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Changes in activities of antioxidant enzymes in sunflower leaves of different ages

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Abstract

Role of superoxide dismutase isozymes and other antioxidant enzymes was studied in relation to leaf age in sunflower (*Helianthus annuus* L. cv. ACC 1508) at pre-flowering and grain filling stages. Relative water content (RWC) did not change much in leaves of different age and at the two stages. Protein content declined continuously from the youngest to the oldest leaf, while chlorophyll (Chl) and carotenoids (Car) contents increased down to $7^{th}/9^{th}$ leaf and declined in subsequent older leaves. Protein, Chl and Car contents were higher at pre-flowering than at seed filling stage. Superoxide dismutase (SOD), its isozymes, and ascorbate peroxidase (APO) and catalase (CAT) activities were highest in the 9^{th} leaf and declined in subsequent older leaves. SOD and APO activities were higher at seed filling, except in oldest senescent (13^{th}, 15^{th}) leaves. Among SOD isozymes, Cu/Zn-SOD and Mn-SOD activities accounted for most of the total SOD, and only marginal activity was observed for Fe-SOD. Peroxidase activity increased from youngest to the oldest leaf at pre-flowering stage and down to 13^{th} leaf at seed filling stage.

Additional key words: ascorbate peroxidase, carotenoids, catalase, chlorophyll, *Helianthus annuus*, oxidative stress, reactive oxygen species, superoxide dismutase, senescence.

Introduction

In plants, senescence symptoms include loss in chlorophyll, carotenoids, proteins, and increase in lipid peroxidation and membrane injury, all of which lead to decrease in photosynthetic capacity (Thompson *et al.* 1987). Senescence associated cell damages have been linked to increase in reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (*e.g.* Thompson *et al.* 1987, Procházková *et al.* 2001). Enzymes superoxide dismutase (SOD) (EC 1.15.1.1), ascorbate peroxidase (APO) (EC 1.11.1.11), glutathione reductase (GR) (EC 1.6.4.2), dehydro-ascorbate reductase (DHAR) (EC 1.8.5.1) and catalase (CAT) (EC 1.11.1.6) are important scavengers of ROS in plants (Asada 1992, Bowler *et al.* 1992, Foyer 1993, Scandalios 1993). Susceptibility of a plant to oxidative stress may depend on the over all balance between factors that increase oxidant generation and those cellular components that exhibit an antioxidant capability (Foyer *et al.* 1994). Activities of various antioxidant enzymes are known to increase in response to drought (Menconi *et al.* 1995, Zhang and Kirkham 1996, Sairam and Saxena 2000, Sairam and Srivastava 2001), high temperature (Upadhyaya *et al.* 1990, Sairam *et al.* 2000), low temperature/chilling (Anderson *et al.* 1995, Doulis *et al.* 1997, Scebba *et al.* 1999) and salinity (Hernandez *et al.* 1994, 1995, 1999, 2000). Contents of ROS are known to increase during leaf senescence, and therefore, it is possible that these changes are associated with lowered antioxidant enzyme activity. There are reports suggesting, both an increase in various antioxidant enzymes (Bueno and del Rio 1992, del Rio *et al.* 1992) as well as a decrease in the activity of these enzymes (Dhindsa *et al.* 1981, Hurng and Kao 1994) during senescence.

The objective of the present investigation has been to study different isozymes of superoxide dismutase and other antioxidant enzymes in sunflower leaves of different ages.

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Abbreviations: APO - ascorbate peroxidase; Car - carotenoids; CAT - catalase; Chl - chlorophyll; POX - peroxidase; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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Materials and methods

Sunflower (*Helianthus annuus* L.) cv. ACC 1508 was sown in cemented pots filled with clay loam soil and farm yard manure in 6:1 ratio on 11 July 2000. Normal recommended culture practices were followed. Samples from leaves of different age from the top (1st, 3rd, 5th, 7th, 9th, 11th, 13th and 15th) were collected in quadruplicate at two stages. Each sample (replicate) was assayed twice, and all data are mean of 8 observations. First sampling was done at the stage of fully developed unopened flower bud, referred as pre-flowering stage and the other 10 d after 100 % anthesis, referred to as seed filling stage. Samples were collected between 09:30 and 10:30. For enzyme essays the washed leaves were dipped in liquid nitrogen to prevent proteolytic degradation of proteins; while other estimations were done in fresh leaf samples.

