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CHARACTERIZATION OF PROTEASES DURING FLOWER SENESCENCE IN GLADIOLUS (*GLADIOLUS GRANDIFLORA* HORT.)

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SUMMARY

Gladiolus is an ethylene-insensitive flowers, where ethylene does not play much role in its senescence. The role of proteases were explored such as metalloprotease, aspartate protease, serine protease and cysteine protease during flower development in gladiolus. Gladiolus spikes were treated with various protease inhibitors *viz.,* 1,10-phenanthroline (10 mM), pepstatin (5 mM), E-64 (0.01 mM), aprotinin (5 mM) and distilled water (control) for 24 hours at 25°C. We concluded from the study that most effective protease of all the protease inhibitors used was that of cysteine protease (E-64) in delaying the process of flower senescence (by 3 days) as compared with control. Among the compounds used, the best compound was salicylic acid (400 µM) which delayed flower senescence by 5 days in comparison to the control. Moreover, salicylic acid also regulates other senescence regulating factors such as ascorbic acid (AsA), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and ROS (O_2^- and H_2O_2) in addition to cysteine protease. Further, loss in fresh weight as well as turgidity was maintained by salicylic acid treatment in comparison to control flowers and other studied treatments. Thus, inhibition of cysteine proteases and enhanced activities of antioxidant enzymes *viz-a-viz* lower activity of oxidative stress could possibly contribute towards the increase in vase life of gladiolus flowers.

***Key words*: Gladiolus, protease inhibitors, proteases, salicylic acid, senescence**

INTRODUCTION

Flower senescence is a final event in the vase life of flower and is a highly regulated process that involves structural, biochemical and molecular changes (Arora *et al.* 2007). Ethylene biosynthesis plays a major role in floral senescence in many species and autocatalytic burst of endogenously produced ethylene initiates senescence and co-ordinates the expression of genes required for the process (Jones *et al.* 1995). However, some species are insensitive to exogenous ethylene as well as endogenous level of ethylene and the floral senescence in these flowers operates independently of ethylene production. Gladiolus is one of the examples for ethylene insensitive flower (Arora and Singh 2004).

Proteolysis, the degradation of proteins, occurs *via* multiple pathways and is of fundamental importance for the normal development, homeostasis and final death of a plant cell (Arora and Singh 2004). The biochemical degradation of proteins through hydrolysis of peptide bonds is caused by the action of proteolytic enzymes or proteases. Protease activity within the cellular compartments may be regulated at various levels by transcription and translational regulation, by post-translational processing and through the action of specific

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protease inhibitor proteins (Solomon *et al.* 1999). Proteolysis is a characteristic of senescence and is required for mobilization of protein reserves to other developing parts of the plants. Therefore, the activity of proteolytic enzymes is essential during plant senescence. These proteolytic enzymes are divided into several groups depending on the specific amino acid-residue in their active sites. Cysteine proteinases (CyPs) have been studied extensively because they appear to play a central role in a wide range of proteolytic functions during senescence (Solomon *et al.* 1999).

Being a plant hormone (Raskin 1992), it has been established that salicylic acid (SA) plays an essential role during ripening and senescence (Wang and Li 2006, Ding *et al.* 2007, Ezhilmathi *et al.* 2007). It behaves in a concentration (≤1 mM) and time dependent manner as shown in *Arabidopsis thaliana* (Rao *et al.* 1997). Similarly, Chen *et al*. (2002) showed that SA at physiologically relevant concentration (≤1 mM) significantly lowered the lipoxygenase (LOX) activity, appreciably delaying the associated increase in superoxide free radical production during the increasing phases of senescence. This raises the possibility that SA may take part in regulation of senescence by restraining the increase in superoxide free radicals.

Recently, application of exogenous SA has been reported to improve the shelf life of mango (Ding *et al.* 2007), sweet cherry (Xu and Tian 2008), cut flowers such as gladiolus (Ezhilmathi *et al.* 2007, Hatamzadeh *et al.* 2012), rose (Alaey *et al.* 2011, Gerailool and Ghasemnezhad, 2011) and grape plants (Wang and Li 2006). So, in this paper we tried to study the effect of application of various inhibitors of proteases (known in the plant system) and SA as a treatment to delay the process of senescence by regulating protease activity and antioxidative properties of gladiolus flower.

