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Influence of exogenous ascorbic acid on vase life and biochemical constituents of chrysanthemum (*Chrysanthemum* × *morifolium.*)

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ABSTRACT

The present investigation was conducted at IARI, New Delhi in the year 2015-16 to examine the effect of ascorbic acid on changes in level of physiological and biochemical constituents during the postharvest life of chrysanthemum cv. Pusa centenary. The study revealed that spraying of ascorbic acid on the surface of cut flowers enhanced the vase life of cut flowers compared to control. Ascorbic acid sprays conserved the chlorophyll a, b and total chlorophyll content in leaves up to 14 days after harvest (S_3). Total carotenoid content in leaves was found highest just after harvest (S_1) and gradually declined as the senescence progressed from S_1 to S_2. Spraying 100 ppm ascorbic acid (T_3) to cut flowers showed higher membrane stability compared to control in S_3. Higher amount of H_2O_2 in control was due to enhanced activity of SOD enzyme as it dismutates superoxide radicals produced over senescence period. Decreased activity of ascorbate peroxidase in control during S_2 and S_3 might have led to excess accumulation of H_2O_2.Significantly higher CAT activity was found after spraying with 100 ppm ascorbic acid at 7 days after harvest (S_2) compared to control. Progress of flower senescence was delayed by the coordinated antioxidant action of SOD, POX and CAT enzymes by maintaining their constant level at S_2 and S_3. This study concludes that spraying ascorbic acid to harvested cut flowers delayed early leaf yellowing and petal senescence in chrysanthemum.

Key words: Antioxidants, Ascorbic acid, Membrane stability, Preservative

Chrysanthemum is a vastly traded cut flower for its high demand in the global floriculture business. Being an ethylene insensitive flower, the postharvest life of chrysanthemum suffers due to premature leaf yellowing and petal wilting (Jain *et al.* 2014). Physiology of flower senescence in chrysanthemum has been studied meticulously by some authors (Elanchezhian and Srivastava, 2001; Chakrabarty *et al.* 2007). Physiological mechanism behind regulation of leaf and petal senescence has been studied using chemical preservatives such as cytokinin (Ferrante *et al.* 2005), alcohols (Petridou *et al.* 2001), aluminium sulphate (Jain *et al.* 2014), minerals etc. Developmental processes during flowering are connected with variations in the levels of antioxidant molecules like polyphenols, carotenoids and vitamin C particularly during advanced senescence stage (Cavaiuolo *et al.* 2013). Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical and hydroxyl radical are responsible for all senescence-related events and their levels are strongly controlled by the mutual action of a series of enzymes and antioxidant compounds.

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Ascorbic acid is water soluble, non-enzymatic antioxidant reported to interact synergistically with α-tocopherol in combating oxidative rupture of lipid membranes during aging of *Chrysanthemum* petals (Bartoli *et al.* 1997). Endogenous level of ascorbic acid is quite essential to regulate the developmental senescence, delay flowering and protection against pathogens (Kotchoni *et al.* 2009). Furthermore, deficiency of endogenous ascorbic acid causes premature senescence in plants due to rapid loss of chlorophyll content in leaves (Barth *et al.* 2006). Therefore an attempt was made to examine the role of ascorbic acid and elucidate the physiological and biochemical mechanism in the regulation of leaf and petal senescence in chrysanthemum flowers.

MATERIALS AND METHODS

Present study was carried out at Division of Floriculture and Landscaping, IARI, New Delhi in the year 2015-16. Cut stems of chrysanthemum cv. Pusa centenary (standard variety) were harvested from the research farm when they were fully open before anthesis during morning hours. Harvested stems were immediately placed in a bucket containing clean water for rehydration and were brought to the laboratory. These stems were cut back to the uniform length of 60 cm and the leaves from the lower 1/3rd portion of the stem were removed and flowers were kept in distilled water. The experiment was laid out in completely

Table 1 Vase life of chrysanthemum cut stems recorded at different ascorbic acid levels

