethyl di thio carbamic acid (DDC) was added. The homogenate was centrifuged at 10,000 g for 15min at 4 ºC in a Remi R-24 centrifuge. An aliquot of 40 μl was mixed with 5 μl phosphate buffer saline and 5 μl of 500 mM N-t-Butyl-α-phenyl nitrone (PBN). The content was vortexed gently and incubated for 45 min. The aliquot was loaded into a quartz capillary tube and ESR spectra were recorded on X-band ESR spectrometer (Varian-E-104 with TM-110 cavity). The instrument settings were as follows-field set 3237 G, scan range 100G, temperature 27 ºC, microwave power 5mW, microwave frequency 9.01 GHz, modulation frequency 100 KHz, receiver gain value $1.25 \times 10^4 \times 10$, modulation 2x1, time constant 2 sec. and scan time 8 minutes. EPR integrated absorption intensity was calculated employing the formula, $I = Kw^2h$. Where, I = integrated line intensity of first derivative signal, K = line shape constant (6.5×10^{-10}), w= width of line and h= height of line. Potassium superoxide and ferrous sulphate were used for the standard spectrum of superoxide and hydroxyl radicals, respectively. Superoxide dismutase enzyme and thiourea were used for confirmation of presence of both radicals (superoxide and hydroxyl radicals) in the system. The chemicals were purchased from Sigma-Aldrich, USA.

Ascorbic acid content: Ascorbic acid was extracted from the control and stressed seeds by the method of Franke (1955). Seeds (100 mg) were ground in mortar and pestle with freshly prepared 10 ml 2% meta-phosphoric acid (2 g in 100 ml) and centrifuged at 10,000 g for 10 min. The supernatant was kept in dark on ice until use. Ascorbate was determined spectrophotometrically at 524 nm (Tonumura *et al.* 1978) by measuring the reduction of DCPIP by a portion of the sample (Hughes 1956). Supernatant (1 ml) was mixed with 1 ml distilled water, 1 ml meta-phosphoric acid, 0.5 ml sodium citrate buffer (pH 2.3) and 1 ml DCPIP. The reagents were added in the same order as described. Absorbance was recorded at 524 nm against blank containing distilled water. The amount of ascorbate present was calculated with reference to a standard curve.

α-Tocopherol content: α-Tocopherol was extracted by the Walker and Slinger (1975) method and estimated by the method of Pearson *et al.* (1970). Seeds (500 mg) were homogenized in 25 ml of absolute alcohol, 0.5 ml 10% alcoholic pyrogallol and few boiling chips. Solution was transferred to a conical flask, refluxed for 5 min, 2.5 ml saturated aqueous KOH was added through the condenser. The solution was again refluxed for another 5 min. The sample was cooled in an ice bath and 25 ml of cold water added along with 25 ml of petroleum ether. Solution was then transferred to 250 ml separating funnel. The lower aqueous phase was decanted for re-extraction with 25 ml of petroleum ether. The ether fraction was collected. Solution was washed 3-4 times with distill water containing alcoholic pyrogallol. Petroleum ether was evaporated in dark and the remaining matter was redissolved in a little benzene (0.2 ml) volume was made up to 10 ml with absolute alcohol (Walker and Slinger 1975).To 1 ml of the above-mentioned solution, 0.2% alcoholic $FeCl_3$ and 1 ml 0.5 % alcoholic α, α-dipyridyl solution were added. Volume was made up to 5 ml with absolute alcohol. After 10 min the absorbance was read at 520 nm (Pearson *et al.* 1970). The amount of α-tocopherol present was calculated from the standard curve.

