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RESEARCH ARTICLE

Characterization of senescence processes in attached flowers of *Calendula officinalis* L. and *Aster novae belgii* L.

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Abstract

Experiments were carried out to characterise senescence processes in attached flowers of *Calendula officinalis* L. and *Aster novae belgii* L. As flowers open and finally senesce, petals show a striking colour change and wilting. Developing flowers of these plants growing in the university botanical garden were divided into four stages viz. half open, fully open, beyond open and advanced senescent stages. The average life span of these flowers from the half open to senescent stage was about seventeen days. Flower diameter and fresh weight of each scape showed a considerable rise between stage I and III followed by a decline in both plants. However, moisture content and membrane stability index exhibited a gradual decline from stage I to IV. Senescence in these flowers was accompanied by rise in petal malondialdehyde (MDA) content which enhanced lipid peroxidation and guaiacol peroxidase (GPOX) followed by the increment in free radical formation. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) play important roles in protecting petal tissue from peroxidation processes. Although activities of CAT and APX were very low at stage I, significant increment was noticed between stage I and III; followed by a sharp decline at stage IV. SOD was unique in having a moderate activity at stage I, registered gradual increment upto stage III and declined thereafter in petals of both plants. Both plants exhibited almost similar trends with respect to various physiological and biochemical changes investigated and compared in petal tissue. Petals from flower stage IV was characterised by low activity of SOD and significant reduction in APX and CAT activities, in comparison to preceding stage.

Introduction

The life span of flowers has a species-specific, limited by an irreversible programme of senescence (Voleti et al., 2000). Flower senescence often considered as the last stage of floral development (Arora, 2008). However, it is not a final event, rather an integral process that allows for the removal of costly tissues (e.g. petals), that otherwise attracted pollinators for sexual reproduction (Arora, 2008). Petals are the floral organs which primarily determine the commercial longevity of flowers (Chakrabarty et al., 2009). Opening and closing of flower petals are highly intricate processes that are regulated by several exogenous and endogenous factors (van Doorn and van Meeteren, 2003). van Doorn (2001) identified three types of flower cessation: by petal wilting or withering, which was either ethylene sensitive or insensitive, and by abscission of turgid petals. The gradual loss in fresh weight is a characteristic symptom of flower senescence, finally leading to visible wilting (Halevy and Mayak, 1979; Borochov and Woodson, 1989). Senescence is characterised by a number of catabolic processes such as degradation of proteins, lipids and nucleic acids; lipid peroxidation and disruption of cell membranes (Shahri, 2011). It is also connected with controlled degradation, remobilisation and re-utilization of cell components (Rubinstein, 2000; O'

Donoghue et al., 2002). This process is under the control of plant hormones that trigger the alteration in the carbohydrate status of petals (Mayak et al., 1972).

Investigations were carried out to characterise the physiological status of petals over the senescence period. *Calendula officinalis* L. and *Aster novae belgii* L. are members of Asteraceae family which undergo senescence independent of the controlling effects of ethylene (Woltering and van Doorn, 1988). These flowering annual species are popular as decorative garden plants. They can also be used as cut flowers, but both have a short vase-life (Khokhar and Mukherjee, 2010 a, b, c; Kaur and Mukherjee, 2013). However, no studies have been reported on the physiology of uncut (attached) flowers at different stages of development. In this study, investigation has been undertaken with unexplored *Calendula* and *Aster* to understand the physiological changes occurring during flower development and senescence with the ultimate aim to improve the postharvest performance of these flowers. We have analyzed changes in flower diameter, fresh weight, membrane stability index and protein content. We also examined the cause of membrane disruption (due to GPOX and lipid peroxidation) and activities of some important antioxidant enzymes such as superoxide dismutase, catalase and ascorbate peroxidase in petals during flower development and senescence.

Materials and methods

Plant materials and growth conditions

Plants of *Calendula officinalis* L. and *Aster novae belgii* L. were grown within the experimental cages of Botany Department, Kurukshetra University, Kurukshetra under natural condition of day and night. The length, breadth and height of the cage were 12×12×2.5 m respectively. Within the cage three experimental plots each were used for growing *C. officinalis* and *A. novae belgii* plants. The dimension of each plot was 3×2 m^2 and was provided with 40 Kg of dung manure. The pH of the soil and soil conductivity were 8.50 and 0.12 dc respectively. The seeds of deep orange-flowered variant of *C. officinalis* and purple-flowered variant of *A. novae belgii* were sown in the pulverized seed beds of the cage in rows during September-October and flowers were produced from January-March. The spacing between adjacent plants and between rows was 30 cm. Uniformity in length and flower head diameter was maintained while flowers were tagged in mid-January. Variations in day/night temperature were 12-15 ºC/4-7 ºC. Pesticides were not used at any stage of growth and development. Water was provided to these plants at 3-day intervals during vegetative and flowering periods. The tagged flowers were harvested at different developmental stages viz. half open, fully open, beyond open and advanced senescent stages (Plate 1).