Treatment	Vase life (days)
T_1 - Control	17.667±0.33
T_2 - Ascorbic acid 50 ppm	19.333±0.33
T_3 - Ascorbic acid 100 ppm	20.333±0.33
T_4 - Ascorbic acid 150 ppm	19.667±0.33
CD (P=0.05)	1.10

randomized design with four treatments replicated thrice having five stems per replication. The basal portion of the cut stems (2 cm) was recut under water and these cut stems were kept in test tubes (100 ml) containing distilled water as per treatment combinations (Table 1). The open space between stem and test tube rim was covered using non-absorbent cotton to avoid moisture loss through evaporation. The experiment was conducted under laboratory conditions having the illumination of fluorescent lights up to 16 h and temperature of 20±2°C. Freshly prepared ascorbic acid (*L*-ascorbic acid) solutions were uniformly sprayed on to petals and leaves daily according to treatments. The level of physiological and biochemical constituents in leaves and petals were analyzed during different vase life period i.e. S_1 (0 days after harvest), S_2 (7 days after harvest), S_3 (14 days after harvest) and S_4 (at the end of vase life i.e. senescence) and recorded observations were subjected to analysis of variance through Duncan's Multiple Range Test using SPSS software.

Vase life (days): Vase life of cut flowers was calculated by counting the number of days after harvest till the termination of vase life. Wilting of outer three rows of ray florets was the criteria for termination of vase life.

Chlorophyll a, b, total chlorophyll and carotenoid content (mg/g fw): Leaf chlorophyll content was measured by a non-maceration method using DMSO (Dimethyl Sulphoxide) described by Hiscox and Israelstam (1979). Chlorophyll a, b and total chlorophyll were calculated by using formulae given by Arnon (1949). The total carotenoid content was calculated by the formula given by Lichtenthaler and Wellburn (1983).

Analysis of oxidants and antioxidant metabolites: Measurements of oxidative stress like ion leakage (MSI%) and hydrogen peroxide content (H_2O_2) and antioxidant activities of Superoxide dismutase (SOD), Ascorbate peroxidase (APOX), Peroxidase (POX) and Catalase (CAT) enzymes were estimated from petals. Leakage of ions from the petals was estimated according to Sairam *et al.* (1997) and expressed as membrane stability index (MSI) percentage. H_2O_2 content was analyzed by estimating titanium-hydro peroxide complex which absorbs at 415 nm (Rao *et al.*1997). Enzyme extract for SOD, APOX, POX and CAT was prepared by homogenizing the weighed amount of petal samples (1 g) with ice-cold 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD, CAT, and POX, and 0.5 mM EDTA and 1 mM ascorbic acid in case of APOX) with pre-chilled mortar and pestle. The homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 20 min at 15000×g and the supernatant was transferred to 3 ml eppendorf tubes and used as enzyme extract in estimation. The protein concentration of enzyme extract was estimated by Bradford reagent method (Bradford 1976). SOD activity (units/mg protein/min) was assayed based on the formation of blue coloured formazone by nitro-blue tetrazolium (Dhindsa *et al.* 1981). CAT activity (mmol/mg protein/min) was estimated by recording the absorbance of H_2O_2 at 240 nm in UV-range (Aebi 1984). POX activity (mmol/mg protein/min) was assayed based on the increase in absorbance due to the oxidation of guaiacol to tetra-guaiacol (Castillo *et al.* 1984). Ascorbate peroxidase reduces hydrogen peroxide to water with the help of ascorbic acid. The assay is based on the decrease in absorbance of ascorbic acid at 290 nm (Nakano and Asada 1981).