RESULTS

The germination and vigour (Table 1 and Table 2) were adversely affected by field weathering. Germination was reduced drastically from 100% at physiological maturity to 57 and 83% in cultivars JS 71-05 and Punjab-1 after four weeks of field weathering, respectively. However, vigour reduced from 88% at physiological maturity to 42% in cv. JS 71-05 and from 96 to 64% in Punjab-1 after four weeks of field weathering, respectively. As against this the germination and vigour reduced slowly in unweathered seeds of both cultivars during four weeks storage at laboratory conditions. Germination was reduced from 100% to 98% in both cultivars while vigour reduced from 88 to 64% in JS 71-05 and 96 to 72% in Punjab-1.

Table 1. Effect of different degree of field weathering (one week to four weeks) on germinability (standard germination) of seeds of soybean [*Glycine max* (L.) Merr.] cultivars JS 71-05 and Punjab-1.

Treatments	**JS 71-05**					**Punjab-1**				
	0 Week* (PM)	**1 Week**	**2 Week**	**3 Week**	**4 Week**	**0 Week (PM)**	**1 Week**	**2 Week**	**3 Week**	**4 Week**
Unweathered	100 ± 0[#]	100 ± 0	98 ± 2	98 ± 1	98 ± 1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	98 ± 1
Field weathered	100 ± 0	93 ± 3	85 ± 2	73 ± 2	57 ± 3	100 ± 0	97 ± 2	92 ± 3	85 ± 3	83 ± 2

[*] Observations were taken at physiological maturity (PM or 0 weeks) to four weeks of field weathering and storage at laboratory conditions for corresponding periods.

[#] Values are percent ± SE (*n*=4) and represent the mean of two years (2000 and 2001) observations.

Table 2. Effect of different degree of field weathering (one week to four weeks) on vigour in soybean [*Glycine max* (L.) Merr.] cultivars JS 71-05 and Punjab-1.

Treatments	Vigour class §	JS 71-05					Punjab-1				
		0 Week*	1 Week	2 Week	3 Week	4 Week	0 Week	1 Week	2 Week	3 Week	4 Week
Unweathered	High vigour	80 ± 2 #	68 ± 5	62 ± 5	36 ± 4	28 ± 6	86 ± 4	78 ± 2	62 ± 5	60 ± 5	48 ± 6
	Medium vigour	8 ± 6	14 ± 6	22 ± 4	40 ± 5	40 ± 4	10 ± 2	12 ± 2	20 ± 2	14 ± 6	24 ± 5
	Low vigour	4 ± 2	10 ± 5	4 ± 4	8 ± 4	14 ± 6	4 ± 2	8 ± 4	12 ± 2	20 ± 5	22 ± 6
	Non viable	8 ± 3	8 ± 2	12 ± 5	16 ± 5	18 ± 5	0 ± 0	2 ± 2	6 ± 4	6 ± 4	8 ± 4
Field weathered	High vigour	80 ± 2	58 ± 5	42 ± 4	36 ± 4	4 ± 5	86 ± 4	50 ± 5	42 ± 6	34 ± 6	28 ± 2
	Medium vigour	8 ± 6	20 ± 5	22 ± 3	24 ± 4	38 ± 6	10 ± 2	38 ± 4	20 ± 4	26 ± 4	36 ± 6
	Low vigour	4 ± 2	6 ± 4	14 ± 6	8 ± 6	10 ± 5	4 ± 2	10 ± 4	28 ± 5	24 ± 6	8 ± 6
	Non viable	8 ± 3	16 ± 4	22 ± 4	32 ± 4	48 ± 4	0 ± 0	2 ± 2	10 ± 5	16 ± 4	28 ± 6

§ Vigour class represent the percentage of high, medium, low vigour and non-viable seeds of unweathered and field weathered seeds.

* Observations were taken at physiological maturity (PM or 0 weeks) to four weeks of field weathering and storage at laboratory conditions for corresponding periods.

\# Values are percent ± SE (*n*=4) and represent the mean of two years (2000 and 2001) observations.

