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Lipid peroxidation and peroxide-scavenging enzymes in cotton seeds under natural ageing

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Abstract

The present study was carried out to contribute to our knowledge of the mechanism of seed deterioration in two cotton (*Gossypium hirsutum* L.) cultivars (HS6 and H1098) during natural ageing. The seeds were sealed in polythene bags and stored at 25 ± 1 °C for 3, 6, 9, 12 and 18 months. In both the cultivars, germinability decreased whereas membrane deterioration assayed as electrical conductivity of the seed leachates increased with storage period. The decrease in germinability was well correlated with increased accumulation of H_2O_2 and malondialdehyde content. The activities of peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase decreased with ageing. Seeds of cv. H1098 were more susceptible to ageing than HS6.

Additional key words: ascorbate peroxidase, catalase, germination, glutathione reductase, *Gossypium hirsutum*, H_2O_2, malondialdehyde, superoxide dismutase.

Introduction

Seed possesses the highest vigour at the time of its physiological maturity, and gradually decreases with storage period. The climatic conditions of humid tropical regions accelerate the seed ageing and the seed viability is lost during prolonged storage. The rate of seed deterioration varies among plant species, cultivars and storage conditions (*e.g.* Subba Rao *et al.* 1996, Dhakal and Pandey 2001). The exact causes of seed viability loss are still unknown, but the viability is affected by pre-harvest climatic conditions, seed type, seed structure, seed health, temperature, relative humidity and seed moisture content (Abba and Lovato 1999). One important factor governing the rate of viability loss is lipid peroxidation (Wilson and McDonald 1986) in consequence of formation of an increased amount of free oxygen radicals.

Several protective mechanisms including free radical and peroxide scavenging enzymes, such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) have been evolved within the seed (Bowler *et al.* 1992, McDonald 1999). SOD is a key enzyme in the regulation of amount of superoxide radical and peroxides. Hydrogen peroxide can react in the Haber-Weiss reaction forming hydroxyl radicals (Bowler *et al.* 1992) that cause lipid peroxidation. CAT and POD are implicated in removal of H_2O_2 (Fridovich 1986). Similar to CAT, APX and GR could also play a part in the control of endogenous H_2O_2 through an oxido-reduction cycle involving glutathione and ascorbate without undergoing conversion to a destructive radical (Nishikimi and Yagi 1996). The removal of H_2O_2 through a series of reactions is known as an ascorbate-glutathione cycle in which ascorbate and glutathione participate in a cyclic transfer of reducing equivalents resulting in the reduction of H_2O_2 to H_2O using electrons derived from NADPH (Gillham and Dodge 1986, Smith *et al.* 1989).

Seeds of cotton rapidly deteriorate because of higher linoleic acid content. However, reports on biochemical basis of seed deterioration in cotton are very few. Hence, the present studies were conducted to investigate seed germinability and the corresponding activities of peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase in cotton seeds of two cultivars viz. HS6 and H1098 subjected to natural ageing.

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; GR - glutathione reductase; MDA - malondialdehyde; POD - peroxidase; SOD - superoxide dismutase

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Materials and methods

Freshly harvested seeds of cotton (*Gossypium hirsutum* L.) cvs. HS6 and H1098 were obtained from the Cotton Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. Seeds were sealed in polythene bags and stored at 25 ± 1 °C for upto 18 months. The seeds were sampled at 0, 3, 6, 9, 12 and 18 months of storage.

For germination, 3 samples of 50 seeds were placed on paper towels (pre-soaked with water) and kept in the germination chamber at 25 ± 1 °C in dark. The number of seeds that germinated was evaluated on 9th day. Seeds with 0.5 cm radicle emergence were considered as germinated. For membrane permeability, 3 samples of 50 seeds each were soaked in 75 cm^3 of deionized water and kept in the incubator at 30 ± 1 °C for 24 h and electrical conductivity of the seed leachates was recorded using a conductivity meter (*NPC 360D*, Naina, India). The conductivity was expressed as dS m^{-1} $seed^{-1}$.