Sampling

Flowers of different stages were harvested in the morning hours. Flower petals were plucked with the help of forceps from the basal part of flower, washed and dried in the folds of filter paper and samples were made in such a manner that atleast three replicates were available for most of the estimations and biochemical analysis. The diameter of the flower head was recorded by a millimeter scale and each data was based upon 10 randomly selected replicates. After recording the fresh weight, samples were put in the deep freezer till their analysis. In case of ascorbate peroxidase (APX) and superoxide dismutase (SOD), analyses were carried out immediately after collecting petals.

Flower fresh weight, moisture content and membrane stability index

Changes in moisture content as well as membrane stability index (MSI) were determined at each flower development stage for both species. Fresh weight of each flower (only flower head) was measured immediately after harvest.

Dry mass was determined by drying 100 mg of the petal sample in oven at 70 ºC for 24 h until reaching a constant weight. Moisture content was determined as the difference between fresh and dry mass.

Membrane stability index (MSI) was calculated on the basis of electrolyte leakage of petals. The electrolyte leakage was measured by recording electrical conductivity of leachates in double distilled water (DDW) at 40 °C and 100 °C (Deshmukh et al., 1991). Two similar discs (0.1 gm) from petals were cut to uniform size and placed in two separate test tubes containing 10 ml of DDW. One disc was kept at 40 °C for 30 min. and the other at 100 °C for 15 min. and their respective electric conductivities C_1 and C_2 were measured with a conductivity meter (Digital Conductivity Meter, HP G-3001) by the following formula:

$$\text{MSI}\ (\%) = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100$$

Determination of peroxidase (GPOX) activity and malondialdehyde (MDA) content

Total peroxidase (GPOX) activity was measured by the method of Maehly (1954). One hundred milligram of sample was homogenized with ice cold phosphate buffer (pH-7.0) and centrifuged in a Remi centrifuge (R-8C) at 5000 rpm for 10 min. Supernatant was taken out as an enzyme source and its final volume was raised to 10 ml with the above

ice cold phosphate buffer. The reaction set was prepared by mixing 2 ml of enzyme source, 2 ml of phosphate buffer (pH-7.0), 2 ml of guaiacol (20 mM) and 2 ml of H_2O_2 (10 mM) in sequence. In the blank set 2 ml of enzyme source, 2 ml of phosphate buffer (pH-7.0) and 4 ml of DDW were added. These sets were kept undisturbed at room temperature exactly for 10 min. The absorbance of the reaction set was taken in a uv-vis spectrophotometer (Specord-205, Analytic Jena, Germany) at 420 nm. Specific activity of peroxidase was expressed in terms of mg protein per 10 min. Protein was estimated from the same extract following the procedure of Bradford (1976) with Coomassie brilliant blue dye G-250 at 595 nm using above mentioned model of spectrophotometer.

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content following the method of Heath and Packer (1968). Two hundred milligram of petal sample was homogenized in 2 ml of 50 mM phosphate buffer (pH 7.0).The homogenate was centrifuged at 8000 rpm for 20 min in a Remi centrifuge (R-8C).To 0.5ml aliquot of the supernatant, 2 ml of 5 g L^{-1} thiobarbituric acid (TBA) in 200 g L^{-1} trichloroacetic acid (TCA) was added. The mixture was heated at 90 ºC for 30 min in the water bath and then quickly cooled in an ice water bath. After centrifugation at 8000 rpm for 10 min the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption of each sample at 600 nm was also recorded and subtracted from the absorption recorded at 532 nm. The concentration of MDA, an end product of lipid peroxidation was calculated according to its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Determination of superoxide dismutase (SOD) activity and protein

SOD activity was estimated by measuring the decrease in absorbance of superoxide- nitroblue tetrazolium chloride complex by the enzyme (Giannopolitis and Ries 1977). Fifty milligram of petals homogenized in 2 ml of 0.1 M EDTA-phosphate buffer (pH-7.8) and the final volume was raised to 100 ml with DDW. This was centrifuged at 15000 g and resultant supernatant was used as crude extract. A reaction mixture containing 0.1 ml enzyme extract, 0.9 ml of double distilled water (DDW), 0.5 ml of 300 mM sodium carbonate (Na_2CO_3), 0.5 ml of 378 µM p-nitroblue tetrazolium chloride (NBT), and 0.5 ml of 78 mM L-methionine were taken in test tubes in triplicate from each sample. The tubes without enzyme extract served as standard. The reaction was started by adding 0.5 ml of riboflavin (7.8 µM) and keeping the tubes below a light source of 100 µ mol photon $m^{-2}s^{-1}$ PFD from fluorescent lamp for 15 min. The reaction was stopped by switching off the light and placing the tubes in darkened box. The tubes without enzyme developed the maximum colour. A non-irradiated complete reaction mixture, without colour served as a blank. The initial rate of reaction was measured as the difference in increase in absorbance at 560 nm in the absence or presence of extract proportional to the amount of enzyme. The unit of SOD activity was obtained as that amount of enzyme which under experimental conditions caused 50 % inhibition of the NBT photoreduction as observed in the absence of enzyme.

