Full Length Research Paper

Oxidative stress and antioxidant activity as the basis of senescence in *Hemerocallis* (day lily) flowers

Debasis Chakrabarty[1], Arvind Kumar Verma[2] and Subodh Kumar Datta[3]

[1]Plant Molecular Biology and Genetic Engineering, National Botanical Research Institute, Rana Pratap Marg, Lucknow-226001, India.
[2]Floriculture Section, National Botanical Research Institute, Lucknow-226001, India.
[3]Bose Institute, Madhyamgram Experimental Farm, Jessore Rd., 24-Parganas (N), Kolkata 700 129, India.

Senescence can be viewed as a developmental change that leads to the death of plant tissues. Naturally senescence is generally accepted as being genetically programmed. Petals are the floral organs which primarily determine the commercial longevity of flowers and as a consequence, it is necessary to study the physiological, biochemical and genetic processes that occur during floret senescence. Most of the early work on flower senescence focused on ethylene sensitive flowering plants. It has also been reported that lipid peroxidation and membrane damage are involved in deterioration of petals of several ethylene sensitive or insensitive plants. However, little information is available on the actual role of oxidative stress and the protective enzymatic systems with their corresponding isoenzymes in relation to progression of flower senescence in plants. One important bulbous ornamentals (*Hemerocallis*) was used as experimental materials in order to establish a proposed model of petal senescence based on the changes in antioxidant enzymes behavior over the senescence period. Hemerocallis flower blooms and fades at the end of same day. The vase life parameters were evaluated at 21°C and 16 h illumination (36 mol m^{-2} s^{-1}) under laboratory condition. The stage of flower bud development was arbitrarily divided into 4 different developmental stages. Different parameters selected for the studies were changes in fresh weight, antioxidant enzymes like ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and Guaiacol peroxidase (G-POD), membrane integrity, H_2O_2 etc. Significant decrease in petal fresh weight, increase in lipid peroxidation, decrease in membrane stability index, decline in APX activity, progressive increase in SOD activity, changes in GR activity and increase in endogenous H_2O_2 were observed over the senescence period. All the parameters have been critically analysed and compared over the senescence period to understand the petal senescence in day lily.

Key words: Hemerocallis, cut flower, petal senescence, antioxidant.

INTRODUCTION

Cut flower is one of the major components of floriculture industry. Type of cut flower and its quality depends upon customer choice. Vase life of cut flower is most attractive and economic components of cut flower. Flower senescence is a developmental change leading to death of petal tissues. Senescence is a genetically programmed step wise biochemical changes which varies from flower to flower. Efforts are going on world wide to understand the physiological and biochemical changes related to senescence. For such programmed cell death, information related to the role of reactive oxygen species and cellular enzymatic antioxidant defense mechanism that have been reported from time to time in different ornamentals with special reference to the role of ethylene in ethylene sensitive flowering crops (Sylvestre et al., 1989; Paulin et al., 1986; Baker et al., 1978). Panavas et al. (1999) reported a genetically based program that leads to senescence and cell death in daylily petals. In daylily petals, exogenous H_2O_2 stimulates ion leakage and endogenous levels of H_2O_2 increase along with thiobarbituric acid reactive substances (TBARS) before flower

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*Corresponding author. E-mail: subodhskdatta@rediffmail.com, subodhskdatta@yahoo.com.

+0 h   +8 h   +16 h   +24 h

Figure 1. Developmental stages of Hemerocallis (day lily) flower.

opening (Panavas and Rubinstein, 1998). Since up regulation of endogenous H_2O_2 would help to stimulate senescence, it is important to determine the activity of H_2O_2 regulatory enzymes. However, little information is available on the actual role of oxidative stress and the protective enzymatic systems with their corresponding isozymes in relation to progression of flower senescence (Bartoli et al., 1995; Hossain et al., 2006). Time taken to understand the physiological and biochemical change, through experiment, depends upon the total senescence period of a particular flower from flower bud initiation to fading of flower (2 - 10 days) . The daylily flowers provide an excellent material for the study of petal senescence and cell death because this flower crop is not only non-climacteric but also it completes its reproductive stage that is, flower bud opening to fading in one day which may be used as an excellent material for understanding different biochemical processes (organ senescence and cell death) within short time.