For malondialdehyde (MDA) and total peroxide contents determinations, 200 mg naked seeds were ground in 5 cm^3 of 5 % trichloroacetic acid (TCA) at 4 °C and centrifuged at 8 000 *g* for 15 min. The supernatants were used for MDA and peroxide determination by the methods of Heath and Packer (1968) and Sagisaka (1976), respectively.

Soluble enzymes were extracted from 18 h-imbibed seeds. Decoated seeds (200 mg) were ground in a chilled mortar and homogenized with 10 cm^3 of 0.1 M phosphate buffer (pH 7.8) containing 0.2 g polyvinylpyrrolidone (PVP), 10 mM β-mercaptoethanol, 10 mM KCl, 1 mM $MgCl_2$ and 1 mM EDTA. The homogenate was centrifuged twice at 15 000 *g* for 15 min at 4 °C. The supernatant was filtered and used for the enzyme assay. Soluble proteins, extracted from seeds were determined using the method described by Lowry *et al.* (1951).

Peroxidase activity was assayed essentially according to the method of Shannon *et al.* (1966). The reaction mixture contained 0.1 cm^3 of enzyme extract, 2 cm^3 of 0.1 M sodium-acetate buffer (pH 4.5) and 0.5 cm^3 of *O*-dianisidine solution (0.2 % in methanol, freshly prepared). The reaction was initiated with the addition of 0.1 cm^3 of 0.2 M H_2O_2. The change in absorbance (A) was recorded at 470 nm at an interval of 15 s for 2 min. One unit of POD was defined as 0.1 ΔA_{470} min^{-1}.

Catalase activity was estimated by the method of Aebi (1983). The reaction mixture contained 0.6 cm^3 of enzyme extract, 0.1 cm^3 of 10 mM H_2O_2 and 2 cm^3 of 30 mM potassium phosphate buffer (pH 7.0). The absorbance was read at 240 nm immediately after addition of enzyme extract at an interval of 15 s for 2 min. The blank was without enzyme extract. One unit of CAT was defined as 0.1 ΔA_{240} min^{-1}.

Ascorbate peroxidase activity was assayed by the method of Nakano and Asada (1981). The reaction mixture contained 0.1 cm^3 enzyme extract, 0.2 cm^3 of 0.5 mM ascorbic acid in 50 mM potassium phosphate buffer (pH 7.0) and 2 cm^3 of 50 mM potassium phosphate buffer (pH 7.0). The reaction was started by addition of 0.06 cm^3 of 1 mM H_2O_2 and absorbance decrease was recorded at 265 nm after every 15 s. Ascorbate peroxidase activity was calculated using the coefficient of absorbance 2.8 $mmol^{-1}$ cm^{-1}.

Glutathione reductase activity was assayed by the method of Goldberg and Spooner (1983). The reaction mixture contained 0.1 cm^3 of enzyme extract, 2.5 cm^3 of 120 mM phosphate buffer (pH 7.2) and 0.1 cm^3 of both EDTA (0.015 mM) and oxidized glutathione (0.065 mM). After 5 min 0.05 cm^3 of NADPH (9.6 mM) was added and mixed thoroughly. The reaction was monitored after every 15 s at 340 nm. One unit of GR was defined as 0.1 ΔA_{340} min^{-1}.

Superoxide dismutase activity was assayed by the method of Giannopolitis and Ries (1977). The reaction mixture contained 3 cm^3 of 0.1 M phosphate buffer (pH 7.8) containing 1.3 μM riboflavin, 13 mM methionine, 63 μM nitroblue tetrazolium and 0.1 cm^3 of enzyme extract. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Glass tubes containing the mixture were exposed to light (50 μmol m^{-2} s^{-1}). Identical tubes, which were not illuminated, served as blanks. After illumination for 10 min, they were covered with black cloth and absorbance was measured at 560 nm. Log A_{560} was plotted as a function of volume of enzyme extract used in reaction mixture. From the resultant graph, volume of enzyme extract corresponding to 50 % inhibition of the photochemical reaction was obtained. One unit of SOD was defined as the enzyme activity, which inhibited the photoreduction of NBT to blue formazan by 50 %.