RESULTS AND DISCUSSION

Vase life was found maximum (20.33 days) when flowers were sprayed with 100 ppm ascorbic acid (T_3) compared to control (17.66 days). However, vase life of T_3 was at par with treatments T_2 and T_4 (Table 1). Ascorbic acid sprays on cut stems have positive influence on postharvest life of chrysanthemum. Abri *et al.* (2013) reported vase life of rose can be enhanced using ascorbic acid. Bedour *et al* (2011) found that combined foliar spray of 200 ppm ascorbic acid and 200 ppm thiamine ensured maximum keeping quality in gladiolus. Ascorbic acid in holding solution increased number of opened flowers in tuberose (Anjum *et al.* 2001). Supplementation of ascorbic acid to cut flowers might have elevated endogenous ascorbic acid, thereby maintaining freshness and quality of cut flowers for longer time.

In the present investigation, ascorbic acid sprays were proved beneficial in preserving leaf chlorophyll pigments during the postharvest life (Table 2). Chlorophyll a content was recorded to slightly increase at S_3 compared to S_1. It was found maximum (5.46 mg/g fw) 7 days after harvest when sprayed with 100 ppm ascorbic acid compared to control. However, it was found at par with other ascorbic acid levels in S_2 and S_3. Correspondingly, chlorophyll b content values were found non-significant. Total chlorophyll content progressively increased 14 days after harvest and reached maximum (8.22 mg/g fw) in S_3 when cut flowers were sprayed with 50 ppm ascorbic acid (T_2). Ascorbic acid spray at 50 ppm maintained the stability of total chlorophyll up to 14 days after harvest as indicated in Table 2. Highest total carotenoids (1.91 mg/g fw) were found at S_3 when cut stems were sprayed with 50 ppm ascorbic acid and thereafter declined at the end of vase life. The partial decline in chlorophyll pigments 7 days after harvest might be due to lipid peroxidation of chloroplast membranes or the formation of hydroperoxides of fatty acids (Farouk 2011). Application of ascorbic acid directly to leaves might have stabilized the chlorophyll at S_3 and S_4 by elevated photosynthesis and photo-assimilation. Chloroplasts are

Table 2 Effect of ascorbic acid on chlorophyll (a, b and total chlorophyll) and carotenoid values

Treatment/Stage		Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Total Chlorophyll (mg g fw)	Total Carotenoid content (mg/g fw)
S_1	T_1	4.63^{cd}*	2.11^{cdef}	6.73^{c}	1.63^{efg}
	T_2	5.01^{ef}	1.73^{abcd}	6.73^{c}	1.74^{efg}
	T_3	4.95^{ef}	2.02^{cdef}	6.97^{c}	1.75^{efg}
	T_4	5.22^{e}	1.92^{bcde}	7.14^{c}	1.60^{defg}
	Mean	4.95	1.94	6.89	1.68
S_2	T_1	3.71^{ab}	1.75^{abcd}	5.46^{ab}	1.26^{abcd}
	T_2	5.23^{e}	2.13^{def}	7.36^{cd}	1.22^{abc}
	T_3	5.46^{e}	2.16^{def}	7.05^{cd}	1.41^{bcdef}
	T_4	5.01d^{e}	2.06^{cdef}	4.92^{a}	1.55^{cdef}
	Mean	4.85	2.02	6.20	1.36
S_3	T_1	4.20^{bc}	1.64^{abc}	5.84^{b}	1.16^{ab}
	T_2	5.28^{e}	2.25^{ef}	8.22^{e}	1.91^{g}
	T_3	5.40^{e}	2.42^{f}	7.59^{d}	1.58^{defg}
	T_4	5.28^{e}	2.19^{def}	7.13^{cd}	1.35^{bcde}
	Mean	5.04	2.13	7.20	1.50
S_4	T_1	3.58^{a}	1.39^{a}	5.07^{a}	0.95^{a}
	T_2	4.49^{cd}	1.37^{a}	6.97^{cd}	1.68^{efg}
	T_3	4.59^{cd}	1.47^{ab}	5.82^{b}	1.53^{cdef}
	T_4	4.67^{cd}	1.35^{a}	5.34^{ab}	1.42^{bcdef}
	Mean	4.33	1.40	5.80	1.40
CD (P= 0.05)	Stage	0.23	0.22	0.31	0.16
	Treatment	0.23	NS	0.31	0.16
	Interaction	0.46	NS	0.61	0.33