MATERIALS AND METHODS

Plant material

Day lily (*Hemerocallis*) which blooms during April - May was selected as experimental material. Stems were harvested when flower bud showed colour. The basal 2 - 3 cm portion of the stems were recut under water and the stems were put in distilled water. Antibiotic ampicillin was added at a concentration of 100 mg l^{-1} to prevent infection. The vase life parameters were evaluated at 21°C and 16 h illumination (36 mol m^{-2} s^{-1}) under laboratory condition. The stage of bud development (mean of all the buds on the stem, based on the numerical scale) were arbitrarily divided into 4 different developmental stages: stage 1, 0 day after harvest; stage 2, 8 h after harvest; stage 3, 16 h after harvest; stage 4, 24 h after harvest (Figure 1).

Leakage of ions from the petals

Leakage of ions from the petals was measured according to Sairam et al. (1997) and expressed as MSI (membrane stability index) percentage. Petals (0.2 g) were taken in 20 ml of deionized water in

two sets. One set was kept in water bath at 40°C constant temperature for 30 min and its conductivity (C1) was measured using an electrical conductivity meter (DiST 3 Conductivity meter, Hanna make, Portugal). Second set was kept in a boiling water bath (100°C) for 10 min and its conductivity was recorded (C2) . MSI was expressed in percentage using the formula: [1-(C1/C2)] X 100.

Petal relative water content

Petal relative water content was determined and calculated from the following relationship: (W_{fresh} - W_{dry})/(W_{turgid} - W_{dry}) × 100, where W_{fresh} is the weight of freshly harvested sample, W_{turgid} is the turgid weight after saturating the sample with distilled water for 24 h at 4°C, and W_{dry} is the oven-dry (70°C for 48 h) weight of the sample (Weatherley, 1950).

H_2O_2 content

Hydrogen peroxide content of the petal was measured spactophotometrically after reaction with potassium iodide (KI) . The reaction mixture consisted of 0.5 ml of 0.1% trichloroacetic acid (TCA), petal extract supernatant, 0.5 ml of 100 mM K-phosphate buffer and 2 ml reagent (1M KI, w/v in fresh double-distilled water).The blank probe consisted of 0.1% TCA in the absence of petal extract. After 1 h of reaction in darkness, the absorbance at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentration of H_2O_2.

Antioxidant enzyme assay

For determination of antioxidant enzyme activities, 0.5 g of florets was homogenized in 1.5 ml of respective extraction buffer in a pre-chilled mortar and pestle by liquid nitrogen. The homogenate was filtered through four layers of cheesecloth and centrifuged at 22000 g for 20 min at 4°C. The supernatant was re-centrifuged again at 22000 g for 20 min at 4°C for determination of antioxidant enzyme activities. Protein concentration of the enzyme extract was determined according to Bradford (1976).

SOD activity

Petals were homogenized in 1 ml cold 100 mm K-phosphate buffer (pH 7.8) containing 0.1 mm ethylenediamine tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone and 0.5% (v/v) Triton X-100.

For assay of superoxide dismutase (SOD), fresh leaves (1 g) were homogenized in 25 mL PVP with a chilled pestle and mortar. The homogenate was centrifuged at 20,000 g for 20 min. the supernatant was collected and used for the assay of SOD following the method of Beyer and Fridovich (1987). The reaction mixture was prepared by mixing 25 mL of 50 mmol/L potassium phosphate buffer, pH 7.8, 35 mg of L-methionine (9.9 mM/L), 7.5 µl of Triton X-100 (0.025%) and 4 mg NBT (57 µmol/L). Aliquots (1 mL) of this mixture were delivered into small glass tubes, followed by 20 µL of enzyme extract and 10 µL of Riboflavin (4.4 mg/100 ml). The cocktile was mixed and then illuminated for 7 min in an aluminum foil-lined box, containing two 20 w florescent tubes. A control tube in witch the sample was replaced by 20 µL of buffer was run in parallel and the A_{560} was measured in all tubes. The test tubes containing the reaction mixture were exposed to light immersing the glass tubes in a cylindrical glass container three fourth filled with clean water and maintained at 25°C and placed in between two 20 w florescent tubes. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per min. Fifty percent of inhibition was taken as equivalent to 1unit of SOD activity.