Leaf relative water content (RWC) was estimated by recording the saturated mass (SM) of 0.5 g fresh leaf samples by keeping in water for 4 h, followed by drying in hot air oven till constant dry mass (DM) is achieved (Whetherley 1950):

$$RWC = [(FM - DM)/(SM - DM)] \times 100$$

Chlorophyll and carotenoids contents were estimated by extracting 0.05 g of the leaf material in 10 cm^{-3} dimethylsulfoxide (DMSO) (Hiscox and Israelstam 1979). The samples were heated at 65 °C for 4 h and then the absorbance was recorded at 665, 645 and 480 nm spectrophotometrically (*Systronics*-166, Ahmedabad, India). Chlorophyll and carotenoids contents were calculated as per Arnon (1949) and Lichtenthaler and Wellburn (1983). Soluble protein content was estimated as per Lowry *et al.* (1951) using bovine serum albumin as standard.

Enzyme extract for SOD, APO, POX, and CAT was prepared by freezing the leaf samples (1 g) in liquid nitrogen followed by grinding with 10 cm^3 extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). Brie was passed through 4 layers of cheesecloth and filtrate was centrifuged at 15 000 *g* for 20 min and the supernatant was used as enzyme extract.

SOD activity was estimated by following prevention of an increase in A_{560} due to the formation of formazon by superoxide anion with nitroblue tetrazolium chloride (Dhindsa *et al.* 1981). Three cm^3 of the reaction mixture contained 13 mM methionine, 25 mM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 cm^3 enzyme. Reaction was started by adding 2 μM riboflavine and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a blank. To distinguish SOD isoforms, Cu/Zn-SOD, Fe-SOD and Mn-SOD, the sensitivity of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to hydrogen peroxide (5 mM) were used, whereas Mn-SOD is unaffected (Yu and Rengel 1999). Separate controls (lacking enzymes) were used for total SOD and inhibitor studies. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme.

Ascorbate peroxidase was assayed by recording the decrease in absorbance due to ascorbic acid oxidation at 290 nm (Nakano and Asada 1981). The 3 cm^3 reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM H_2O_2 and 0.1 cm^3 enzyme. The reaction was started with the addition of hydrogen peroxide. Absorbance was measured at 290 nm in a UV-visible spectrophotometer (model *M 36*, *Beckman*, USA).

Catalase activity was assayed by estimating residual hydrogen peroxide by forming titanium-hydroperoxide complex (Teranishi *et al.* 1974). The 3 cm^3 reaction mixture contained 0.1 mM phosphate buffer (pH 7.0), 6 mM H_2O_2 and 0.2 cm^3 enzyme extract. The reaction was stopped after 5 min by the addition of 2 cm^3 of titanium reagent, which also formed yellow titanium-hydroperoxide complex with residual hydrogen peroxide. After 30 min aliquot was centrifuged at 10 000 *g* for 10 min. Absorbance of supernatant was recorded at 410 nm.

Peroxidase activity was assayed as increase in absorbance due to the formation of tetra-guaiacol (Castillo *et al.* 1984). The 3 cm^3 reaction mixture contained 16 mM guaiacol, 2 mM H_2O_2, 70 mM phosphate buffer (pH 6.1) and 0.1 cm^3 enzyme extract diluted 10 times. Absorbance due to the formation of tetraguaiacol was recorded at 470 nm.

Results were analyzed by analysis of variance and standard error of mean was calculated.

Results

Relative water content (RWC) (Fig. 1*A*) did not vary much both at the two stages as well as in leaves of different age. However, slightly higher RWC was recorded in the fully mature (7th) leaf from the top, and decreased in the lower leaves.

Protein content (Fig. 1*C*) was highest in the youngest

(1st) leaf, and decreased gradually in the older leaves, minimum being in the lowest pale-green (15th) leaf. Protein content was higher at pre-flowering stage in all the leaves as compared to seed filling stage.

Total chlorophyll (Chl) content (Fig. 1*B*) increased from youngest (1st) leaf to the 7th and 9th leaves at pre-flowering and seed filling stages respectively, except for a dip in the 5th leaf at both the stages. From seventh/ninth leaf onward their Chl content decreased gradually, with the lowest Chl content recorded in the bottom leaf (15th). Chl content in all the leaves was significantly higher at pre-flower opening stage than the seed filling stage.

Carotenoid (Car) content was higher at pre-flowering stage than the seed filling stage (Fig. 1*D*). At pre-flowering stage maximum Car content was observed in the 7th leaf, followed by gradual decline in subsequent leaves. At seed filling stage the Car content was highest in the 3rd leaf, while 5th, 7th and 9th leaves showed no differences in Car content, followed by decrease in lower leaves.