Protein content was estimated by the method of Bradford (1976) using Coomassie Brilliant blue G-250. Bovine Serum Albumin (BSA) was used for making a standard curve for protein estimation.

Ascorbate peroxidase (APX) activity

The ascorbate peroxidase activity was measured by the method of Janda et al., (1999). One hundred mg of plant sample was crushed in one ml of 100 mM HEPES/sodium hydroxide (NaOH) buffer of pH 7.6 containing 8.8 mg acid ascorbate in pre-cooled pestle and mortar. This was centrifuged at 10,000 rpm at 4º C for 5 min. and resultant supernatant was used as crude extract. The reaction mixture was prepared by adding 50 µl ascorbate in DDW, 1ml 50 mM HEPES buffer, 50 µl plant extract and 100 µl 3 mM H_2O_2. In the mixture without enzyme, no crude extract was added, instead of it 50 µl 50 mM HEPES buffer was added which served as blank. The final reaction mixture was 120 µl. The change in absorbance was read at 290 nm in the cuvette using uv-vis spectrophotometer. Specific activity of APX was expressed in terms of mg protein per min. Protein was estimated from the same extract following the procedure of Bradford (1976) with Coomassie brilliant blue dye G-250 at 595 nm in an uv-vis spectrophotometer as mentioned above.

Catalase (CAT) activity

The supernatant that was used for APX activity was also used to determine catalase activity (CAT) following the method of Aebi (1984). The reaction mixture was prepared by adding 1.5ml of 50mM HEPES buffer, 1.2 ml of 150mM H_2O_2 and 30 µl petal extract. In the mixture without enzyme, no crude extract was added, instead of it 50 µl 50 mM HEPES buffer was added. The change in absorbance was read at 490 nm in the test tube cuvette using uv-vis spectrophotometer. Specific activity of catalase was expressed in terms of per mg protein. Protein was estimated from the same extract following the procedure of Bradford (1976) as described earlier.

Statistical analysis

Data in the tables and figures are expressed as means ±standard error. A mean of three readings was taken in every replication excepting flower head diameter. In biochemical estimation, three aliquots were used for each replication. Statistical analysis was done using Statistical Packages for Social Sciences (SPSS) version 8.0. One-way ANOVA was used to test whether there was a significant difference in various estimations.

Results and discussion

Morphological observations

The relative morphological characteristics of petals and the flower development stages are shown in Plate 2. Developmental stages of flowers that can be recognized are: (I) half open stage (cup shaped), (II) fully open stage (saucer shaped), (III) beyond open stage (ray florets attain maximum diameter) and (IV) advanced senescent stage (flower head closed; ray florets wilted; petals inrolling). Time taken for the appearance of flower stage I from the buds was 6 days thereafter stage II was noticed after another 4-day. Transformation of stage II to III took 6 days and stage III to IV additional 7 days.

Flower diameter and fresh weight increased as the flower development progressed from stage I to stage III and thereafter decreased to stage IV in both species (Table 1). At the end of stage III, *C. officinalis* flowers showed complete inrolling of petals starting from lateral sides to the middle, while in *A. novae belgii* petals rolled downward towards the abaxial side. Flower moisture content and membrane stability index declined from stage I to IV in both species; however, the reduction was greater in *C. officinalis* than *A. novae belgii* (Table 1). A reduction in flower diameter, fresh weight, moisture content and membrane stability index was noticed by Shahri et al., (2011) in *Helleborus orientalis* Lam.cv. Olympicus. An increase in membrane permeability is a characteristic attribute of senescing plant tissues (Ferguson and Simon, 1973; Suttle and Kende, 1980). The loss of membrane integrity, the last and irreversible phase of senescence, is closely related with modifications of lipids, principally due to peroxidation (Paulin et al., 1986).

Lipid peroxidation and specific activity of peroxidase increased from initial to final stages of flower development in both species as noticed in petals (Fig 1a and 2a). Lipid peroxidation not only threatens the integrity and function of membranes and membranous proteins but also produce a variety of toxic aldehydes and ketones (Valentine et al., 1998; Wilhelmova et al., 2006). MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero, 1990). Specific activity of GPOX in petals of *C. officinalis* and *Aster* increased from stage I to IV (Fig 1a and 2a). Parish (1968) suggested that increase in peroxidase activity was one of the most reliable indicators of maturity. Enhanced peroxidase activity was associated with an increase in the level of peroxides and free radicals, which reacted with cellular constituent (Fridovich, 1995) and are probably involved in the promotion of senescence (Brenen and Frenkel, 1977). Peroxidase activity and lipid peroxidation increase in both *Chrysanthemum* (Bartoli et al., 1995) and daylily (Chakrabarty et al., 2009) during senescence.