For germination, the experiment was arranged as randomized complete block design with three replicates. Percentage data were arcsine transformed before analysis. All the data were subjected to an analysis of variance and LSD values were calculated at $P \leq 0.05$.

Results

Cotton seeds of two cultivars showed good germination at the beginning of experiment. Loss of viability, expressed by percentage germination, accompanied the natural ageing of seeds (Fig. 1*A*). No significant decrease

in germination was observed in 3-month-old seeds, but thereafter, this value declined progressively in both the cvs. The two cultivars showed different rate of ageing with H1098 showing the more rapid decline in germination as compared to cv. HS6. Electrical conductivity of the seed leachates increased significantly after three months of storage in both the cultivars of cotton. Significantly higher leakage of electrolytes was observed in cv. H1098 as compared to HS6 after 3 months of storage (Fig. 1*B*).

Malondialdehyde and total peroxide content increased significantly with natural ageing time from 3 - 18 months in both the cultivars (Fig. 1*C,D*). A rapid increase in MDA and H_2O_2 was observed after 6th month of storage. At the end of 18th month, the accumulation of MDA was significantly higher in H1098 as compared to cv. HS6. However, no significant difference in the accumulation of H_2O_2 was observed at this stage.

The activities of POD (Fig. 2*A*), APX (Fig. 2*B*), SOD (Fig. 2*C*), CAT (Fig. 2*D*) and GR (Fig. 2*E*) decreased progressively with the seed ageing. The decrease in the activities of these enzymes was more pronounced in cv. H1098.

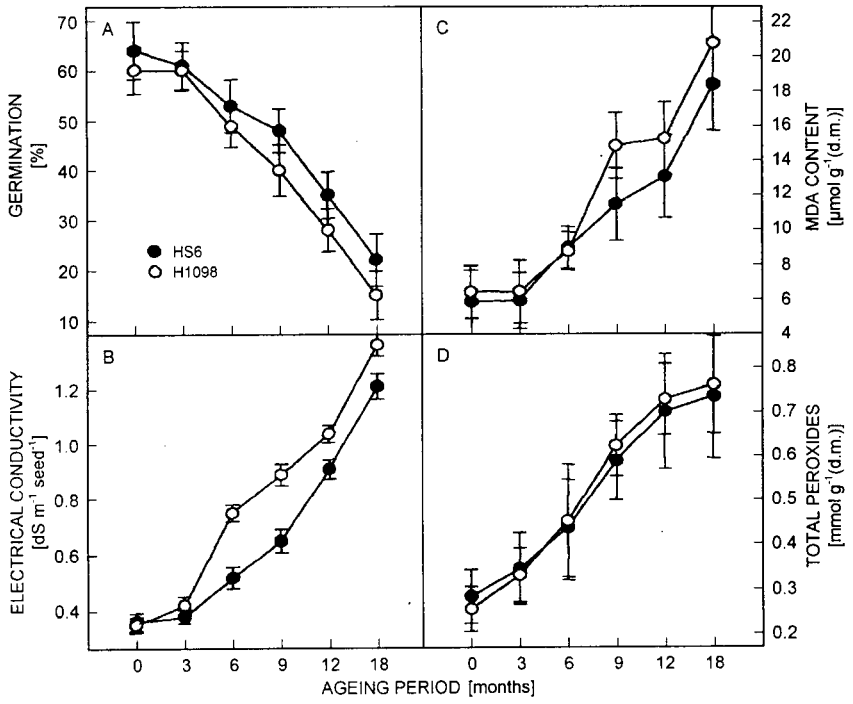

Fig. 1. Changes in germination (*A*), electrolyte leakage (*B*), malondialdehyde (MDA) content (*C*), and total peroxides (*D*) in naturally aged cotton seeds. Means ± SE.