Total SOD activity (Fig. 2*A*) from 1st to 11th leaves was higher at seed filling stage than at pre-flowering stage, however the decrease in activity in 13th and 15th leaves was more steep at seed filling stage, and the activity in the last two leaves was lower than observed at pre-flower opening stage. The peak of SOD

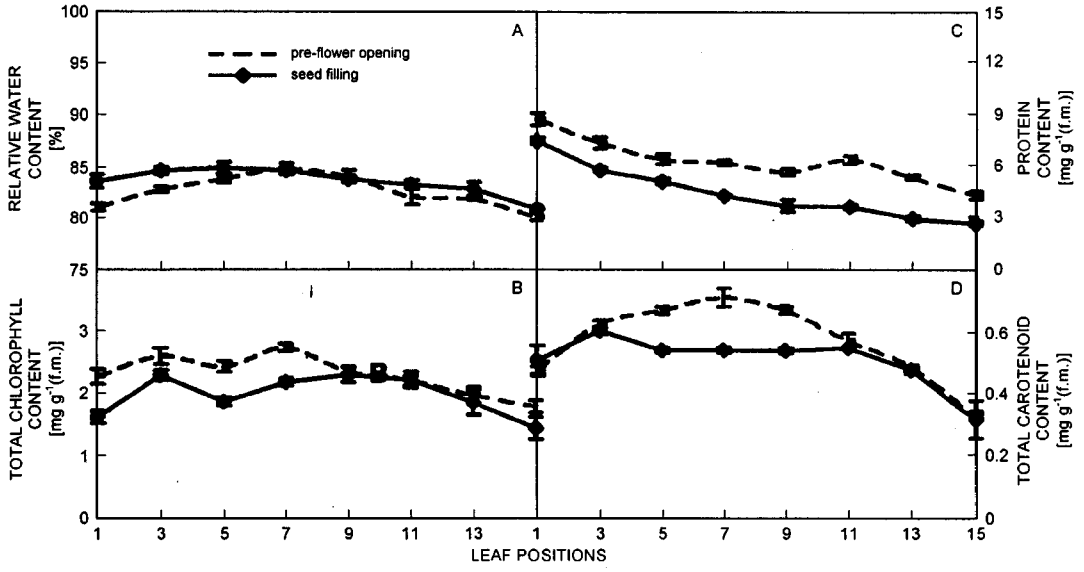

Fig. 1. Relative water content (*A*), chlorophyll content (*B*), protein content (*C*), and content of carotenoids (*D*) in different leaves of sunflower at various stages of development. *Vertical bars* indicate SE of mean ($n = 8$).

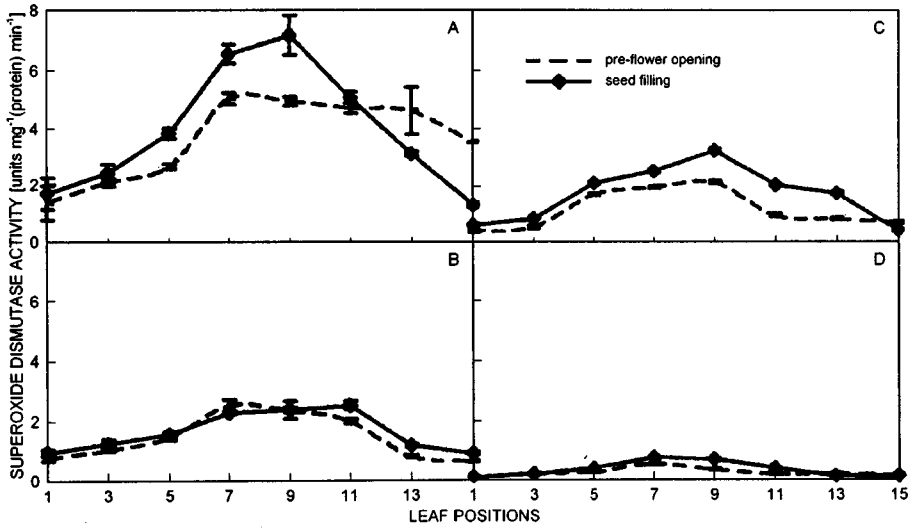

Fig. 2. Activity of total superoxide dismutase (SOD) (*A*), Mn-SOD (*B*), Cu/Zn-SOD (*C*) and Fe-SOD (*D*) in different leaves of sunflower at various stages of development. *Vertical bars* indicate SE of mean ($n = 8$).

activity at seed filling stage observed in the 9th leaf was higher than the peak activity observed in the 7th leaf at pre-flower opening stage.