Free radicals are known to participate in plant senescence (Dhindsa et al., 1981). Several enzymes such as SOD, CAT and GPOX are involved in the scavenging of free radicals in plant systems (Monk et al., 1989). In our experiment with *C. officinalis* and *Aster*, flower development was characterised by increment in superoxide dismutase activity and protein content while both declined with the onset of senescence (Fig. 1b and 2b). In carnation petals, specific activity of SOD decreased after full blooming (Droillard et al., 1989) whereas in daylily, the activity increased with the onset of senescence (Panavas and Rubinstein, 1998). The decrease in protein levels during senescence has been suggested to be due to decreased synthesis and/or increased degradation (Celikel and van Doorn, 1995). In *Hemerocallis*, a sharp decrease in protein levels has been shown to precede the visible symptoms of senescence (Lay-yee et al., 1992).

Ascorbate peroxidase (APX) activity increased about 874 per cent from stage I to II, registered almost 4.8 fold increment between stage II and III; followed by a sharp fall at stage IV (79.7 n mol mg^{-1} protein min^{-1}) in *C. officinalis* petals. In *A. novae belgii* petals, however, APX registered a rise of about 1424 per cent from stage I to III followed by sharp decline at stage IV (18.8 n mol mg^{-1} protein min^{-1}). Stage wise trend in the activity of catalase in both *C. officinalis* and *A. novae belgii* resembles to the pattern of APX in *C. officinalis* (Fig 1c and 2c). Senescent petals of both plants have revealed very low activity of APX and CAT. Available information with cut flowers/inflorescence indicates the increment in CAT activity during flower development upto flower opening in petals of several species (daylily: Panavas and Rubinstein, 1998; Chakrabarty et al., 2009; *Iris*: Bailly et al., 2001; *Gladiolus*: Yamane et al., 1999; carnation: Zhang et al., 2007). Although CAT activity declined in *Gladiolus* and carnation petals during senescence, increment has also been reported in Iris and daylily. Reports also reveal higher APX activity in young flowers and lower values in senescent ones (Gladiolus: Hossain et al., 2006; daylily: Panavas and Rubinstein, 1998; carnation: Zhang et al., 2007; *Iris*: Bailly et al., 2001). However, Bartoli et al., (1995) reported rise in APX and CAT activities during senescence of carnation petals. CAT and APX perform key roles in detoxification of H_2O_2 (Scandalios, 1993). However, there is also number of other enzymes involved in ROS scavenging (Rogers, 2012). Membrane bound APX scavenge the H_2O_2 which was produced by the action of SOD on the superoxide radical (O_2^-). The ascorbate peroxidase activity was found to be directly correlated with the reduction in free radical induced membrane damage (Nakano and Asada, 1981).