Discussion

Germination of the seeds decreased with ageing in two cotton cultivars after three months of storage (Fig. 1*A*). Higher decrease in germination was observed in cv. H1098 than in HS6 indicating that cv H1098 was more susceptible to natural ageing than HS6. Seed deterioration can be explained by the findings that ageing of seeds leads to lipid peroxidation that subsequently causes membrane perturbation (Ponquett *et al.* 1992, Chang and Sung 1998). Such changes in the membrane of aged seeds lead to electrolyte leakage. In these studies also, the decrease in germinability correlated well with increased electrolyte leakage (Fig. 1*B*), which reflected the loss in membrane integrity. This phenomenon may be an indicator of an inability of seeds to maintain coherent membranes, resulting in reduction of germinability (Chang and Sung 1998). Woodstock *et al.* (1985) also reported that loss of membrane functional integrity was a major factor responsible for the reduction of germination potential in cotton seeds.

Free radical induced damage plays a key role in seed deterioration during ageing (Pinhero *et al.* 1998). Enhanced lipid peroxidation, mediated by free radicals and peroxides is one of the probable reasons for seed viability loss during storage (Sung 1996). Cotton seeds are prone to free radical damage due to higher linoleic acid content (Taneja *et al.* 1991). Determination of MDA is a convenient method of quantifying the extent of lipid peroxidation, especially in oil rich seeds having high

linoleic and linolenic acid content (Chang and Sung 1998). In the present studies, level of MDA and H_2O_2 content increased during natural ageing in both the cultivars of cotton (Fig, 1*C*,*D*). The enhanced lipid peroxidation was indirectly supported by increased peroxide accumulation (Fig. 1*D*). These results taken in connection with reduced germinability (Fig. 1*A*) indicate that increased lipid peroxidation might explain the loss of vigor and viability of cotton seeds.