Accumulation of free radicals: Signals of oxyradicals in seed homogenate were similar to signals obtained by the auto oxidation of KO_2 at different concentration in chemical system (data not shown). At physiological maturity there were no signals, but in case of field weathering, signals increased rapidly and then became less intense, while in unweathered seeds signals increased slowly in both cultivars. In JS 71-05, which is comparatively highly susceptible for field weathering signal become maximum (778.4 x 10^{-10} a.u.) within one week of field weathering, while in comparatively tolerant cultivar, i.e. Punjab-1 signal intensity became maximum (662 x 10^{-10} a.u.) after 2 weeks of field weathering and then decreased up to four weeks of field weathering gradually (Figs. 1a, and 1b).

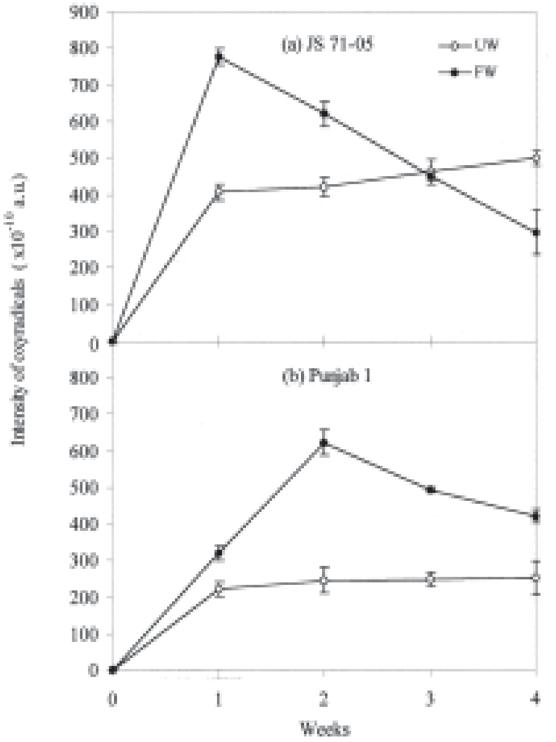

Fig. 1. Effect of field weathering on oxyradical germination in seeds of soybean [*Glycine max* (L.) Merr.] cv. (a) JS 71-05 and (b) Punjab-1. Values are mean of two years (2000 and 2001) observations. Each value represents the average of 10 individual spectra obtained by EPR. Vertival bars indicate ± SE. UW: unweathered, FW: field weathered

Level of ascorbic acid: The level of ascorbic acid (AsA) was highest (i.e. 0.60 mg/g dry wt in JS 71-05 and 0.61mg/g dry wt. in Punjab-1) at physiological maturity (PM). Beyond physiological maturity a decline in ascorbic acid was observed when the seeds were subjected to one to four weeks of field weathering as well as in the seeds harvested at PM and stored in the laboratory for the corresponding period of four weeks. However, the reduction in AsA content was significantly higher for seeds subjected to field weathering as compared to unweathered seeds. Considerable varietal differences in the levels of AsA were also observed. In JS 71-05 Ascorbic acid content became lowest (0.24 mg/g dry wt.) in one week field weathering and increased up to 0.39 mg/g dry wt. in four weeks of field weathered seeds, while in unweathered seeds of this variety, reduced level of ascorbic acid was observed up to four weeks of storage at laboratory conditions. However, in Punjab-1 ascorbic acid content became lowest after two (0.15 mg/g dry wt.) and four weeks (0.019mg/g dry wt.) of field weathering while, in unweathered seeds it reduced up to 0.22 mg/g dry wt. after four weeks of storage at laboratory conditions (Figs. 2a and 2b).

