Oxyradical accumulation and rapid deterioration of soybean seeds due to field weathering

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The effect of field weathering on oxyradical accumulation and subsequent changes were studied in the seeds of soybean [Glycine max (L.) Merr.] cv. JS 71-05. Electron spin resonance (ESR) quantification of oxyradical revealed that field weathering plays an important role in acceleration of their accumulation. One week of weathering increased the accumulation of oxyradicals to almost 2-fold and triggered the deteriorative cascade, by enhancing the lipid peroxidation and membrane perturbation, leading to cell death in seed tissues and poor germinability and vigour of soybean seeds. Thus, the weather conditions at the time of physiological maturity to harvesting of crop are very crucial and the field weathering plays a critical role for the maintenance of seed quality.

Keywords: Electron spin resonance (ESR) spectra, field weathering, membrane perturbation, lipid peroxidation, oxyradicals, spin trap, soybean, seed vigour

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The accumulation of reactive oxygen species (ROS) is one of the earliest symptoms against biotic and abiotic stresses. Like other environmental stresses, such as drought, high temperature, chilling, high light intensity, UV-radiation, air pollutant and pathogens, field weathering may also cause oxidative stress and generate ROS. "Field weathering" is generally termed for the deterioration of seeds from physiological maturity till harvesting in the field itself, when seed is attached with mother plant. It is mainly caused by the adverse weather conditions i.e., high temperature, intermittent rainfall and high relative humidity and their fluctuations. Oxyradicals particularly superoxide radical (O2·-) and hydroxyl radical (OH·) are produced in almost every cell compartment during normal metabolism, such as biosynthesis, cell defense, intra- and inter-cellular signaling. These free radicals along with other ROS are indispensable for the signal transduction for many physiological processes as well as are the toxic factor for life. Free radicals, if not scavenged are known to adversely affect the cell functioning by initiating the lipid peroxidation, protein modification/denaturation, DNA and RNA damage along with the membrane perturbation, which could lead to cell death.

Electron spin resonance (ESR) spectroscopy is an important technique for the study of free radicals in the plants. The reaction of labile free radicals with spin trap such as PBN (N-t-butyl-α-phenyl nitronate), resulting in the formation of long-lived nitroxide radical, can easily be identified by ESR. Oxyradicals are thought to be the primary radicals, generated in the plants. Reactions of these initial radicals, with cellular biomolecules, such as lipids lead to the formation of secondary carbon and oxygen-center radicals, which propagate in chain reaction manner and impair the normal cell physiological functioning and finally resulting to cell death.

The significance of free radicals in physiologically matured seeds and their relationship to seed vigour is poorly understood. There has been considerable speculation that oxyradicals could play an important role in seed deterioration. The most frequently
reported cause of seed deterioration is lipid peroxidation, due to generation of oxyradicals either by auto-oxidation or by oxidative enzymes, such as lipoxygenase present in many seeds. Lipid peroxidation and subsequent formation of some organic radicals are responsible for natural and accelerated aging. Although role of oxyradicals in seed germination processes has been studied, there is no report about oxyradical accumulation during field weathering, which results in rapid seed deterioration.

In the present study, an attempt was made to determine the oxyradicals accumulation in physiologically matured soybean [Glycine max (L.) Merr.] seeds and subsequent effect on the quality, in response to field weathering. The aim was to understand the mechanism of fast deterioration of soybean seeds in field itself at physiological maturity to harvesting of crop.

Materials and Methods

Plant material and weathering treatment

The soybean [Glycine max (L.) Merr.] cv. JS 71-05 was planted in Kharif season in year 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 3 m × 4 m 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., Bavistin and Diathane M @ 3 g/kg seeds and also with Rhizobium culture @ 5 g/kg seeds. A basal dose of N:P:K @ 20, 60 and 20 kg/ha was applied. The plants were kept free of weeds and insects by hand weeding and application of recommended insecticides, respectively.

The daily weather data was also recorded during the crop season. The weather data of sampling period from the date of physiological maturity i.e., 22 September for year 2000 and 15 September for year 2001 to till 4 weeks field weathering is presented in Fig. 1A, B & C.

In order to ensure the same stage of pods, at seed initiation (R5 stage), about 2000-3000 pods were tagged in each plot. The unweathered seeds and the seeds with varying degree of field weathering were obtained as described and collected as follows:

Unweathered seeds

From tagged pods, some pods were collected (500 from each block) at physiological maturity (R7 stage) and were subjected to natural drying (up to 14% moisture content) in laboratory, before they were hand-threshed to obtain unweathered seeds. The harvest seeds were stored in paper bags at laboratory temperature (25 ± 2°C) till further analysis.