Plate 1 Flowers of *Calendula* and *Aster*

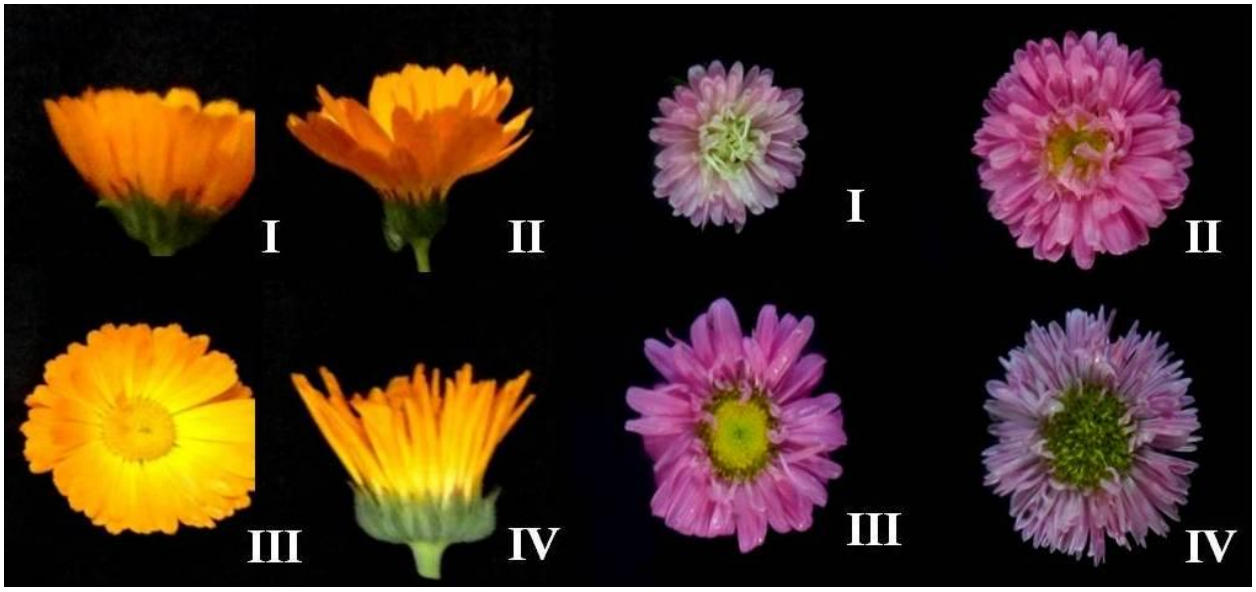

Plate 2 Developmental stages studied in Calendula and Aster: I. Half open stage; II. Fully open stage; III. Beyond open stage; and IV. Advanced senescent stage.

Table 1 Flower diameter, fresh weight, moisture content and membrane stability index in uncut *Calendula officinalis* L. and *Aster novae belgii* L. flowers at different stages. Means followed by different letters are significantly different at 0.05 % level, using DMRT.
* Each value indicates mean of three replicates.

Flower	PARAMETERS	Ist stage	IInd stage	III rd stage	IVth stage
***Calendula officinalis* L.**	Flower diameter (cm)	2.82 ± 0.09^{c}	5.04 ± 0.09^{b}	7.76 ± 0.10^{a}	4.3 ± 0.10^{b}
	Fresh weight (gm)	1.1082 ± 0.1^{d}	2.756 ± 0.09^{b}	3.593 ± 0.09^{a}	1.930 ± 0.1^{c}
	Moisture content (%)	94.8 ± 2^{a}	88.36 ± 1^{b}	75.2 ± 5^{c}	65.3 ± 1^{d}
	Membrane stability Index (%)	189.5 ± 0.96^{a}	178.3 ± 0.71^{b}	166.55 ± 0.82^{c}	134.7 ± 0.97^{d}
***Aster novae belgii* L.**	Flower diameter (cm)	2.9 ± 0.05^{c}	4.3 ± 0.17^{b}	6.65 ± 0.17^{a}	4.9 ± 0.30^{b}
	Fresh weight (gm)	1.5 ± 0.10^{c}	2.87 ± 0.09^{ab}	3.2 ± 0.1^{a}	2.1 ± 0.12^{b}
	Moisture content (%)	91.5 ± 2^{a}	84.5 ± 0.6^{b}	81.5 ± 0.6^{b}	78.2 ± 2^{c}
	Membrane stability Index (%)	88.5 ± 0.45^{a}	81.2 ± 0.2^{b}	71.68 ± 0.31^{c}	54.5 ± 0.28^{d}

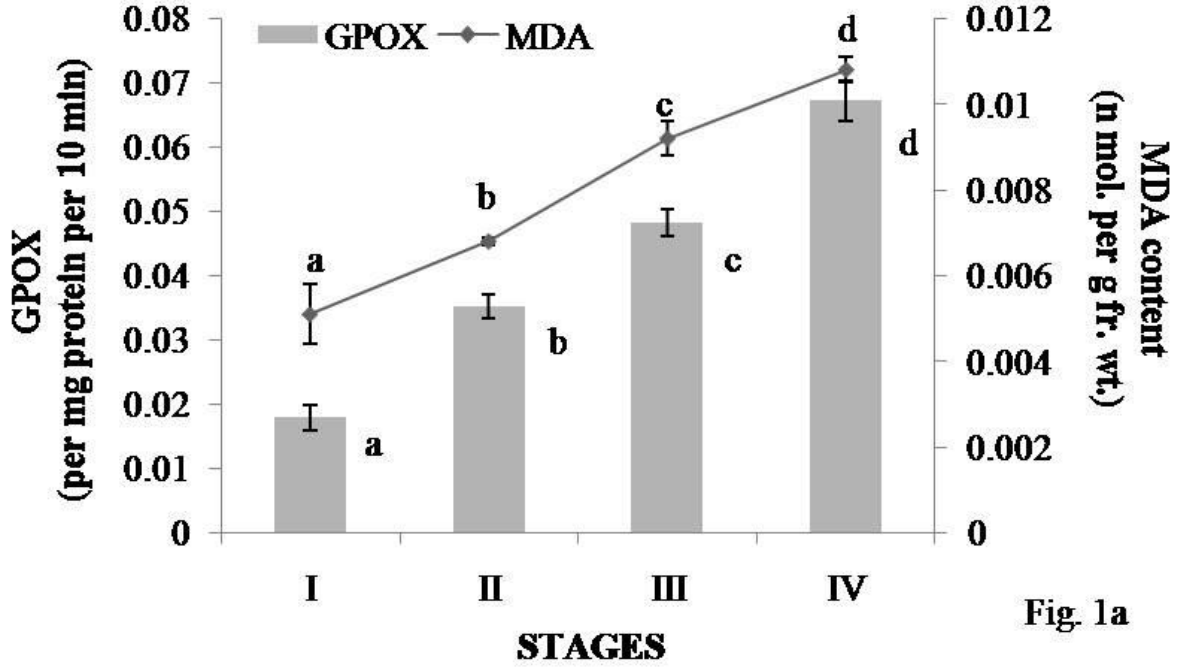

Fig. 1a Specific activity of GPOX and MDA content in petals of uncut *Calendula officinalis* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