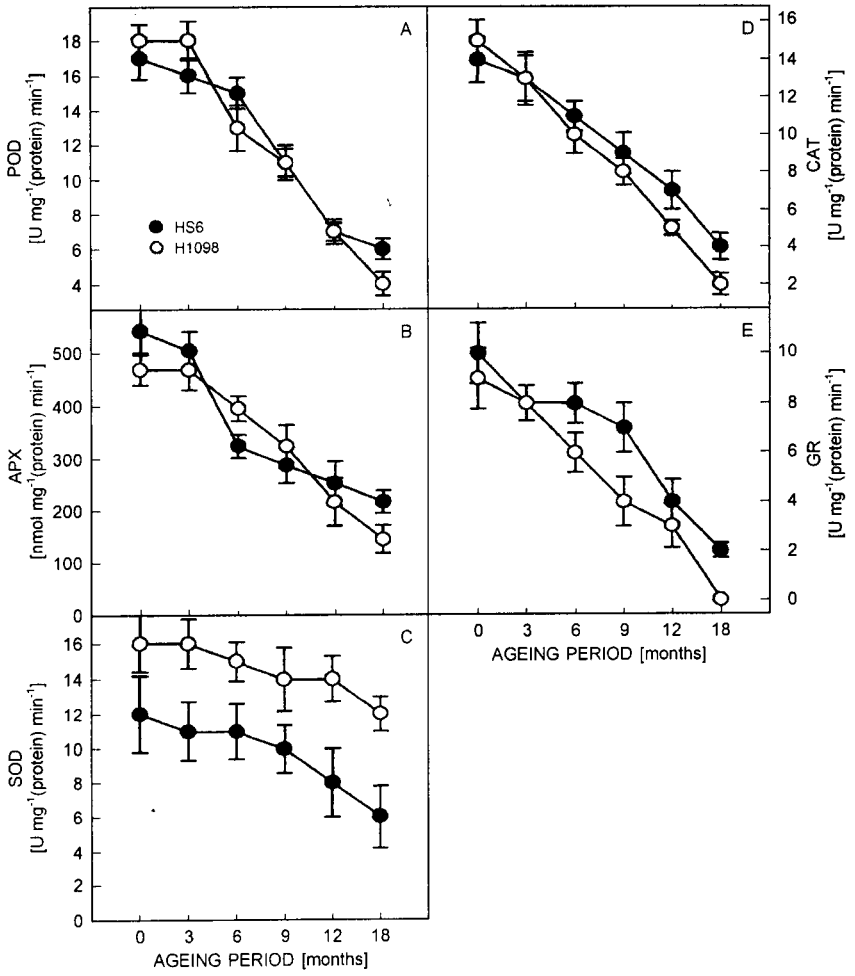

Fig. 2. Changes in activities of peroxidase (*A*), ascorbate peroxidase (*B*), superoxide dismutase (*C*), catalase (*D*), and glutathione reductase (*E*) in naturally aged cotton seeds. Means ± SE.

The increased levels of MDA and peroxide in aged cotton seeds might also result from ageing-induced inhibition of free radical and peroxide scavenging activity of antioxidant enzymes. Thus, the removal of peroxide in aged seeds might be too slow to prevent damage caused by peroxide (Bailly *et al.* 1996). In the present studies also, the activities of various peroxide-scavenging enzymes (POD, APX, SOD, CAT, and GR; Fig. 2) decreased in parallel with decrease in germination and increased content of MDA and H_2O_2 under natural ageing. The loss of enzyme activities could be responsible for the higher H_2O_2 accumulation in aged seeds (Chang and Sung 1998). Similarly, Sung and Chiu (1995) reported increased lipid peroxidation and decreased activities of various peroxide-scavenging enzymes with natural ageing in soybean seeds. Chang and Sung (1998) reported decrease in the activities of CAT and POD in sweet corn during ageing. The loss of enzyme activity could be due to ageing induced deterioration in soluble proteins by Amadori and Maillard reaction (Wettlaufer and Leopold 1991, Machado Neto *et al.* 2001).

H_2O_2 is partially detoxified by CAT (which is active at higher H_2O_2 concentration) in peroxisomes but the part of H_2O_2 in the cytosol as well as in chloroplast is degraded by APX (Klapheck *et al.* 1990). In this study, activity of APX decreased with ageing. SOD activity which is involved in the regulation of intracellular concentration of superoxide radical and H_2O_2 (Gutteridge and Halliwell 1990) also decreased with ageing (Fig. 2*C*; Sung 1996, Scebba *et al.* 2001).

Reduced glutathione is an important non-enzymatic

antioxidant that can act as a direct free radical scavenger (McDonald 1999). Glutathione is a water-soluble antioxidant found in the cytoplasm that can eliminate hydroxyl radical and singlet oxygen radicals. Reduced glutathione is regenerated by GR from oxidized form of glutathione in a reduction reaction that consumes NADPH (Smith *et al.* 1989). In the present studies, activity of GR decreased under natural ageing (Fig. 2*E*). This decrease in activity would result in decreased level of reduced glutathione, a potent free radical scavenger (Bailly *et al.* 1996). In these studies, no significant differences were observed between two cultivars in germination parameters. However, a higher electrolyte leakage was noticeable for H1098 as compared to HS6. Cv. H1098 showed a higher accumulation of MDA and H_2O_2 content as well as maintained the lower activities of various antioxidant enzymes as compared to HS6. The results support the hypothesis that cotton seed deterioration occurs due to membrane lipid peroxidation caused by the imbalance in the reactive oxygen scavenging system.

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