MATERIALS AND METHODS

Gladiolus grandiflora cv. Snow Princess flowers were harvested early in the morning from a commercial grower field. The spikes were cut to a uniform length of 15 cm in distilled water and all the leaves were removed except the bract like leaf below the floret, and placed in holding vase solutions. Spikes were placed in test tubes (25 mm diameter) containing 30 ml of vase solution for 24 hours. Vase solutions were replaced with water after its consumption. The tubes were plugged with non-absorbent cotton to prevent evaporation losses from the surface of vase solution and kept at a room temperature of 20 ± 2°C, relative humidity 70 ± 5% under continuous illumination (range 400–700 nm) of 20 Wm^{-2}.

Vase life: The vase life of gladiolus spikes was calculated by counting the days from treatment to the initial stage of senescence, but up to the stage when they remained still acceptable for marketing.

Weight loss percentage (WLP): The WLP of gladiolus spikes was calculated by considering the differences between initial weight on the day of treatment and final weight on the day of initial stage of senescence divided by their initial weight. Weight loss percentage data represented in tabular form as the extended days of vase life by different inhibitors and treatments.

Ascorbic acid content determination: Ascorbic acid (AsA) was determined using the dye method (Albrecht 1993). Petal tissues were homogenized with 3% metaphosphoric acid (HPO_3). Aliquot was titrated with a standard dye solution (2, 6-dichlorophenol-indophenol) to a pink colour. The ascorbic acid content was expressed as mg per 100 g of fresh weight.

Enzyme extraction: The flower petals were homogenized (2:1 buffer volume: fresh weight) in a mortar with a pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediamine-tetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinylpoly-pyrrolidone (Azevedo *et al.* 1998). The homogenate was centrifuged at 10,000×g for 30 min at 4°C and the supernatant was used as enzyme extract.

Assay

Ascorbate peroxidase (EC 1.11.1.11) activity: Ascorbate peroxidase (APX) activity was assayed according to the method of Nakano and Asada (1981). The reaction mixture consists of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM

H_2O_2 and 0.2 ml of enzyme extract in a total volume of 3 ml. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (Extinction coefficient 2.8 mM^{-1} cm^{-1}). The APX activity was expressed as µmole ascorbate oxidized per minute per mg protein (µmole min^{-1} mg^{-1} protein).

Guaiacol peroxidase (EC 1.11.1.7) activity: Guaiacol peroxidase (GPX) activity was based on the determination of guaiacol oxidation at 470 nm by H_2O_2. The reaction mixture consist of 100 µl of guaiacol (1%) prepared in ethanol, 100 µl H_2O_2 prepared in distilled water,100 µl of enzyme extract and total volume made up to 3 ml with distilled water. The change in absorbance was recorded at 470 nm immediately after adding H_2O_2 over period of 5 min with an interval of 60 seconds (Zieslin and Ben-Zaken 1993). The enzyme activity was calculated using the extinction coefficient of tetraguaiacol of 470 nm (Extinction coefficient 26.6 mM^{-1} cm^{-1}) and expressed as µmole guaiacol oxidized per minute per mg protein (µmole min^{-1} mg^{-1} protein).

Superoxide radical (O_2^-) determination: Superoxide radical (O_2^-) in the sample was assayed by the method of Wang and Lou (1990). One millilitre of enzyme extract was mixed with 1 ml of 1 mM hydroxyl ammonium chloride and then incubated for 30 min at 30°C. One millilitre of incubated solution was then added to 1 ml of 17 mM 3-aminobenzenesulfonic acid and 1 ml of 7 mM 1-naphthylamine and then further incubated for 20 min at 30°C. The absorbance of the solution was monitored at 530 nm. O_2^- production was expressed as nmol min^{-1} mg^{-1} protein.

Hydrogen peroxide (H_2O_2) determination: The content of H_2O_2 was determined according to the method Alexieva *et al*. (2001). Flower petal was homogenized in 0.1% (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000×g for 15 min at 4°C, 200 µl of the supernatant was mixed with 200 µl of 100 mM potassium phosphate buffer (pH 7.0) and 800 µl of 1M KI. The absorbance was read at 390 nm. H_2O_2 content was determined using H_2O_2 as a standard and it was expressed as µmole per g fresh weight (µmole g^{-1} fw).