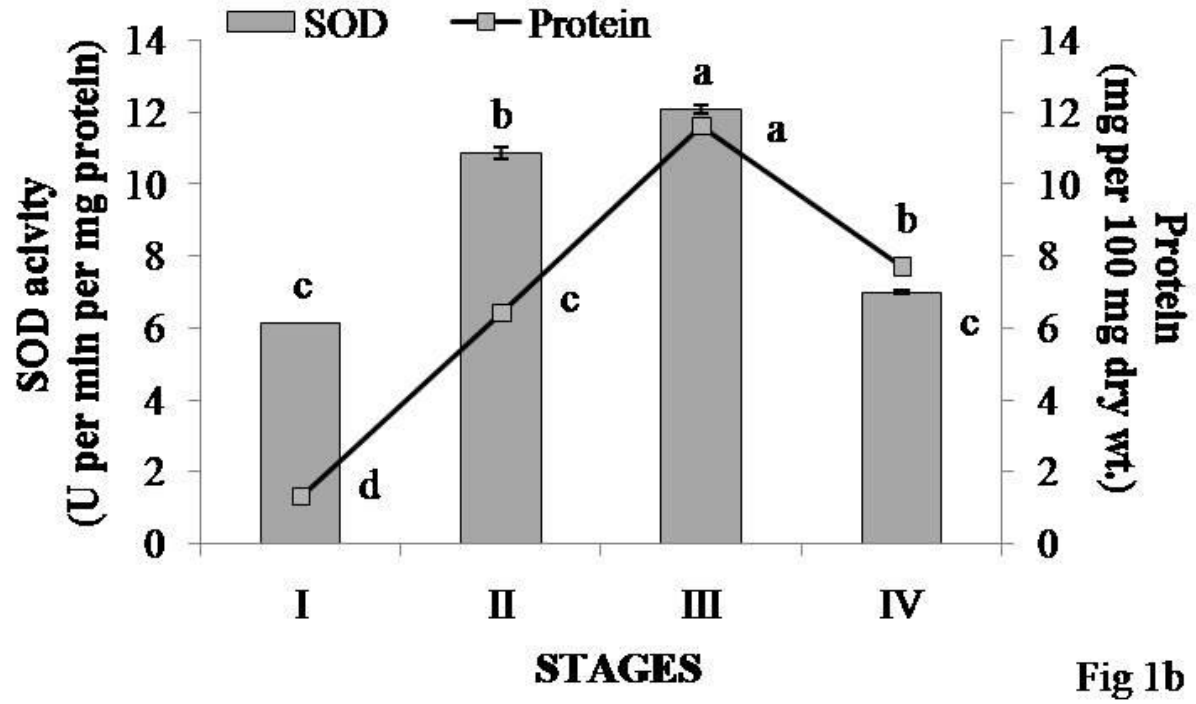

Fig. 1b Protein content and SOD activity in petals of uncut *Calendula officinalis* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