APX activity

Ascorbate peroxidase (APX) was assayed as described by Nakano and Asada (1981). The reaction mixture contained 25 mmol/L potassium phosphate (pH 7.0), 0.1 mmol/L EDTA 0.25 mmoL/L ascorbic acid and 1 mmol/M H_2O_2. The reaction was stated at 25°C by the addition of H_2O_2 after adding the enzyme extract containing 50 µg of protein. The decrease at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8 mmol/L^{-1} cm^{-1}.

CAT activity

Catalase (CAT) activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm, taking at 240 nm as 43.6 mol/L^{-1} cm^{-1} (Patterson et al., 1984) . The reaction mixture contained 50 mmol/L potassium phosphate (pH 7.0), 0.1 mmol/L H_2O_2. The reaction was run at 25°C for 2 min, after adding the enzyme extract containing 20 µg of protein, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

G-POD activity

Guaiacol peroxidase (G-POD) activity was measured spectrophoto-metrically at 25°C by following the method of Tatiana et al. (1999). The reaction mixture (2 ml) consisted of 50 mmol/L potassium phosphate (pH 7.0), 1 mmol/L H_2O_2, and 2.7 mmol/L (0.25% V/V) guaiacol. The reaction was started by the addition of an enzyme extract equivalent to 50 µg protein. The formation of tetraguaiacol was measured at 470 nm (= 26.6 mmol/ L^{-1} cm^{-1}).

Measurement of lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdihyde (MDA) produced by the thiobarbituric acid (TBA) reaction, as described by Dhindsa et al. (1981). Fresh leaves (0.2 g) of control and Nacl treated plants were homogenized in 2 mL of 20% (w/v) Trichloro acetic acid (TCA). The homogenate was centrifuged at 3500 g for 20 min. 1.5 mL of 20% TCA containing 0.5% (w/v) TBA

and 100 µL 4% butylated hydroxytoluene (BHT) in ethanol were added to 0.5 mL of the aliquot of the supernatant. The mixture was heated at 95°C for 30 min and was then quickly cooled in ice. The contents were centrifuged at 10,000 g for 15 min and the absorbance was measured at 532 nm. The value of non-specific absorption was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mmol/ L^{-1} cm-^{1}.

Native polyacrylamide gel electrophoresis (PAGE) and activity stain

Native PAGE was performed at 4°C, 180 V, following Laemmli (1970) . For SOD and APX, the enzyme solutions were subjected to native PAGE with 10% polyacrylamide gel. Activity stain for each enzyme was carried out as follows. APX activity was detected by the procedure described by Mittler and Zilinskas (1993). The gel equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min was incubated in a solution composed of 50 mM sodium phosphate (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gel was washed in the buffer for 1 min and submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 10 - 20 min with gentle agitation in the presence of light. SOD activity was detected by the procedure described by Beauchamp and Fridovich (1971). The gel equilibrated with 50 mM K-phosphate buffer (pH 7.8) containing 2.8×10^{-5} M riboflavin, 0.028 M N,N,N',N'-tetramethyl ethylenediamine (TEMED) for 30 min. The gel was washed in distilled water for 1 min and submerged in a same solution (mentioned above) containing 2.45 mM NBT for 10 - 20 min with gentle agitation in the presence of light, the enzymes appeared as colourless bands in a purple background.

Experimental design

Experiments were set up in a completely randomized design and repeated three times with four replicates. Data were subjected to Duncan's multiple range test (DMRT) using SAS program (Version 6.12, SAS Institute Inc., Cary, USA). A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Fresh weight of the flower increased until stage 3 and decreased thereafter (Figure 2A). Results on relative water content (RWC) recorded in different stage of senescent flower and a significant decline in RWC was observed at stage 4 (Figure 2B). Significant increase in malondialdehyde (MDA) content was observed after stage 2 (Figure 2C). MDA, a decomposition product of polyunsaturated fatty acids hydroperoxides, has been utilized very often as a suitable biomarker for lipid peroxidation (Bailly et al., 1996), which is an effect of oxidative damage. This increase in lipid peroxidation over the senescence period was also confirmed by the high degree of membrane deterioration expressed as decrease in MSI. A steady decrease in MSI percentage upon the progression of flower senescence in day lily indicates the gradual loss of membrane's ability of selective ion leakage (Figure 2D).