Field-weathered seeds

Among these tagged pods, about 500 pods (from each block) were harvested at weekly interval up to 4 weeks after physiological maturity (R7 stage) and hand-threshed and stored similarly as above.

Spin trapping of oxyradicals

Oxyradicals were determined as follows: The dry seeds (200 mg) were powdered (each seed was crushed with steel pestle then powdered) and immediately homogenized in 1.98 ml phosphate buffer saline (PBS) (100 mM, pH 7.4 containing 100 μM EDTA) and 20 μl of 100 mM di-ethyl dithiocarbamic acid (DDC) was added. The homogenate was centrifuged at 10,000 g for 15 min at 4°C in a Remi R-24 centrifuge. An aliquot of 40 μl was mixed...
with 5 μl PBS and 5 μl of 500 mM N-t-butyl-α-phenyl nitro oxide (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 100 G, temperature 27°C, microwave power 5 mW, microwave frequency 9.01 GHz, modulation frequency 100 KHz, receiver gain value 1.25 x 10^4 x 10, modulation 2 x 1, time constant 2 s, and scan time 8 min.

ESR integrated absorption intensity was calculated employing the following formula: 
\[ I = K w^2 h, \]
where \( I \) = integrated line intensity of first derivative signal, \( K \) = line shape constant (6.5 x 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 the presence of both radicals (superoxide and hydroxyl radicals) in the system. All the chemicals used in ESR study were purchased from Sigma-Aldrich, USA.

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content spectrophotometrically. MDA determination was carried out as described\(^{38}\). The dry seeds (100 mg) were powdered and immediately homogenized in 5 ml of 50 mM phosphate buffer (pH 7.2) and centrifuged at 5000 g for 5 min. An aliquot of 0.5 ml supernatant was added to 4 ml TBA-TCA reagent (0.25% TBA in 10% TCA) and incubated at 95°C for 30 min and again centrifuged at 10,000 g for 15 min at 4°C. Absorbance was read at 532 nm and value for the non-specific absorption was read at 600 nm. The amount of MDA (A_532-A_600) present was calculated from a calibration curve using MDA (Merck, Germany) as a standard.

Electrical conductivity

The electrical conductivity (EC) of leachate from whole imbibed unweathered and field-weathered seeds was performed. Twenty-five seeds from each treatment in triplicate were weighed and placed in a plastic beaker with 50 ml of double-distilled water. The beakers were kept in an incubator at 30°C for 24 hr, after which conductivity was measured using a Systronics conductivity meter (Model 306, India) and the mean values expressed as μS cm\(^{-1}\) g\(^{-1}\) seed ml\(^{-1}\).

Standard germination

The test was performed on 50 seeds from each treatment in triplicate by rolled paper towels method\(^{29}\). The paper towels were kept at 29°C in a seed germinator (Reico seed germinator, India) at 90% RH. Seedlings were counted after 5 (1st count) and 8 days (final count), respectively.

Vigour test (Tetrazolium test)

The seed vigour was tested by tetrazolium test\(^{30}\), which is based on the activity of dehydrogenase enzymes that catalyze the reactions in glycolysis and the citric acid cycle in living cells of seed tissues. The 2,3,5-triphenyl tetrazolium chloride (TTC) interferes with the reduction processes of the living cells by accepting a hydrogen ion and gives red coloured, stable, non-diffusible substance triphenylformazan. The faint red colour in the seed tissue is a positive indicator of its viability by indirectly detecting the respiratory activity at the cellular level. The intensity (darkness) of red colour according to the level of diffusion of TTC is indicator of membrane perturbation. So, for assessment of vigour, two replicates of 50 seeds from each treatment were kept in wet paper towels for 16 hr at 29°C. These pre-conditioned seeds were incubated in 0.075% TTC solution for 180 min at 40°C in dark.

The seed vigour was evaluated according to their topographical staining. The differently stained seeds were grouped in the following vigour classes\(^{30}\): Class I: very high vigour seeds (stained superficially with glossy faint red colour, Plate 1 A & B), class II: medium vigour seeds (stained deeply with intense red colour, Plate 1 C & D), class III: very low vigour seeds (typical white lesions of dead tissues on embryonic axis and more than 50% of cotyledons, Plate 1 E & F) and class IV: non-viable seeds (completely unstained and chalky white fragile seeds, Plate 1 G & H).