T_1– Control; T_2– 50 ppm Ascorbic acid; T_3– 100 ppm Ascorbic acid; T_4 – 150 ppm Ascorbic acid, S_1– 0 days after harvest; S_2– 7 days after harvest; S_3-14 days after harvest; S_4– At the end of vase life. *Mean separation within columns by Duncan's multiple range test @ 5% level.

Table 3 Effect of ascorbic acid on total H_2O_2 content and membrane stability index

Stage/Treatment		Membrane stability index (%)	Total H_2O_2 content (µmol/g)
S_1	T_1	71.78$^{de\#}$	1.32^{a}
	T_2	71.86^{de}	1.287^{a}
	T_3	71.45^{de}	1.25^{a}
	T_4	71.43^{de}	1.33^{a}
	Mean	71.63	1.29
S_2	T_1	86.51^{f}	2.47^{ef}
	T_2	84.80^{f}	1.82^{b}
	T_3	86.15^{f}	2.06^{b}
	T_4	80.69^{f}	2.09^{c}
	Mean	84.54	2.11
S_3	T_1	59.50^{b}	3.01^{g}
	T_2	61.68^{b}	2.38^{de}
	T_3	72.68^{de}	2.16^{cd}
	T_4	68.55^{cde}	2.32^{cd}
	Mean	65.60	2.47
S_4	T_1	74.76^{e}	3.80^{h}
	T_2	50.88^{a}	2.44^{def}
	T_3	68.13^{cd}	2.38^{de}
	T_4	62.81^{bc}	2.69^{f}
	Mean	64.14	2.83
CD (P= 0.05)	Stage	2.61	0.13
	Treatment	2.61	0.13
	Interaction	5.22	0.26

T_1– Control; T_2– 50 ppm Ascorbic acid; T_3– 100 ppm Ascorbic acid; T_4 – 150 ppm Ascorbic acid, S_1– 0 days after harvest; S_2– 7 days after harvest; S_3– 14 days after harvest; S4- At the end of vase life, #Mean separation within columns by Duncan's multiple range test @ 5% level.

prone to H_2O_2 toxicity as they are the main centers of its production. Elevated H_2O_2 concentration in leaves could have triggered leaf senescence causing oxidative damage to cell membrane and disrupt ion of cellular metabolism (Sairam and Srivastava 2000).

Membrane stability index was significantly increased from the day of harvest to 7 days after harvest (S_2) whereas it declined gradually in S_3 (Table 3). Spraying different levels of ascorbic acid maintained membrane stability in petal cells S_2 and S_3. Specifically, spraying 100 ppm ascorbic acid (T_3) to cut flowers showed higher membrane stability compared to control in S_3 indicating slow deterioration of cell membrane. Low membrane stability in control at S_3 might be due to membrane deterioration accompanied by loss of membrane phospholipids and neutral lipids. Higher concentration of ascorbic acid might have inhibited the production of reactive oxygen species. Bartoli *et al.* (1995) reported that lipid peroxidation and membrane damage are the main reasons for deterioration of chrysanthemum cut flowers. The reason of minimum ion leakage may be ascribed to the antioxidant action of ascorbic acid against free radicals.

Total H_2O_2 content was recorded minimum (1.82 µmol/g) in cut flowers sprayed with 50 ppm ascorbic acid (T_2) at S_2 compared to control (T_1) (Table 3). Lower levels of H_2O_2 were recorded in S_3 following sudden increase in S_4 showing advanced petal wilting. Results suggest that hydrogen peroxide level gradually increased in ascorbic acid treatments while it was rapid in control. Higher amount of H_2O_2 in control was due to enhanced activity of SOD enzyme as it dismutates superoxide radicals produced over senescence period. It has been proposed that there is a significant association between free radicals produced during cell metabolism and senescence of flowers. Ascorbate is highly essential for the removal of H_2O_2 accumulated

in chloroplasts (Foyer *et al.* 1994). Decreased activity of ascorbate peroxidase in control during S_2 and S_3 might have led to over-accumulation of H_2O_2 (Hossain *et al.* 2006).