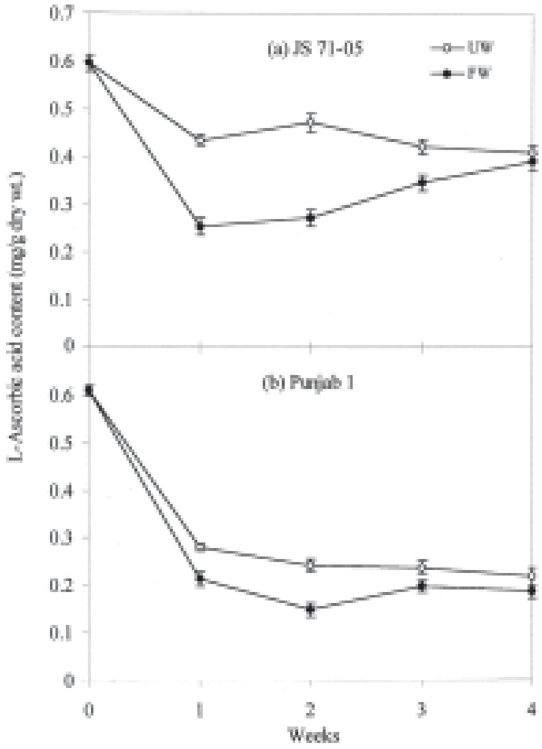

Fig. 2. Effect of field weathering on level of ascorbic acid in seeds of soybean [*Glycine max* (L.) Merr.] cv. (a) JS 71-05 and (b) Punjab-1. Values are mean of two years (2000 and 2001) observations. Vertical bars indicate ± SE (n=4). UW: unweathered, FW: field weathered

Level of α-tocopherol: A gradual reduction (from 4.90 to 3.99mg/g dry wt. and 6.97 to 5.5 mg/g dry wt. in JS 71-05 and Punjab-1, respectively) was observed in unweathered seeds of both the cultivars during first four weeks of storage at ambient conditions. However, the pattern of α-tocopherol differed considerably in the seeds subjected to field weathering. In cultivar JS 71-05, after one week of field weathering α-tocopherol was reduced to 3.36 mg/g dry wt. and increased thereafter. A gradual increase was observed two (5.55 mg/g dry wt.) to four weeks (7.30 mg/g dry wt.) of field weathering. In Punjab-1 the lowest level of 4.98 mg/g dry wt. was observed in the seeds subjected to two weeks of field weathering after which its levels increased in the seeds subjected to three (6.6 mg/g dry wt.) and four weeks (7.40 mg/g dry wt.) of field weathering (Figs. 3a & 3b).

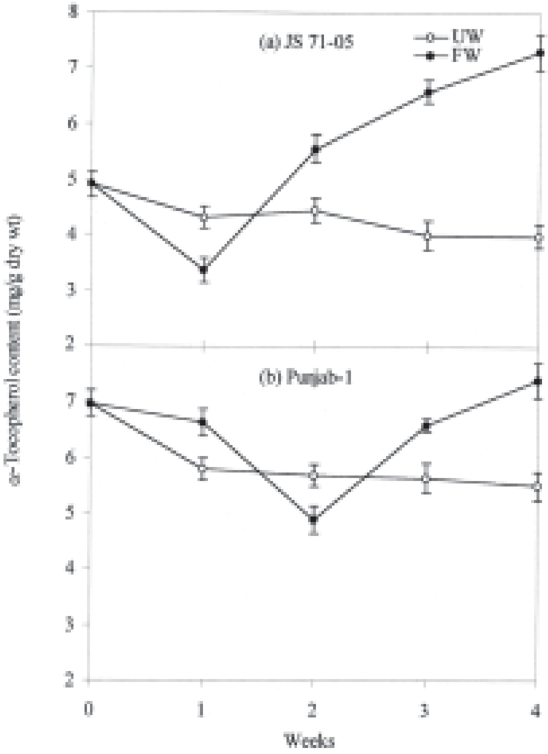

Fig. 3. Effect of field weathering on α-tocopherol content in seeds of soybean [*Glycine max* (L.) Merr.] cv. (a) JS 71-05 and (b) Punjab-1. Values are mean of two years (2000 and 2001) observations. Vertical bars indicate ± SE (n=4). UW: unweathered, FW: field weathered