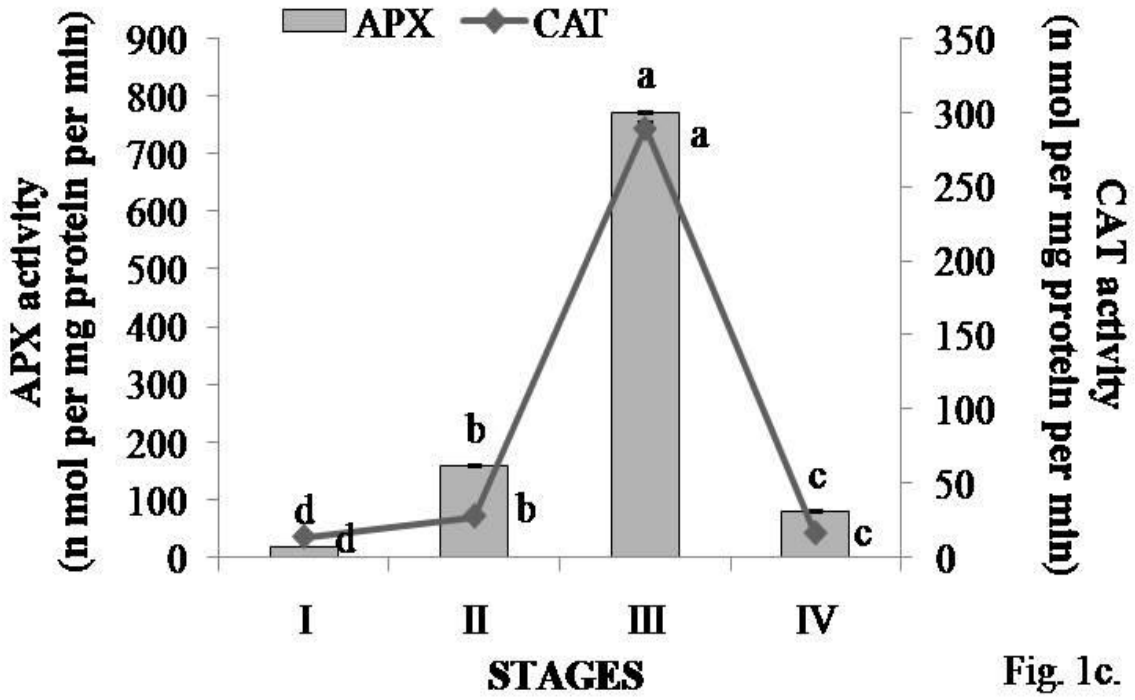

Fig. 1c CAT and APX activity in petals of uncut *Calendula officinalis* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

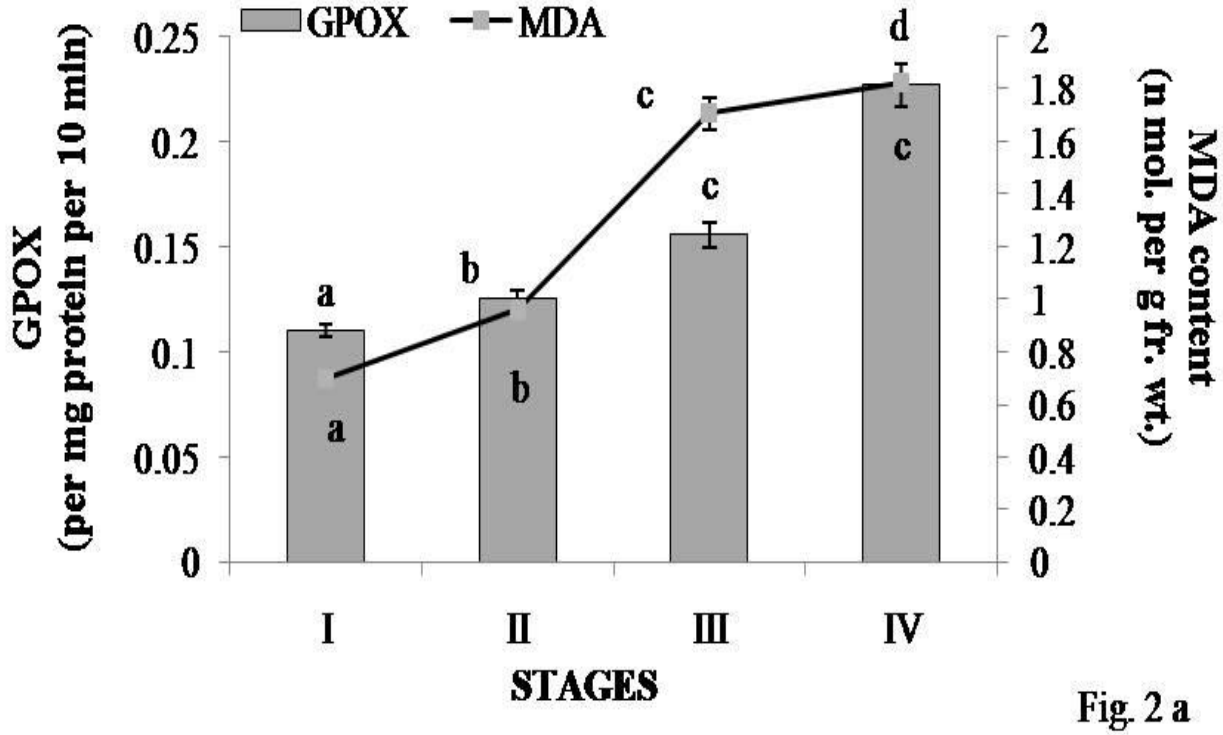

Fig. 2a Specific activity of GPOX and MDA content in petals of uncut *Aster novae belgii* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