Results and Discussion

The field weathering in soybean seeds under tropical environment is attributed to intermittent rainfall, high temperature, humidity and their fluctuation and pathogen attack during physiological maturity and post-physiological maturity period of seeds\(^{9,10,31}\). These stresses along with high light intensity and drought etc. are known to induce the formation of ROS, such as superoxide radical (O\(_2^-\)), hydroxyl radical (OH\(^-\)) and hydrogen peroxide (H\(_2\)O\(_2\))\(^{32}\).
The observations of two consecutive years did not differ significantly, hence the data were pooled together. The data showed that field weathering plays a key role in the enhancement of oxyradicals accumulation in soybean seeds. The oxyradical-PBN adduct gave characteristic ESR spectrum (Fig. 2A) i.e., triplet-of-doublet with hyperfine coupling constant ($a^N = 15.2 \text{ G}$ and $a^H = 2.8 \text{ G}$). In the present study, no detectable signals were observed in unweathered seeds at physiological maturity. However, as the plants were left for field weathering for 1 week, the accumulation of oxyradicals was induced and became almost 2-fold ($778.4 \times 10^{-10} \text{ a.u.}$), immediately within 1 week, as compared to unweathered seeds ($407.20 \times 10^{-10} \text{ a.u.}$). As the duration of field weathering increased from 2 to 4 weeks, the intensity of oxyradicals continuously decreased, however, in case of unweathered seeds the intensity of oxyradicals gradually increased in laboratory storage conditions (Figs 2A and B). The exact reason for decreased intensity of oxyradicals after an immediate outburst is not clear, but it may be due to their conversion into other radicals and/or secondary radicals\cite{33} or due to their consumption in the deteriorative events\cite{34}.

The accumulation of oxyradicals initiates the lipid peroxidation, and propagates in a chain reaction manner, by generating other free radicals and reactive intermediates\cite{19}. Oxyradicals (particularly $\text{OH}^+$) initiate the oxidation reaction by abstracting the hydrogen from methylene (-CH$_2$-) part of lipids and convert it into lipid radical (L$^\cdot$). The presence of L$^\cdot$ in the biomembranes readily leads to the chain reaction and formation of several intermediary radicals, such as lipid peroxyl radical (LOO$^\cdot$), lipid hydroperoxides (LOOH), and alkoxyl radicals (LO$^\cdot$)\cite{35,36} and finally production of carbonyl compounds including MDA.

In the present investigation, along with the intensity of oxyradicals, the increase in the MDA content also changed similarly (Fig. 3). At physiological maturity, the MDA content was very low (175.9 $\mu$M/g seed). But, it was almost doubled (351.8 $\mu$M/g seed) within 1 week of field weathering, as compared to unweathered seeds (184.2 $\mu$M/g seed). After initial maximum value, the MDA content decreased as the duration of field weathering increased from 2 to 4 weeks. However, in case of unweathered seeds, the MDA content increased very slowly up to 4 weeks of storage in laboratory conditions. Changes in production of other volatile aldehyde compounds, such as butanal, pentanal and hexanal, besides the MDA were also observed earlier with the weathering stress\cite{37,38}. The reason for reduction in the MDA content after initial increase in field-weathered seeds is yet to be investigated.
The accumulation of oxyradicals and lipid peroxidation play an important role in the membrane perturbation by rearrangement or by changing of the membrane lipid composition. In the present study, we observed a significant increase in the electrical conductivity (EC) of seed leachate. Electrolyte leaching, measured as EC of the whole imbibed seeds has been used as an indicator of the membrane perturbation in the cells of seed tissue. The increase in EC is mainly due to the change in the membrane permeability of seeds, which is generally indirectly related with the quality of seed. Earlier studies also showed that ROS affect the structural and functional properties of cell membranes including inactivation of the membrane bound protein and an increase in the membrane permeability. In case of field-weathered seeds, the EC increased as the duration of field weathering increased. After 4 weeks of field weathering, the EC became almost 4-fold i.e., it increased from 3.78 μS cm⁻¹ g⁻¹ seeds ml⁻¹ at physiological maturity to 12.2 μS cm⁻¹ g⁻¹ seeds ml⁻¹ after 4 weeks of field weathering. However, in unweathered seeds, it increased very slowly from 3.78 to 4.58 μS cm⁻¹ g⁻¹ seeds ml⁻¹ during the 4 weeks of storage in laboratory conditions.

