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ACTIVITY OF ANTIOXIDANT ENZYMES IN LEAVES AND BRACTS OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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SUMMARY

A study was conducted to examine the activity of superoxide dismutase (SOD) and its isozymes, ascorbate peroxidase (APOX) and peroxidase (POX) in bracts and in the upper most 1st and 3rd leaves of sunflower at flower opening stage (50 days after sowing) and seed filling stage (70 DAS). The upper most leaf has smaller amounts of chlorophyll and carotenoids and lower activity of SOD, its isozymes, ascorbate peroxidase and peroxidase than the third leaf at flower-opening stage but increased significantly at seed filling stage. Relative water content (RWC) did not differ significantly in any of the leaves at the two stages. Soluble protein content did not differ at the 1st stage but was higher in 1st leaf at seed filling stage. Bracts in upper and lower rows contained significantly lower contents of chl a, chl b, total chl and carotenoids as compared to leaves at both the stages. The antioxidant enzyme activities were also very low in bract. Among SOD isozymes (Mn-SOD, Fe-SOD and Cu/Zn-SOD), only Cu/Zn-SOD contributed largely to the total SOD activity in bracts, while Mn-SOD and Fe-SOD activities were very rudimentary. Ascorbate peroxidase (APOX) activity decreased in bracts at the seed-filling stage, while peroxidase activity increased. The role of antioxidant enzymes in maintenance of protein and pigment contents in bracts and leaves has been discussed.

***Key words*: Ascorbate peroxidase, bracts, isozymes, pigments, sunflower, superoxide dismutase.**

INTRODUCTION

Water deficit and senescence associated damage to macromolecules and pigments have been linked with increasing levels of reactive oxygen species (ROS) (Thompson *et al*. 1987). ROS such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) are cytotoxic in nature and damage proteins, lipids, pigments and DNA (Halliwell and Gutteridge 1999). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX) and glutathione reductase (GR) scavenge the ROS in plants (Bowler *et al.* 1992, Asada 1992, Foyer 1993). Activities of various antioxidant enzymes are known to increase in response to drought (Zhang and Kirkham 1996, Sairam and Srivastava 2001), temperature (Upadhayaya *et al.* 1990, Jagtap and Bhargava 1995, Sairam *et al*. 1997, 2000), and salinity (Hernandez *et al*. 1993, 1995, 2000, Sairam and Srivastava 2002, Sairam *et al.* 2002). A positive relationship between cell antioxidant activity and the levels of protein and pigments (Prochazkova *et al.* 2001, Gillham and Dodge 1987, Leipner *et al*. 1999) and rate of photosynthesis (Kaiser 1979, Foyer 1993, Leipner *et al*. 1999) has been reported in various plant species. The relationship between cell antioxidant system and oxidative stress in relation to leaf senescence has been

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reported in maize (Prochazkova *et al*. 2001) and sunflower (Sairam *et al.* 2003).

Bracts found in sunflower head (inflorescence) are photosynthetically active and contribute to assimilate supply (Laxman and Srivastava 2000 a, b). However, the rate of photosynthesis by bracts is very low. The present study was under taken to examine the causes of low physiological efficiency of sunflower bracts, which are comparatively younger in comparison to the uppermost leaf, in relation to antioxidant activity.

MATERIALS AND METHODS

Sunflower (*Helianthus annuus* L.) cv. ACC 1508 was sown in cemented pots (40 x 40 x 45 cm) filled with clay loam soil and FYM in 6:1 ratio on July 11, 2000. Pots were fertilized with N, P and K @ 120, 60, 60 kg ha^{-1}, supplied in the form of urea, single super phosphate and muriate of potash, respectively, before sowing. Initially six seeds were sown in each pot, which were thinned to four plants per pot after 15 days of germination. Normal recommended inter-culture practices were followed. Samples from 1st and 3rd leaves from top and bracts were collected in quadruplicate at two stages marked as flower-opening (50 DAS) and seed-filling (70 DAS) stages. Each sample (replicate) was assayed twice. For sampling purpose, bracts were divided into upper two rows (or upper bracts) and lower two rows (lower bracts). Samples were collected between 9.30 a.m. and 10.30 a.m. and brought to the laboratory in ice bucket. For enzyme assays the washed leaves and bracts were dipped in liquid nitrogen to prevent proteolytic degradation of proteins, while other estimations were done in fresh leaf samples.

Relative water content (RWC) of leaves and bracts was determined following the method described by Whetherley (1950). Chlorophyll and carotenoids were extracted in DMSO following the method of Hiscox and Israelstam (1979). The absorbance of extract was recorded at 665, 645 and 470 nm. The chlorophyll a and b and carotenoids contents were calculated using standard methods (Arnon 1949, Lichtenthaler and Wellburn 1983). Soluble protein content was estimated following Lowry *et al.* (1951).