DISCUSSION

Field weathering has negative effects on seed quality (Egli *et al*. 1979, Burris 1980, Bhatia 1996, Yadav *et al*. 2003), is also evident from the present study (Table 1 and 2). The loss of seed germination and vigour due to field weathering is associated with rapid lipid peroxidation and membrane perturbation mediated by rapid generation of ROS such as oxyradicals (Yadav *et al*. 2006). Also, the degree of deterioration differed among the varieties and between unweathered and field-weathered seeds and, these differences could be explained on the basis of amount of lipid peroxidation and oxyradical accumulation. We know that a trickle of reactive oxygen species is continuously produced in all cellular compartments as a byproduct of normal cellular metabolism (Alvarez *et al*. 1988, Brisson *et al.* 1994, Dixon *et al.* 1996) and that cell survival will depend upon adequate protection (Wise and Naylor 1987). All aerobic forms of life have evolved multiple defense lines, which include both scavenging enzymes and non-enzymatic antioxidants. Such multiplicity is required because reactive oxygen species (ROS) are produced in different cellular and extra cellular components and also because reactive species differ in properties such as diffusibility, solubility and propensity to react with various biological molecules to act in both aqueous and membranal phases. Seed cells also contain a complex system of antioxidant defenses to protect against the harmful consequences of activated oxygen species. A large number of enzymatic and non-enzymatic factors have been reported which have different capabilities to neutralize ROS (Alscher and Hess 1993). Some low molecular weight antioxidants, viz. ascorbic acid and α-tocopherol function as water-soluble and lipid-soluble chain breaking antioxidants, respectively and protect lipid, proteins and membranes from oxidative damages (Burton *et al.* 1982, Lee *et al*., 1984, Yu 1994). Despite the existence of such complex antioxidant defense system, the deleterious effect of oxyradicals was evident in the seeds indicating an upset in the balance of ROS and quenching ability of the cells. Therefore, besides oxyradicals, the levels of above antioxidants may further illustrate the reasons for deterioration of soybean seeds during field weathering as well as differences observed in the rate and extent of deterioration among the varieties.

Ascorbic acid is an important antioxidant and reacts with hydrogen peroxide, superoxide radical ($O2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and lipid hydroperoxides (Yu 1994). It also acts as a substrate for ascorbate peroxidase enzyme. Tappel (1977) proposed the synergism between ascorbic acid and α-tocopherol. Ascorbic acid regenerates the α-tocopherol when it is oxidized to α-chromanoxyl radicals (α-toc$^{\cdot}$), during scavenging of reactive oxygen species. In this study, at the physiological maturity maximum level of ascorbic acid was observed in both cultivars, while interestingly, there were no oxyradical signals observed at that time (cf. Fig. 1a, b and Fig. 2a, b). In case of field-weathered seeds, the maximum reduction in the level of AsA was observed within one week of weathering in JS 71-05 and two weeks of weathering in Punjab-1 (Fig. 2a and b). This decline in AsA coincided with the maximum levels of oxyradicals in the seeds of these varieties (Fig. 1a and b). Following a maximum decline, a slight increase was observed in the level of ascorbic acid in the seeds subjected to longer duration of weathering, but at this stage the level of oxyradicals had also reduced in the seeds of both the cultivars. In the same experiment, in unweathered seeds that were harvested at physiological maturity and were stored in laboratory for the corresponding period of four weeks, a continuous decline in ascorbic acid content and a concomitant rise in the level of oxyradicals were observed. The reduction in available Ascorbate pool of seed reveals that it may be either utilized for detoxification of ROS or for regeneration of α-tocopherol. The reduction in ascorbic acid was higher in tolerant variety than in susceptible indicating higher levels of detoxification of ROS.