Mn-SOD activity did not vary significantly at the two stages (Fig. 2*B*). The activity was higher in the 7th, 9th and 11th leaves with decline on both sides, *i.e.*, in very young and older leaves. The activity was slightly higher at seed filling stage in all the leaves. Cu/Zn-SOD activity (Fig. 2*C*) was significantly higher at seed filling stage in most of the leaves, except in 1st and 15th leaves where it was slightly higher at pre-flowering stage but the differences were non-significant. At both the stages Cu/Zn-SOD activity increased till the 9th leaf, which showed peak activity, followed by a decline in subsequent lower leaves. Fe-SOD activity (Fig. 2*D*) was lowest of the three SOD isoforms. However, the activity was marginally higher at seed filling stage, and the 7th leaf expressed higher activity than the younger and older leaves at both the stages.

APO activity increased from youngest (1st) leaf to 7th/9th leaf at pre-flowering and seed filling stages respectively (Fig. 3*A*), and thereafter declined to lowest in the bottom leaf (15th) at both the stages. From 1st to 11th leaf higher activity was recorded at seed filling stage, while in 13th and 15th leaves activity was higher at pre-flower opening stage.

CAT activity (Fig. 3*B*) also increased up to 9th leaf at both the stages, followed by gradual decline in activity in subsequent lower leaves. The differences in CAT activity at the two stages were non-significant, the two stages overlapping each other, except in 9th and 11th, which showed more activity at pre-flower opening stage.

POX activity continued to increase from youngest leaf down to 15th leaf at pre-flower opening stage, and to 13th leaf at seed filling stage (Fig. 3*C*). There were non-significant differences in POX activity for 1st and 3rd leaves at pre-flower opening and seed filling stages, but from 5th to 13th leaf significantly higher POX activity was observed at seed filling stage, while 15th (senescent) leaf showed more activity at pre-flower opening stage.

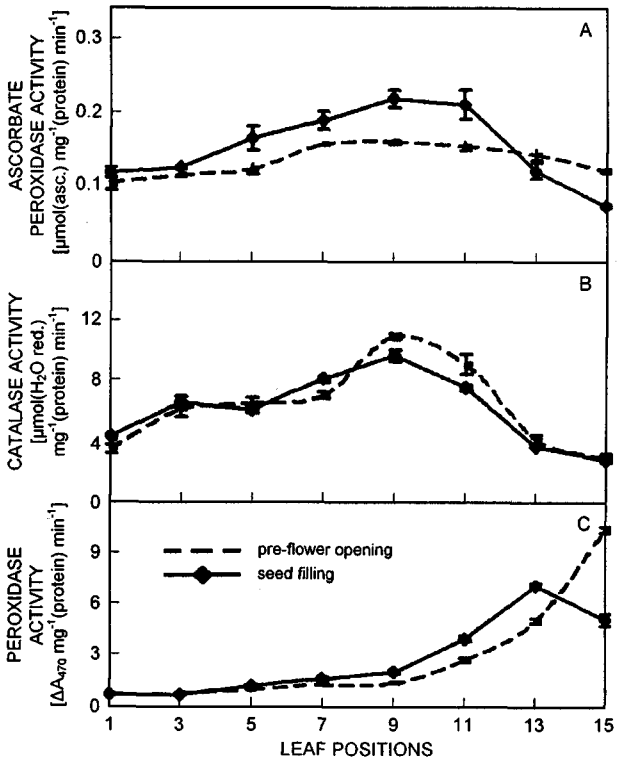

Fig. 3. Ascorbate peroxidase (*A*), catalase (*B*), and peroxidase (*C*) activity in different leaves of sunflower at various stages of development. *Vertical bars* indicate SE of mean ($n = 8$).

Discussion

Protein content decreased continuously from youngest leaf to the oldest one, suggesting that protein turnover rate, *i.e.*, synthesis/degradation was more towards degradation. This could be due to a continuous and linear increase in oxidative stress from young leaf to oldest leaf. Procházková *et al.* (2001) have reported a linear increase in H_2O_2 accumulation and lipid peroxidation in flag leaf of maize with age. Chl and Car contents on the other hand increased from the youngest leaf (1st) to mature (7th/9th) leaves and decreased there after, indicating the onset of senescence. The lower Chl and Car contents in leaves of different ages at seed filling stages indicate that senescence has already set in at seed filling stage. Loss in pigment and protein contents with ageing has been reported by various workers (Dhindsa *et al.* 1981, Olsson 1995, Procházková *et al.* 2001), and might be associated with an increased production of ROS and lipid peroxidation (Dhindsa *et al.* 1981, Hurng and Kao 1994, Ye *et al.* 2000, Procházková *et al.* 2001).