Enzyme assay

Enzyme extraction for superoxide dismutase, ascorbate peroxidase and peroxidase was done by freezing the leaf samples (1 g) in liquid nitrogen followed by grinding with 10 ml extraction buffer containing 0.1 M phosphate buffer, pH 7.5, 0.5 mM EDTA and 1 mM ascorbic acid. Brie was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15,000 g and the supernatant was used as enzyme extract.

Superoxide dismutase activity was estimated by recording the decrease in optical density of nitro-blue tetrazolium dye by the enzyme (Dhindsa *et al.* 1981). To distinguish SOD isoforms, viz. 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). Complete reaction mixture plus KCN (3 mM) was used to inhibit Cu/Zn-SOD, while complete reaction mixture plus 3 mM KCN and 5 mM H_2O_2 were used to inhibit both Cu/Zn-SOD and Fe-SOD activities. Separate controls (lacking enzymes) were used for the total SOD and inhibitor studies. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as the 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 optical density due to ascorbic acid at 290 nm (Nakano and Asada 1981). Absorbance was measured at 290 nm in a UV-visible spectrophotometer (model M 36, Beckman, Ca., USA). Peroxidase activity was assayed as increase in optical density due to the formation of tetra-guaiacol (Castillo *et al.* 1984). Data were analysed statistically for analysis of variance and standard error of mean.

RESULTS AND DISCUSSION

Antioxidant systems are essential for the living organism in aerobic environment. Oxygen is potentially toxic since it can be transformed by metabolic activity into more reactive forms such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^\bullet$) (Halliwell and Gutteridge 1999),

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which are collectively known as reactive oxygen species (ROS). Superoxide dismutase (SOD), an essential component of defense mechanism of the living organisms has received special attention in plants under environmental adversity since ROS are produced during many stress conditions (Bowler *et al.* 1992). Based on their metal cofactor SOD isozymes are classified into three types, i.e. Cu/Zn-SOD found in cytoplasm with or without Fe-SOD, Mn-SOD in mitochondria matrix and Fe-SOD in chloroplast stroma with or without Cu-Zn-SOD (Hernandez *et al.* 1993, 1995, 2000, Sairam and Srivastava 2002).

Total superoxide dismutase (SOD) activity was more in the 3rd leaf at flower-opening stage but decreased at seed-filling stage as compared to the 1st leaf. Though, at flower-opening stage SOD activity in the upper and lower bracts was almost identical but at seed-filling stage the upper bracts showed more activity than the lower bracts (Fig. 2 A). Activity pattern of the three SOD iso-forms in leaves and bracts was similar to that of the total SOD activity. Cu/Zn SOD activity did not differ in two leaves at flower-opening stage, but the 1st leaf showed a comparatively higher activity at seed filling stage. Mn-SOD activity was comparatively more in the lower leaf at flower-opening stage and in the upper leaf at seed-filling stage. Fe-SOD activity in leaves was very rudimentary. Cu/Zn-SOD activity accounted for maximum of total SOD activity in leaves and bracts. In bracts, activity of both Mn-SOD and Fe-SOD were very rudimentary. Sairam and Srivastava (2002) have also reported that Cu/Zn-SOD accounted for maximum of total SOD in wheat leaves. The upper bracts showed higher activity at seed filling stage (Fig. 1 A, B, C, D).

Ascorbate peroxidase (APOX) followed a pattern similar to that of SOD, as leaves showed a higher activity than bracts. APOX activity was more in the lower leaf (3rd) than in the upper leaf at both stages, and the activity level was higher at seed-filling stage. In bracts, the APOX activity decreased from flower-opening stage to seed-filling stage and the upper bracts maintained slightly higher APOX activity at both stages (Fig. 2A). Peroxidase (POX) activity in leaves increased at seed-filling stage. The third leaf showed more activity than the first leaf at both the stages. Magnitude of POX activity was comparatively higher

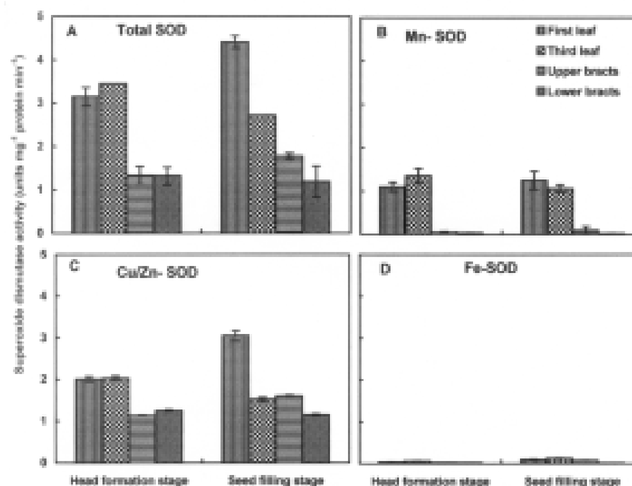

Fig. 1. Total superoxide dismutase (SOD) activity (A), Mn-SOD (B), Cu-Zn-SOD (C) and Fe-SOD (D) activity in bracts and leaves of sunflower (vertical bars indicate ± SEm).