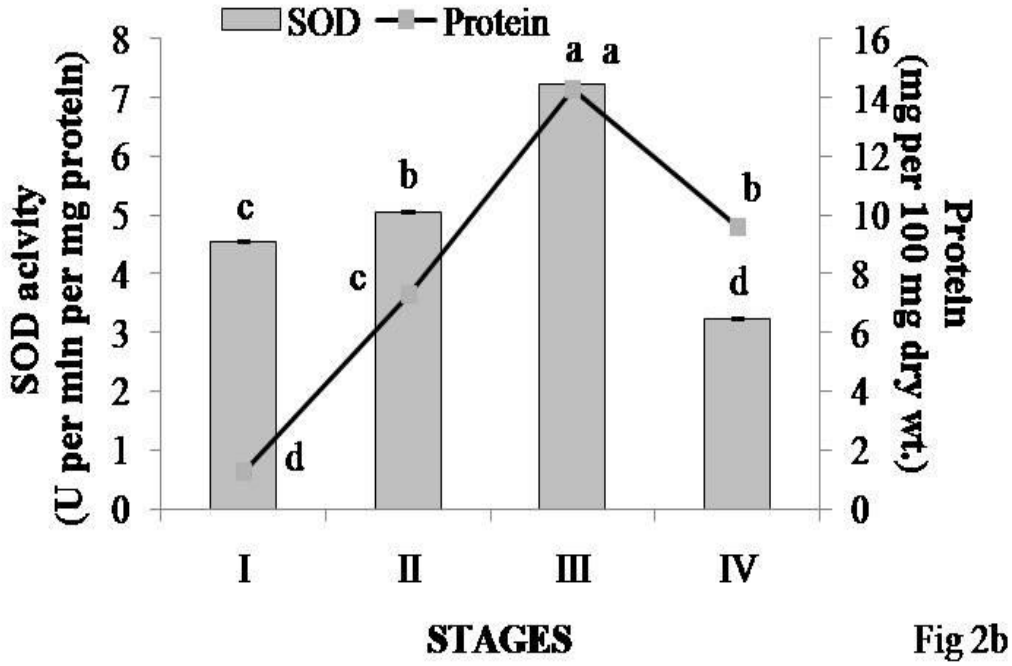

Fig. 2b Protein content and SOD activity in petals of uncut *Aster novae belgii* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

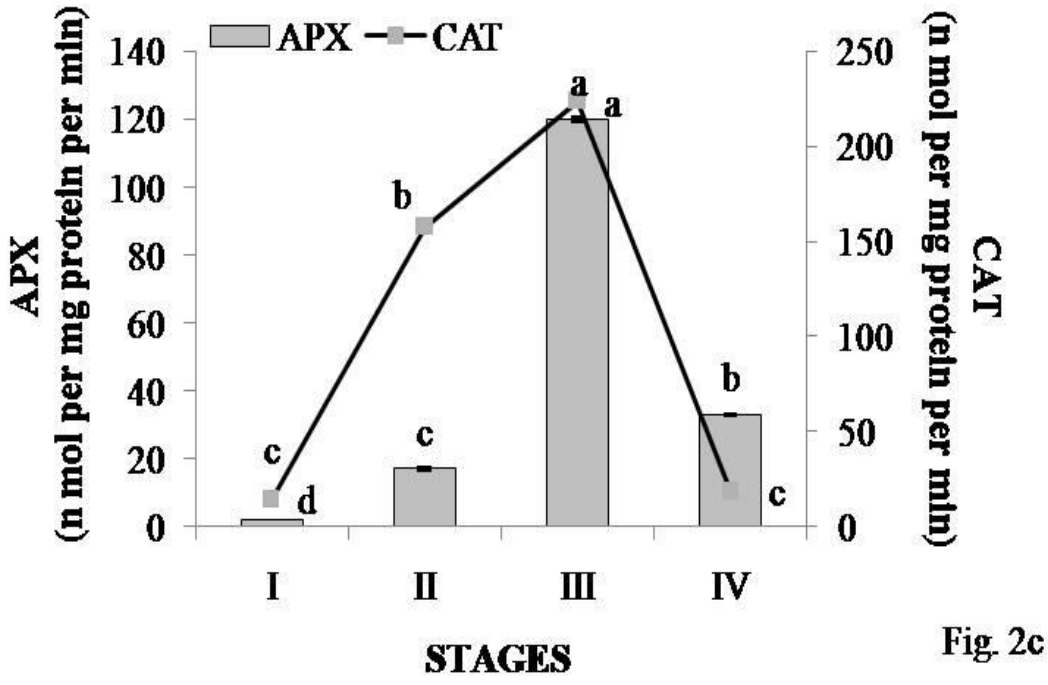

Fig. 2c CAT and APX activity in petals of uncut *Aster novae belgii* L. flowers. Different letters in the figure represent values are significantly different at 0.05 % level, using DMRT.

Conclusions

An investigation on the flower development of *C. officinalis* and *A. novae belgii* has revealed characteristic morphological changes in shape, size, weight and diameter of flowers. Further, petals of both the plants were characterised in having very low activity of SOD, an important enzyme at stage I which increased significantly at stage II and III. To counteract the lipid peroxidation and membrane damage, enzymes like APX and CAT appear in the petal tissue. These antioxidant enzymes remained effective till stage III of the flower development. The onset of senescence (flower stage IV) was characterised by a decline in SOD and very low activities of APX and CAT.

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