During senescence marked changes occur in the bio-chemical and biophysical properties of the cell mem-
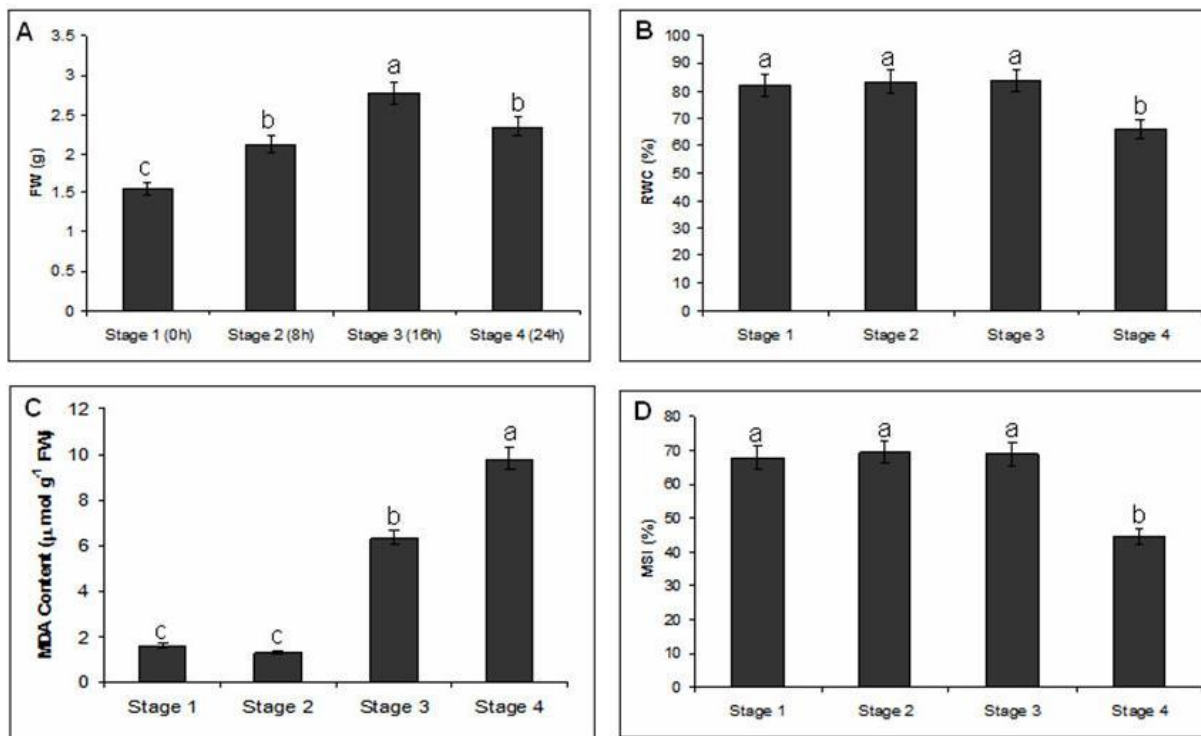
Figure 2. Fresh weight (FW), relative water content (RWC), malondialdihyde (MDA) content and percentage MSI changes of day lily flower during different stages of senescence. Different letters in each bar differ significantly according to DMRT test (P 0.05).
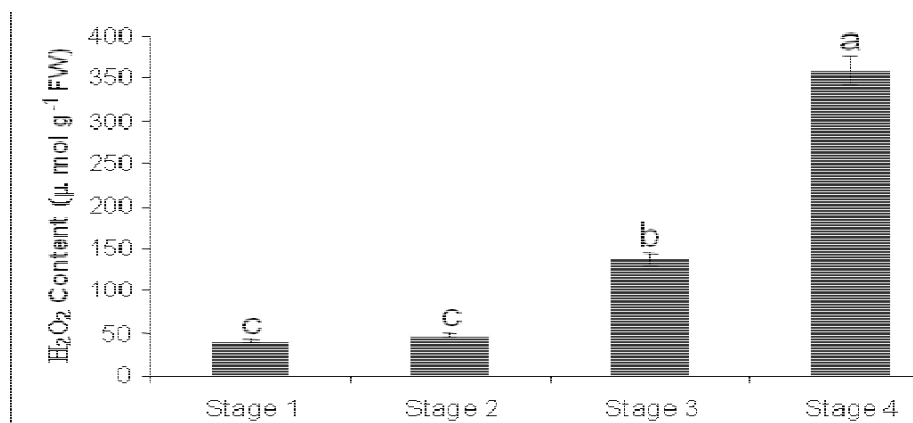