Determination of protein concentration: The protein content of all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis: All treatments were replicated thrice. Data represent the means and standard error mean and the experimental design was completely randomized. Data were further subjected to analysis of variance and means were compared using least significance difference at $P \leq 0.05$ level.

RESULTS AND DISCUSSION

The effect of protease inhibitors on delay of visible senescence and the vase life of gladiolus flowers in terms of fresh weight was tested *in vitro*. The compound that are known inhibitor of metalloproteases (1-10 phenanthroline) and aspartate protease (pepstatin) had no effect on the vase life of gladiolus flowers and did not delay the time to visible senescence (Table 1, Fig. 1A). Aprotinin, the endoprotease inhibitor of serine protease delayed senescence by about 1.5 days and prolonged the vase life of gladiolus flowers by 7.5 days (Table 1, Fig. 1B.). E-64 the known endoprotease inhibitor of cysteine protease delayed senescence by 3 days and prolonged the vase life of gladiolus flowers by 9 days (Table 1, Fig. 1C). Salicylic acid a well-known antisenescent compound in various fruits and flowers, and also inhibitor of cysteine protease as reported earlier by

Table 1. Effect of four different kinds of inhibitors: 1, 10-phenanthroline (metalloprotease inhibitor), pepstatin (aspartate protease inhibitor), aprotinin (serine protease inhibitor), E-64 (cysteine protease inhibitor) and salicylic acid (cysteine protease as well as ROS inhibitor) on vase life of gladiolus flower. All data are the means ± SE of three replications. LSD ($P \leq 0.05$).

Treatment	Vase life (in days)
Control (Distilled water)	6.0±0.81
1,10-phenanthroline (10 mM)	6.3±0.82
Pepstatin (5 mM)	6.0±0.81
Aprotinin (5 mM)	7.5±0.65
E-64 (0.01 mM)	9.0±0.40
Salicylic acid (400 µM)	11.0±0.81

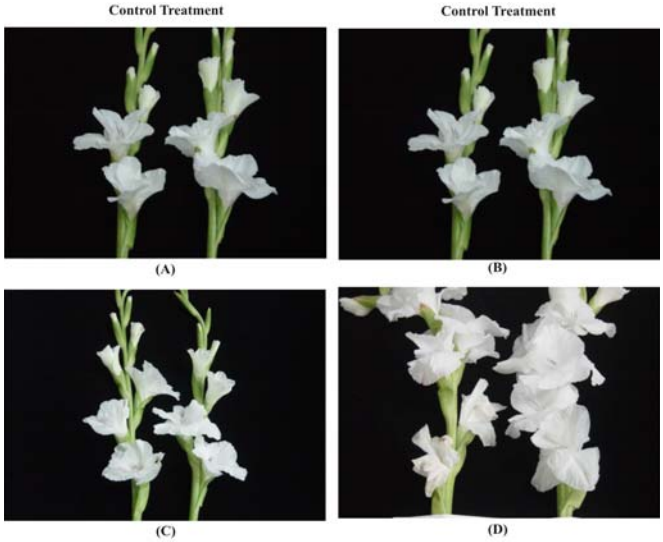

Fig. 1. Effect of four different kinds of inhibitors: (A) 1, 10-phenanthroline (metalloprotease inhibitor) at 6 days after harvest, (A) pepstatin (aspartate protease inhibitor) at 6 days after harvest, (B) aprotinin (serine protease inhibitor) at 7.5 days after harvest, (C) E-64 (cysteine protease inhibitor) at 9 days after harvest and (D) salicylic acid (cysteine protease as well as ROS inhibitor) at 11 days after harvest on visible senescence of gladiolus flower.

our group (Ezhilmathi *et al.* 2007), delayed senescence by around 5 days and prolonged the vase life of gladiolus flowers by 11 days (Table 1, Fig. 1D). Visible senescence of gladiolus florets is preceded by a sharp increase in endoprotease activity. The onset of this increase showed a positive correlation with the onset of the visible senescence symptoms. The data (Table 1 and 2) indicate that cysteine and serine proteases are involved close to the maximum of endoprotease activity. A number of known protease inhibitors substantially reduced the increase in vase life and delayed the time to visible senescence in gladiolus spikes. It might be possible that the timing of the rise in endoprotease activity will be correlated well with the timing of the visible senescence symptoms.