in bracts vis-à-vis SOD and APOX. The upper bracts showed a greater POX activity than the lower bracts and the activity level further increased at seed-filling stage (Fig. 2B).

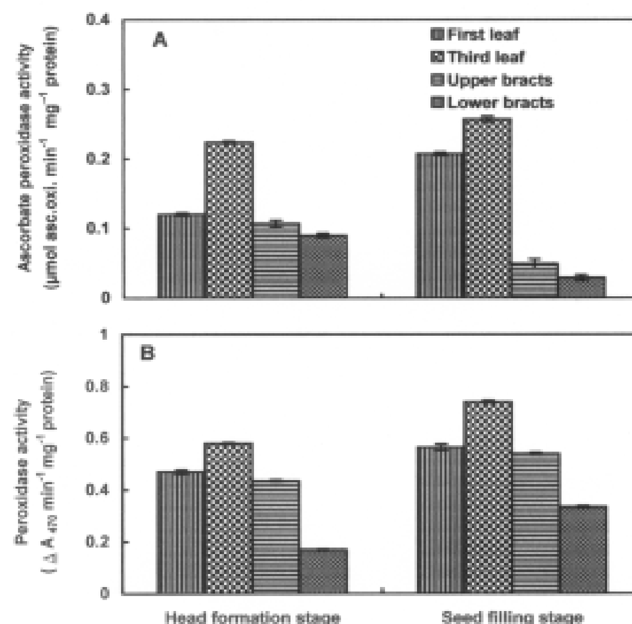

Fig. 2. Ascorbate peroxidase activity (A) and peroxidase activity (B) in bracts and leaves of sunflower (vertical bars indicate ± SEm).

Relative water content (RWC) in the 1st and the 3rd leaves did not differ significantly at flower-opening stage.

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However, at seed-formation stage it decreased in the lower (3rd) leaf by 2 – 2.5%. RWC in the upper and lower rows of bracts did not differ significantly at flower-opening stage but at seed-formation stage the upper bracts showed higher RWC (6 – 8%) than lower bracts. At both stages RWC was generally less in bracts than in leaves (Fig. 3A). Protein content in the two leaves did not differ at flower-opening stage. However, at seed-filling stage the 1st leaf maintained a higher protein content than the lower leaf. Bracts showed lower protein content than leaves which further declined at seed filling-stage. The upper bracts had a slightly higher protein content than lower bracts (Fig. 3B). Chlorophyll a, b and total chlorophyll contents were lower in the 1st leaf, as compared to the 3rd leaf at flower-opening stage, however at seed-filling stage the trend was reversed. Bracts generally had lower

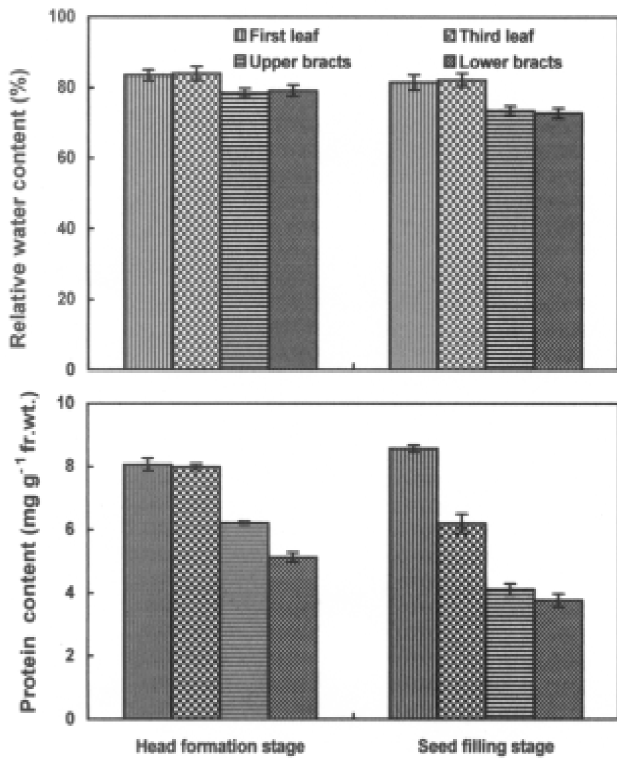

Fig. 3. Relative water content (A) and protein content (B) in bracts and leaves of sunflower (vertical bars indicate ± SEm).

chlorophyll content than leaves. Upper bracts had slightly higher chlorophyll than lower ones. At seed filling-stage the upper row of bracts showed significantly higher chlorophyll content than the lower bracts (Fig. 4 A, C, D). Carotenoids contents in the leaves showed a pattern similar to chlorophyll contents. However, carotenoids content was negligible in upper bracts. It further declined at seed-filling stage, compared to flower-opening stage (Fig. 4 D).