Figure 3. Changes in H_2O_2 content in the petals of day lily flower during different stages of senescence. Different letters in each bar differ significantly according to DMRT test (P 0.05).

branes. These result from losses of membrane phospholipids, increases in neutral lipids, increases in sterol to phospholipid ratio, and increases in the saturation: unsaturation index of fatty acids (Lesham, 1992; Thompson et al., 1998). Membrane polyunsaturated fatty acids are prone to oxidation either by enzymatic means like lipoxygenase (LOX) or through autoxidative events (nonenzyme catalyzed). Increase in lipid peroxidation, usually estimated as MDA content, accompanies the

increase in lox activity and the products of peroxidation are considered to perturb membrane function (Leverentz et al., 2002). It has already been reported that LOX activity may play a positive role in promoting senescence through oxidative membrane damage as seen in day lily (Rubinstein, 2000) and gladiolus (Peary and Prince, 1990; Hossain et al., 2006).

ROS content (H_2O_2 content) increased with age, highest being at the final stage of sampling (Figure 3). Singlet
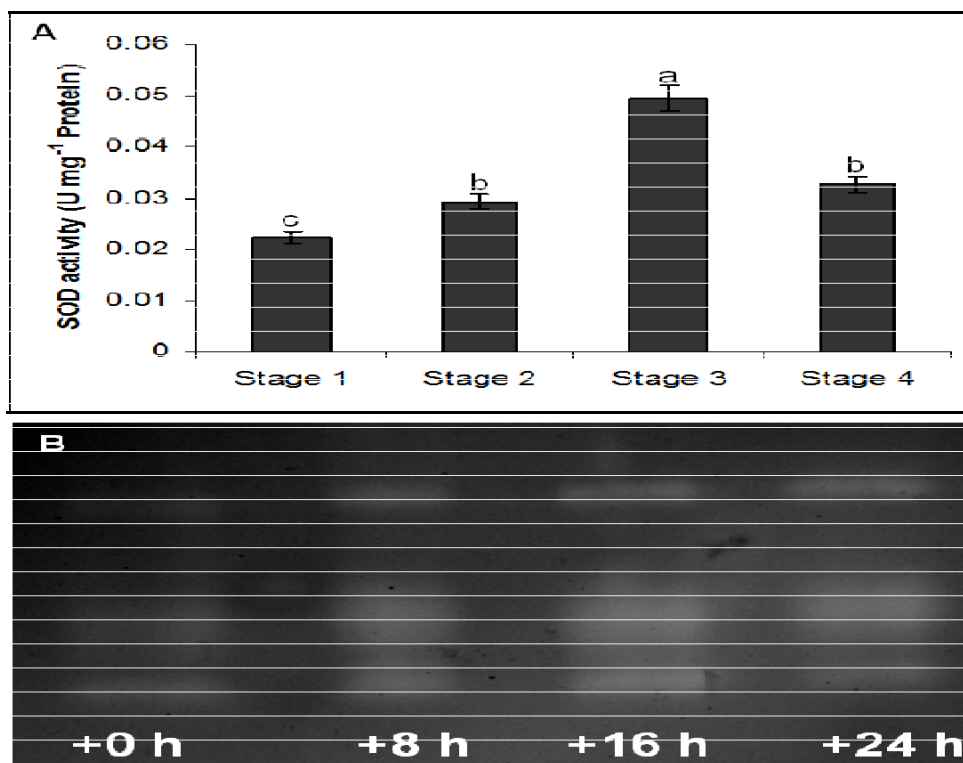
Figure 4. Changes in the level of SOD activity (A) and isozymes patterns of SOD (B) in the petals of day lily flower during different stages of senescence. Different letters in each bar differ significantly according to DMRT test (P 0.05).