Flower development and senescence, relative fresh weight and solution uptake were affected by SA treatments. In our study, maximum vase-life was obtained in solution which contained 400 µM SA and it also increased water uptake and fresh weight of spike, and thereby, caused a delay in decline of fresh weight and senescence as was also found in gladiolus by Ezhilmathi *et al*. (2007) with a solution containing 100 ppm 5-sulfosalicylic acid + 4% sucrose. Treatment with sodium benzoate and *n*-propyl gallate delayed slightly the onset of wilting in gladiolus florets (Yamane *et al.* 1999). Singh *et al*. (2008) showed that vase solution containing GA_3 (50 mg/L), followed by BA (50 mg/L) with sucrose (50 g/L) significantly enhanced the vase life of gladiolus flowers.

Generally, the relative fresh weight of spikes kept in aprotinin, E-64 and SA vase solutions were greater than those flowers kept in distilled water (control) and 1–10 phenanthroline, and pepstatin, at least during 7, 9 and 11 days after harvest, respectively. The relative fresh weight of control flowers continuously decreased from the sixth day after harvest until death, while in most SA-treated flowers, relative fresh weight gradually increased over the first six days after harvest (Table 2). The highest relative fresh weight was obtained in spikes kept in E-64 (0.01 mM) and SA (400 µM) vase solutions at 7 and 9 days after harvest, respectively. The cumulative solution uptake was significantly increased in vase solutions containing SA and E-64 compared to the control (Table 2) and other treatments. SA in vase solutions significantly increased the cumulative solution uptake than E-64. The highest solution uptake was observed in flowers kept under SA vase solutions which showed the highest relative fresh weight. Vase solution uptake increased to its maximum level at stage 4 (Arora and Singh 2004) thereafter it gradually declined. A gradual decrease in fresh weight was observed over the senescence period in control and treated flower, but cut spikes that were treated with SA (400 µM) suppressed declining of fresh weight in early days of vase life (Table 2) and prolonged the vase life to 11 days. Similar kinds of results have been reported in gladiolus (Hossain *et al.* 2006, Ezhilmathi *et al.* 2007). Lam *et al.* (1987) suggested that SA plays an important role in decreasing transpiration and evaporation of tissues, as well as decreasing respiration, hence preventing from loss of fresh weight in cut flowers.

AsA, APX and GPX activity was higher ($p < 0.05$) in petals of spikes which were treated with SA compared to control at 11 days after harvest (Fig. 2A, B and C).

Table 2. Effect of four different kinds of inhibitors: 1, 10-phenanthroline (metalloprotease inhibitor), pepstatin (aspartate protease inhibitor), aprotinin (serine protease inhibitor), E-64 (cysteine protease inhibitor) and salicylic acid (cysteine protease as well as ROS inhibitor) on weight loss percentage of gladiolus flower. All data are the means ± SE of three replications. LSD ($P \leq 0.05$)

Treatment	Weight loss percentage			
	6 days	7.5 days	9 days	11 days
Control (Distilled water)	14.23±0.79	22.34±0.46	29.41±2.35	41.21±2.87
1,10-phenanthroline (10 mM)	12.34±0.75	21.28±0.57	28.79±2.12	39.78±2.42
Pepstatin (5 mM)	13.97±0.89	21.59±0.48	30.24±1.34	38.96±1.34
Aprotinin (5 mM)	9.98±0.68	13.64±0.35	23.24±0.89	35.43±2.26
E-64 (0.01 mM)	6.34±0.37	9.56±0.54	13.79±0.58	22.86±1.48
Salicylic acid (400 µM)	4.62±0.52	8.97±0.38	11.32±0.76	14.78±0.74

Spikes treated with E-64 senesces at 7 days, so regulation of antioxidant activity was not studied. All SA-treated spikes showed higher AsA, APX and GPX activity at 6, 7.5, 9 and 11 days after harvest compared to the control (Fig. 2.). So interestingly, SA application induced a significant shift in time or flower stage for AsA (Fig. 2A), APX (Fig. 2B) and GPX (Fig. 2C) activity levels when compared to control flowers (water treated). Furthermore, SA enhanced the AsA level, APX and GPX activity from harvest to senescence, hence H_2O_2 (Fig. 2D) and O_2^- level (Fig. 2E) was found reduced in flowers of spikes that kept in SA vase solution as compared with control at 6 days, after that it gradually increases while in control it decreases.