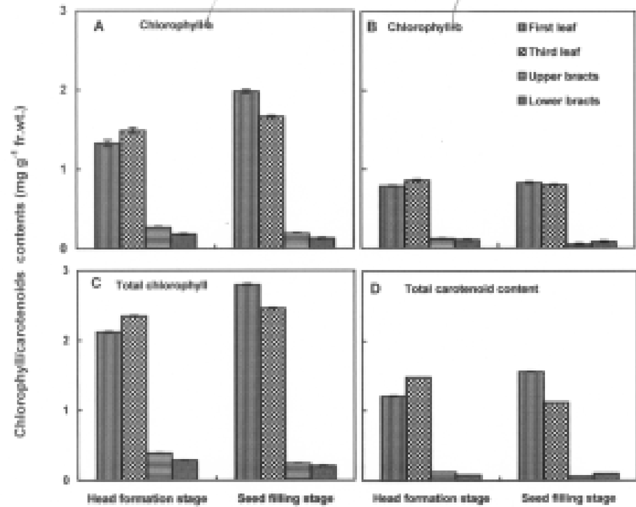

Fig. 4. Chlorophyll a (A), chlorophyll b (B), total chlorophyll content (C) and carotenoids contents (D) in bracts and leaves of sunflower (vertical bars indicate ± SEm).

Results obtained in the present study reveal, that the activities of SOD, its isozymes, ascorbate peroxidase and peroxidase increased in the top leaf during flower-opening stage to seed-filling stage and so did the contents of pigments (chlorophyll and carotenoids) and proteins. Decline in various antioxidant enzyme activities in the lower leaf (3rd) at seed-filling stage is again associated with a decline in chlorophyll, carotenoids and protein contents. Bracts showed comparatively lower SOD and APOX activity than the leaves. Cu/Zn-SOD activity accounted for most of the SOD activity, while Mn-SOD and Fe-SOD were inconspicuous. In bracts too, the total SOD, APOX and POX activities were low in lower bracts at seed-filling stage compared to flower-opening stage. Pigment (chlorophyll and carotenoids) and protein contents similarly showed a significantly lower level in bracts, which further decreased at seed-filling stage. Laxman and Srivastava (2000 a, b) have also shown lower concentrations of pigments and rate of photosynthesis in sunflower bracts than in leaves.

Antioxidant enzymes such as SOD, APOX and POX are involved in the scavenging of ROS, which are produced in various cell organelles. An excess ROS in cell results in the degradation of photosynthetic pigments, protein,

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DNA and lipids (Foyer 1993, Kaiser 1999, Leipner *et al*. 1999). Levels of antioxidant enzymes decide the level of oxidative stress experienced by cells/plants (Foyer 1993, Foyer *et al*. 1994). It is, therefore, possible that lower levels of antioxidant enzymes such as SOD and APOX, which are responsible for scavenging of $O_2^{\cdot -}$ and H_2O_2 in bracts, resulted in an increased oxidative stress and this could be the main factor contributing towards the lower levels of pigments resulting in reportedly low photosynthetic efficiency of bracts (Laxman and Srivastava 2000 a, b). In bracts, almost absence of Mn-SOD, responsible for scavenging of ROS produced in mitochondria due to enhanced respiratory activity during flowering and seed-filling stages (Sairam *et al*. 2003), further supports the hypothesis of oxidative damage to pigments and proteins in bracts resulting in their low photosynthetic efficiency. Another factor, which might be responsible for increased oxidative stress in bracts, is the low content of carotenoids known to scavenge singlet oxygen produced in photosynthetic light reaction at the site of PS II (Krause 1988, Foyer *et al*. 1994, Alscher *et al*. 1997). Very low quantity of carotenoids observed in bracts must have further contributed to the oxidative damage to chloroplast structure, chlorophyll and ultimately the photosynthetic efficiency of bracts. From the present study it can be inferred that the low photosynthetic efficiency of bracts, as reported by Laxman and Srivastava (2000 a, b) was due to the deficiency of antioxidant defence systems in the form of SOD (complete lack of Mn- SOD) and APOX.

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