oxygen, super oxide radical and H_2O_2 are reactive oxygen species that are generated when plant tissues are exposed to a variety of environmental conditions. Among the different ROS, only H_2O_2 is relatively stable and able to penetrate the plasma membrane as an uncharged molecule. Recently, H_2O_2, in addition to being a toxicant, has been regarded as a signaling molecule and a regulator of the expression of some genes in cells. H_2O_2 act as signal that initiates a transduction pathway towards plant cell death came from experiments in soybean cell cultures, in which a short pulse of H_2O_2 was sufficient to activate a hypersensitive cell death mecha-nism (Levine et al., 1994). Hydroperoxyl radicals ($HO_2^{\bullet-}$) that are formed from $O_2^{\bullet-}$ by protomation in aqueous solution can cross biological membranes and subtract hydrogen atoms from polyunsaturated fatty acids and lipid hydroperoxides, thus initiating lipid peroxidation (Halliwell and Gutteridge, 1989). Our results suggest that increased oxidative stress as indicated by increase in H_2O_2 content with plant age results in increased lipid degradation or lipid peroxidation as reflected in increase in MDA content which is further manifested in decline in MSI% observed in senescent florets (Figure 2).

Total SOD activity increased to its maximum on stage 3 and thereafter, it decreased (Figure 4A). Nondenaturing PAGE coupled with activity localization revealed three

SOD isozymes (Figure 4B).

Estimation of APX activity by spectrophotometry also showed a varying pattern. Spectrophotometric analyses showed that APX activity was high in stage 3 (Figure 5A). Examination of APX isozymes revealed three isozymes in day lily petals which expressed differentially in different stages of senescence (Figure 5B). The large increase of APX activity during senescence contributed to their stress tolerance. APX plays a key role in the ascorbate-gluta-thione cycle by reducing H_2O_2 to water using the reducing power of ascorbate and producing monodehydro-ascorbate (MDHA). Our results show that during sene-scence process, as a result of oxidative burst in the cells of florets accumulate H_2O_2 which in turn provoke APX enzymes to express but during the late senescence process the loss of APX activity results in higher concentration of H_2O_2 which might act as a signal molecule for PCD. Similar result also has been reported by Hossain et al., 2006 and suggested that down regu-lation of APX activity seems to be the prerequisite factor for inducing senescence in gladiolus tepal which in turn enhance high level of endogenous H_2O_2 accumulation.