Several physiological- and biochemical-related processes regulate the senescence in flowers (Arora and Singh 2004, Hossain *et al.* 2006). Senescence is closely related with reactive oxygen species (ROS) production, which cause severe damage to cells if they have accumulated to a critical level but not detoxified by an antioxidant system (Arora *et al.* 2002). Changes in antioxidant enzyme activity such as APX, superoxide dismutase (SOD) and catalase (CAT) have been described during senescence and are related to scavenging processes (Alaey *et al.* 2011, Gerailool and Ghasemnezhad 2011). There are several reports on antioxidative defence systems in ornamental plants, such as chrysanthemum (Bartoli *et al.* 1997), daylily (Panavas and Rubinstein, 1998), Iris (Bailly *et al.* 2001) and gladiolus (Ezhilmathi *et al.* 2007, Sairam *et al.* 2011).

In addition to defence, a potential role of SA in response to stresses and gene expression during senescence has been demonstrated (Buchanan-Wollaston *et al.* 2003). Various studies have demonstrated that vase life of flowers is modulated by antioxidants (Ezhilmathi *et al.* 2007, Sairam *et al.* 2011) suggesting the involvement of ROS in senescence. In the present study, the ROS level was found to increase gradually after harvest in control while in SA treated spikes ROS level raised at 9 days after harvest. Similar results were observed in gladiolus (Ezhilmathi *et al.* 2007). An increase in ROS level has been correlated with an increase in cell membrane permeability and senescence in daylily (Panavas and Rubinstein 1998) and rose (Alaey *et al.* 2011, Gerailool and Ghasemnezhad, 2011). Since lipid peroxidation is mediated by ROS (Arora *et al.* 2007), SA may either be directly scavenging ROS and thus decreasing lipid peroxidation, or it may be modulating the activity of antioxidant enzymes (Ezhilmathi *et al.* 2007). In previous studies from our laboratory, Ezhilmathi *et al.* (2007) showed that 5-sulfosalicylic acid could be able to reduce the level of ROS hence lipid peroxidation by enhancing the activities of SOD and CAT. In present study, it was also found that SA increased the level of AsA, enhanced the activities of APX and GPX, reduced the ROS such as H_2O_2 and O_2^- level and ultimately delayed the process of senescence. Our results are similar with previous work with SA treatment where exogenous SA significantly increased the reducing status of ascorbate and glutathione in mango fruits (Ding *et al.* 2007) and it forms a soluble redox buffer in plant cell which is