α-Tocopherol is a principal biochemical antioxidant defense molecule against lipid peroxidation with a capacity to scavenge $O_2^{\cdot -}$, $OH^{\cdot}$ and $^1O_2$ (Fukuzawa *et al*. 1985). Besides being an active *in vitro* chain breaking antioxidant the long chain phytol tail of α-tocopherol allows it to penetrate into lipophilic membranes of cells and organelles where it exerts its antioxidant activity and helps in the prevention of oxidative damage of cell membranes (Burton *et al*. 1982). α-Tocopherol has several possible mode of action, viz. it can act as a chain breaking antioxidant by trapping fatty acyl peroxyl radical formed during the lipid peroxidation (Leung *et al.* 1981), as a reductant of $O_2^{\cdot -}$ to produce H_2O_2 and tocopherol quinone (Nishikimi *et al.*1980) and as a reactant with singlet oxygen (Foote *et al*. 1978). The lipid radicals ($L^{\cdot}$) in biomembranes are primarily scavenged by α-tocopherol that reacts with $OH^{\cdot}$, $LO^{\cdot}$ and $LOO^{\cdot}$ at 10^{10},

10^8 and 10^6 M^{-1} s^{-1}, respectively. The reaction of $HO^{\cdot}$ and $LO^{\cdot}$ with target lipid molecules is so fast that α-tocopherol cannot compete with the lipid oxidation by $HO^{\cdot}$ and $LO^{\cdot}$ effectively. Therefore α-tocopherol terminates the chain reaction mainly via the reaction with $LOO^{\cdot}$ (Niki *et al*., 1987, 1995). The α-chromanoxyl radicals (α-toc$^{\cdot}$) formed during the conversion of oxyradicals to hydroperoxides are reduced back to antioxidant tocopherol by ascorbic acid – glutathione (AsA-GSH) cycle. Thus ascorbic acid and α-tocopherol can act synergistically in the reduction of free radicals formed during any of the stress conditions (Leung *et al*. 1981). Hence, α-tocopherol serves as a powerful antioxidant.

In the present study, the level of α-tocopherol changed with the change in the level of oxyradicals. The level of α-tocopherol decreased with the higher level of oxyradicals accumulation and *vice versa* during field weathering as well as storage in subsequent period (cf. Figs. 1a, b and Figs. 3a, b). In the present study, the decrease in content shows the utilization of it against direct quenching of $O_2^{\cdot-}$ and preventing the seeds against the onset of triggering of deteriorative cascade. After initial reduction in the content of α-tocopherol, an increase was observed during additional field weathering, which may be due to activation of its regeneration from AsA-GSH pathway, but the oxyradicals was not quenched completely. The unquenched oxyradicals may start the deteriorating chain reaction by oxidizing the lipid molecule in to lipid radicals ($L^{\cdot}$). However, the tolerant variety has higher amount of α-tocopherol initially so the outburst of oxyradical was delayed up to two weeks. While in the susceptible varieties which has comparatively less α-tocopherol initially and besides this the outburst of oxyradicals was so high and fast (e.g. in JS 71-05) that before its regeneration the onset of deteriorative cascade has triggered so in spite of availability of α-tocopherol it could not save the seed from the deteriorative events. The interrelation between oxyradicals and antioxidants observed in this study is novel in the context of rapid seed deterioration due to field weathering. Simontacchi *et al*. (1993) also found a decrease in α-tocopherol content during treatment of soybean axes with paraquat, a free radical generating herbicide. Puntarulo *et al.* (1991) observed a decrease in α-tocopherol content in early stages of germination (when ROS accumulation is usually higher), which reflect the consumption of this antioxidant for quenching of reactive oxygen species. Jain *et al*. (2004) also proved the oxyradical scavenging capacity of α-tocopherol in chemically generated oxyradical system. Present study showed that ascorbic acid and α-tocopherol actively participate in the detoxification of oxyradicals in soybean seeds during field weathering.

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