The antioxidant enzymes such as SOD, APO and CAT activities increased from youngest (1st) leaf to the mature (7th/9th) leaf and declined thereafter, with lowest activity being observed in oldest (15th) leaf. Increases in SOD and CAT activities followed by decline in older leaves have been reported in tobacco (Dhindsa *et al.* 1981), *Arabidopsis thaliana* (Ye *et al.* 2000) and pea (Olsson 1995). Ascorbate peroxidase (APO) is the major enzyme responsible for the scavenging of hydrogen peroxide during water stress (Ye *et al.* 2000, Sairam and Saxena 2000). Catalase (CAT) is also involved in H_2O_2 scavenging during stress (Anderson *et al.* 1995, Comba *et al.* 1998). Higher SOD, APO and CAT activity in mature leaves (7th to 9th) and specifically at seed filling stage might be in response to increased oxidative stress due to higher respiratory rates and energy metabolism requirement at these stages (Aharoni and Lieberman

1979, Dunaeva and Galeeva 1980). Inanaga and Kumura (1974) reported higher respiration rate 44 d after flowering in *Brassica napus*, which declined at subsequent stages. Similarly Fukushima *et al.* (1993) observed higher respiration rate in bolted radish plant than in non-bolted ones, indicating higher energy requirements at bolting stage.

Among the SOD isoforms, Cu/Zn-SOD and Mn-SOD showed higher activity then the Fe-SOD, which showed very rudimentary activity. Mn-SOD is primarily located in mitochondria with some activity also reported in peroxisomes (Hernandez *et al.* 1993, Gomez *et al.* 1999, Sandalio *et al* 1987, del Rio *et al.* 1998). Increases in Mn-SOD activity in mature leaves (7^{th}, 9th), especially at seed filling stage might primarily be triggered by increased oxidative stress experienced in mitochondria due to enhanced respiratory rate. Cu/Zn-SOD has been reported from cytosol, chloroplast and mitochondria (Hernandez *et al.* 1999, 2000, Sehmer and Dizengremel 1998). It is natural that chloroplastic Cu/Zn-SOD would be induced by conditions prevailing in chloroplast, *i.e.*, increased oxidative stress at photosystem 1 due to drought, high temperature, *etc.* However, under conditions of negligible environmental stress it is plausible that rise in Cu/Zn-SOD activity in mature leaves (7^{th}, 9th), especially at seed filling stage was not due to chloroplastic Cu/Zn-SOD but due to mitochondrial Cu/Zn-SOD. Sairam and Srivastava (2002) have reported salinity induced increase in mitochondrial Cu/Zn-SOD activity. Procházková *et al.* (2001) reported gradual increase in Mn-SOD and Cu/Zn-SOD activity, the former being higher, in flag leaf of maize up to 25 to 30 d after tasselling, followed by decline at subsequent stages.

On the other hand, POX showed very low activity from 1^{st} to 9^{th} leaf at both stages, and increased thereafter. The increase in POX activity towards the later stages of senescence could primarily be due to its role in the peroxidation of cell wall polysaccharides to generate phenoxy compounds (Greppin *et al.* 1986).

The results suggest that SOD (Mn-SOD and Cu/Zn-SOD), APO and CAT follow a pattern similar to the pattern of Chl and Car contents in different leaves. This means that in younger leaves the oxidative stress is minimum and the antioxidant demand is also low. Chl and Car contents increased to a peak in 7^{th} - 9^{th} leaves which also showed the highest antioxidant enzyme activities. The decline in SOD, APO and CAT activity in subsequent older leaves is associated with a parallel decline in pigment contents, reflecting an enhanced senescence due to lower antioxidant activity. It has been reported that responses of plant are result of a balance between oxidative stress and antioxidant activity (Foyer *et al.* 1994). It is, therefore natural that increasing oxidative stress normally experienced in mature and older leaves (Dhindsa *et al.* 1981, Hurng and Kao 1994, Ye *et al.* 2000, Olsson 1995, Procházková *et al.* 2001), and decrease in antioxidant activity would result in degeneration of cell macromolecules and pigments, and resultant senescence.

From the foregoing discussion it can be concluded that antioxidant enzymes such as SOD, APO and CAT play an important role in the regulation of senescence processes. Secondly it seems quite plausible to assume that much of the increase in SOD activity observed in mature (7^{th}/9^{th}) leaves could be increase in mitochondrial SOD. However, this aspect needs further experimentation.

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