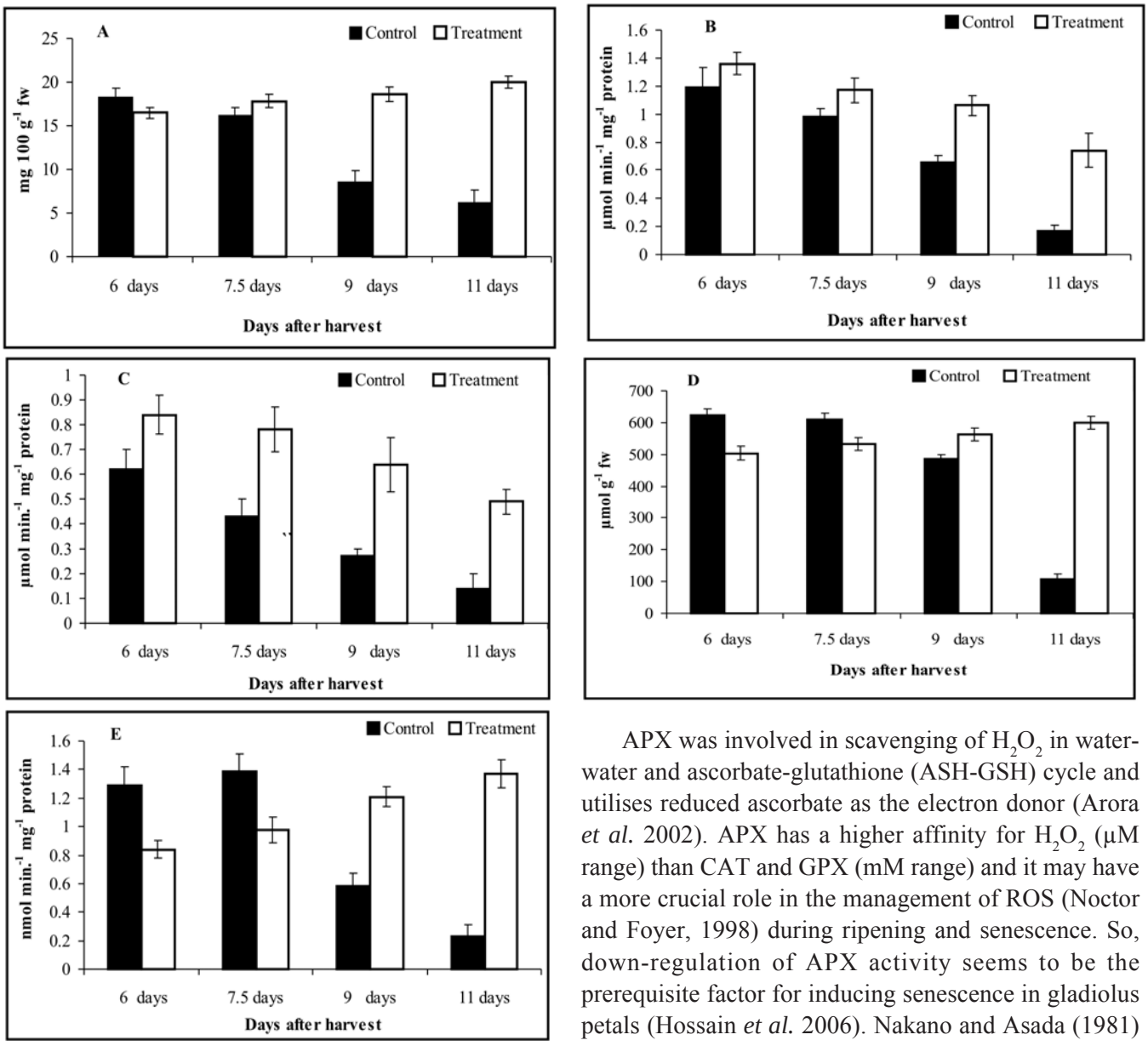

Fig. 2. Effect of salicylic acid (400 µM) on antioxidant and oxidative stress of gladiolus flower. (A) Ascorbic acid (AsA), (B) Ascorbate peroxidase (APX), (C) Guiacol peroxidase (GPX), (D) Hydrogen peroxide (H_2O_2) and (E) Superoxide radicals (O_2^-). All data are the means ± SE of three replications. LSD ($P \leq 0.05$).

important in regulating growth, development and defence against stress in plant (Noctor and Foyer 1998). Report of Wang and Li (2006) suggested that SA increased the reducing potential of ascorbate and glutathione in grape leaves under normal temperature and heat or cold stress.

APX was involved in scavenging of H_2O_2 in water-water and ascorbate-glutathione (ASH-GSH) cycle and utilises reduced ascorbate as the electron donor (Arora *et al.* 2002). APX has a higher affinity for H_2O_2 (µM range) than CAT and GPX (mM range) and it may have a more crucial role in the management of ROS (Noctor and Foyer, 1998) during ripening and senescence. So, down-regulation of APX activity seems to be the prerequisite factor for inducing senescence in gladiolus petals (Hossain *et al.* 2006). Nakano and Asada (1981) have reported a direct correlation between APX activity and the reduction in ROS and also suggested that H_2O_2 produced by the action of SOD on the superoxide radical (O_2^-) is scavenged by membrane bound APX. Xu and Tian (2008) reported that SA in a concentration-dependent manner significantly enhanced GPX activity and increased total protein content of sweet cherry fruit. In *Arabidopsis thaliana,* Rao *et al*. (1997) reported that exogenous SA at lower concentrations enhanced GPX and APX activity in comparison to control.

CONCLUSION

Inhibitor of cysteine proteases (E-64) could be able to prolong the vase-life of gladiolus flowers by 3 days while SA prolongs the vase-life by 5 days. Therefore, we may suggest that SA as an inexpensive natural plant-derived compound has the potential to increase the vase-life of gladiolus flowers.

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