Catalase activity steadily increased during vase life and highest activity was observed at stage 4 (Figure 6). Similarly total peroxidase activity was uniformly increased from young floret to mature stage and thereafter, declined
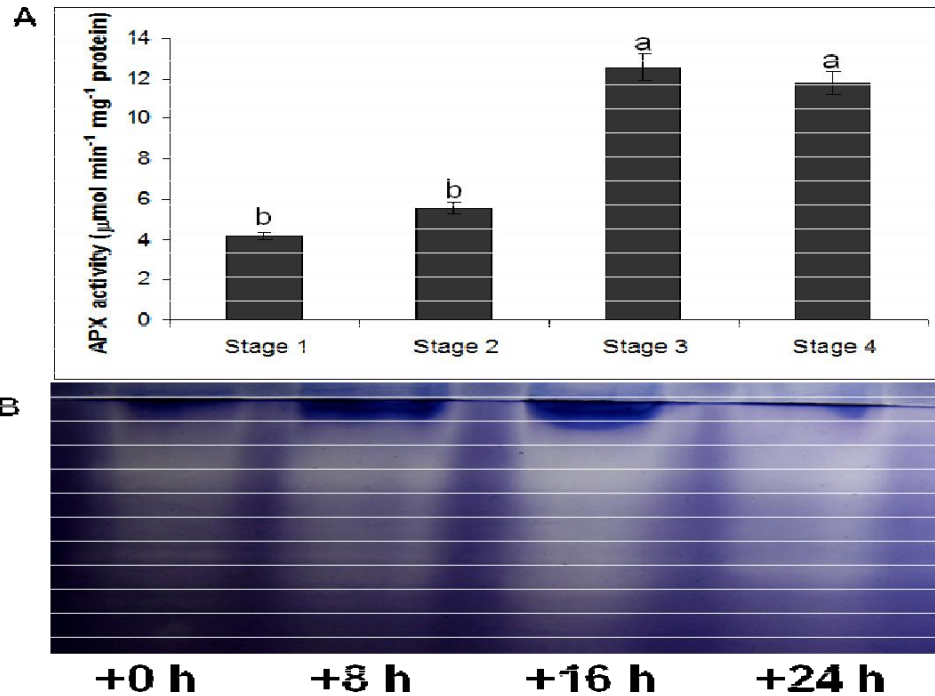
Figure 5 Changes in the level of APX activity and isozymes patterns of APX in the petals of day lily flower during different stages of senescence. Different letters in each bar differ significantly according to DMRT test (P 0.05).
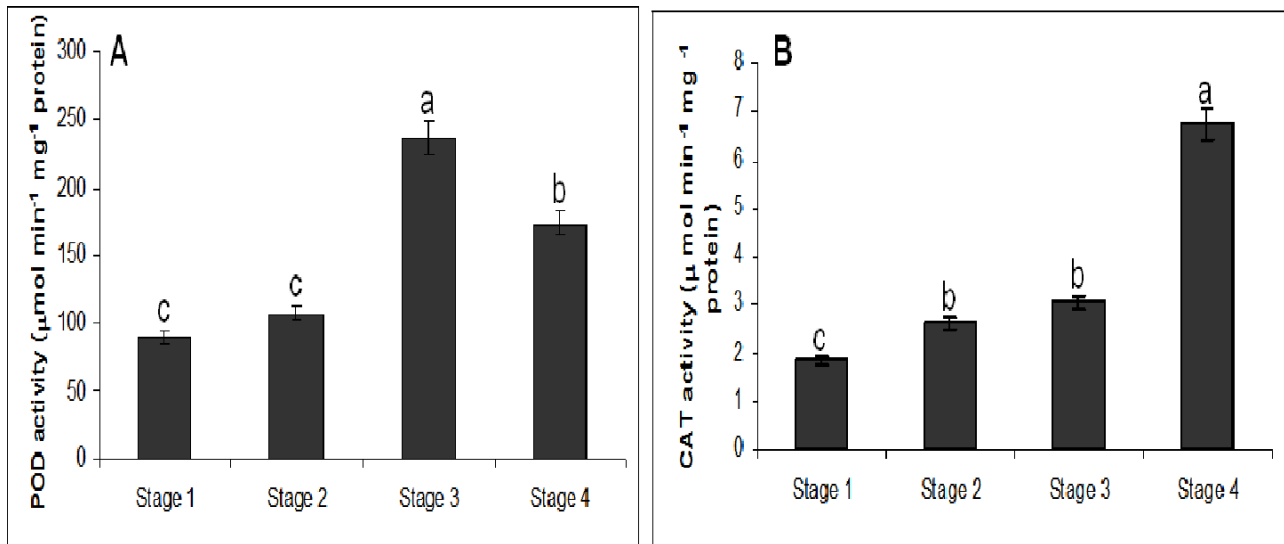

Figure 6. Changes in the level of POD and CAT activity in the petals of day lily flower during different stages of senescence. Different letters in each bar differ significantly according to DMRT test (P 0.05).

(Figure 6). The increased peroxidase and catalase activity that we have observed in our study could reflect a similar process of oxidative stress with the implication of these enzyme activities as part of the antioxidant response against H_2O_2.

From these results it can be concluded that even if there were high antioxidant enzyme activities to remove ROS, it was insufficient to protect the cells from damage. The capacity of the antioxidative defense system is increased during the onset of senescence process but the imbalance between ROS production and antioxidant defenses ultimately led to oxidative damage. It is proposed that a decrease in the activity of a number of enzymes that normally prevent the build up of elevated free radical concentrations might partially account for the process. It is also possible that there might be some role of programmed cell death (PCD) event happening at the same time. Programmed cell death (PCD) is a genetically defined process associated with common morphological and biochemical changes and well established that PCD is an intrinsic part of the life cycle of all multicellular organisms studied so far, including both animals and plants (Xu and Hanson, 2000). The most widely studied PCD is animal cell apoptosis, which is characterized by a distinct set of morphological and biochemical features. Xu and Hanson, 2000 characterized the pollination-induced petal senescence process in *Petunia inflata* and concluded that PCD were found to be present in the advanced stage of petal senescence, indicating that plant and animal cell death phenomena share one of the molecular events in the execution phase. Further analysis will provide more insight about the possible role of PCD along with an imbalance between ROS and antioxidant enzymes during the onset of senescence process.

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