A significant decline in the quality of these seeds in terms of germination (Fig. 5) and vigour (Plate 1 and Table 1) was observed by delaying the harvesting of seeds. Germination was reduced drastically from 100% at physiological maturity to 57% after 4 weeks of field weathering. In contrast, in unweathered seeds, the germination reduced slowly (i.e., 100% to 98%) during 4 weeks of storage at laboratory conditions. Hence, the deterioration in soybean seeds begins immediately after it reaches the physiological maturity under tropical conditions, which further establishes the severity of the problem of seed quality in soybean under tropical and sub-tropical environments.

Earlier, it was reported that the performance potential and vigour decline, as seeds deteriorate, before there is any loss in germination. In the present study, there was a severe decline in vigour of seeds, subjected to different degrees of weathering, as compared to unweathered seeds (Plate 1 and Table 1).
Plate I—Different degrees of seed deterioration in soybean [Glycine max (L.) Merr.] seeds [(A and B): High vigour or vigour class I seeds; (C and D): medium vigour or vigour class II seeds; (E and F): low vigour or vigour class III seeds; (G and H): non-vigour or vigour class IV seeds. Black arrow indicates outer surface and white arrow indicate inner surface of cotyledons]

Table I—Effect of field weathering on vigour of soybean [Glycine max (L.) Merr.] cv. JS 71-05 seeds as assessed by tetrazolium test [Values represent percent ± SE (n= 4)]

<table>
<thead>
<tr>
<th>Vigour class*</th>
<th>0 Week</th>
<th>1 Week</th>
<th>2 Weeks</th>
<th>3 Weeks</th>
<th>4 Weeks</th>
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<tr>
<td></td>
<td>UW</td>
<td>FW</td>
<td>UW</td>
<td>FW</td>
<td>UW</td>
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<tr>
<td>I</td>
<td>80 ± 2</td>
<td>68 ± 5</td>
<td>58 ± 5</td>
<td>62 ± 5</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>II</td>
<td>8 ± 4</td>
<td>14 ± 6</td>
<td>20 ± 5</td>
<td>22 ± 4</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>III</td>
<td>4 ± 2</td>
<td>10 ± 5</td>
<td>6 ± 4</td>
<td>4 ± 4</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>IV</td>
<td>8 ± 3</td>
<td>8 ± 2</td>
<td>16 ± 4</td>
<td>12 ± 5</td>
<td>22 ± 4</td>
</tr>
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</table>

*Vigour classes I, II, III and IV represent the very high vigour, medium vigour, very low vigour and non-viable seeds, respectively. UW, Unweathered; FW, field-weathered seeds

The unweathered seeds were stained glossy faint red colour with TTC solution, due to gradual diffusion of TTC solution through the intact cell membranes of seed tissues. The field weathered/deteriorated seeds stained deeply in intense red colour, where the viable tissues were present, but no staining was observed in the regions, where the tissues deteriorated severely or became dead. The tissues of this region generally appeared loose and yellowish-white coloured (Plate I A-H).

Interestingly, as the duration of weathering increased, there was a decline in the percentage of highly vigorous (vigour class I) seeds and an increase in the percentage of the seeds with low vigour (vigour classes II, III and IV). Also, after staining with tetrazolium salt, the regions of unstained chalky white dead tissues on cotyledons as well as on embryonic axis were increased as the degree of field weathering increased. The exact reason for instantaneous increase in these typical regions of dead tissues by field weathering is not clear, but it may be the result of severe damage caused by the uncontrolled outburst of oxyradicals, as we have observed in this study, which may not be quenched by antioxidant defense system of seed.

In a earlier study, we have observed that the activity of antioxidant enzymes like peroxidase and catalase decreased drastically in soybean genotypes, due to field weathering. The activity of other enzymes, such as superoxide dismutase and ascorbate peroxidase and some antioxidants like L-ascorbic acid and α-tocopherol also decreased drastically in soybean seeds, due to field weathering. It is well-known that the efficient antioxidant defense system protects the cell from the damage by ROS, but if the balance between generation of ROS and their
scavenging gets disturbed, it becomes difficult to stop the deteriorative cascade.

In conclusion, the present study shows that field weathering plays an important role in the accumulation of oxyradicals and fast deterioration of soybean seeds, even if the seeds are in pods and attached with mother plant in the field itself. Only a few days of weathering can double the accumulation of oxyradicals and trigger the deteriorative cascade by enhancing the lipid peroxidation and membrane perturbation several folds, finally leading to cell death, which results in loss of germination potential and vigour of soybean seeds.

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