SOD activity was maximum (7.18 units/mg protein/min) in control (T_1) after harvest; however it was at par with ascorbic acid sprays (Table 4). Ascorbic acid 50 ppm exhibited higher SOD activity (5.74 units/mg protein/min) against control at 7 days after harvest (S_2). Higher SOD activity may be attributed to increased membrane permeability and H_2O_2 generation. SOD levels diminished at S_4 signifying increased petal wilting. Hossain *et al.* (2006) witnessed upsurge in SOD activity at the end of vase life in gladiolus. Similar to our findings, SOD levels were found to decrease with the progression of petal senescence in chrysanthemum (Bartoli *et al.* 1997) and rose (Kumar *et al.* 2008). Reduced SOD activity at S_2 and S_3 compared to S_1 might indicate that CAT and APOX enzymes might be involved in quenching of endogenous H_2O_2.

Table 4 Effect of ascorbic acid on antioxidant enzyme levels

Stage/Treatment		SOD activity (units/mg protein/min)	CAT activity (mmol/mg protein min)	APOX activity (mmol/mg protein/min)	POX activity (mmol/mg protein/min)
S_1	T_1	7.18^{g}*	5.25^{fg}	93.03^{h}	17.74^{ab}
	T_2	6.99^{g}	5.28^{fg}	90.56^{gh}	16.36^{ab}
	T_3	6.92^{g}	5.83^{g}	99.55^{i}	14.22^{a}
	T_4	7.15^{g}	5.98^{g}	90.39^{gh}	14.37^{a}
	Mean	7.06	5.58	93.38	15.6
S_2	T_1	5.63^{ef}	3.85^{c}	41.95^{b}	23.27^{cde}
	T_2	5.74^{f}	4.69+	61.61^{d}	27.10^{ef}
	T_3	5.49^{def}	5.39+	83.09^{f}	23.88^{cde}
	T_4	5.19^{de}	4.97^{ef}	82.68^{f}	22.28^{cd}
	Mean	5.51	4.73	67.33	24.13
S_3	T_1	4.08^{bc}	2.65^{ab}	34.18^{a}	28.51^{f}
	T_2	5.20^{de}	3.93^{cd}	44.43^{b}	26.95^{ef}
	T_3	5.34^{def}	4.24^{cde}	66.56^{de}	21.65^{cd}
	T_4	5.03^{d}	4.07^{cd}	59.66^{c}	25.10^{def}
	Mean	4.91	3.72	51.21	25.55
S_4	T_1	3.09^{a}	1.52^{a}	57.71^{c}	44.54^{g}
	T_2	4.04^{bc}	2.84^{b}	70.15^{e}	28.78^{f}
	T_3	4.49^{c}	2.33^{b}	84.78^{fg}	19.95^{bc}
	T_4	3.97^{b}	2.23^{ab}	71.34^{e}	23.19^{cde}
	Mean	3.90	2.23	70.99	29.11
CD (P= 0.05)	Stage	0.23	0.34	3.24	1.82
	Treatment	0.23	0.34	3.24	1.82
	Interaction	0.47	NS	6.48	3.64

T_1– Control; T_2– 50 ppm Ascorbic acid; T_3– 100 ppm Ascorbic acid; T_4– 150 ppm Ascorbic acid, S_1– 0 days after harvest; S_2– 7 days after harvest; S_3– 14 days after harvest; S4- At the end of vase life, #Mean separation within columns by Duncan's multiple range test @ 5% level.

APOX activity in chrysanthemum petals showed decreasing trend during different stages of vase life (Table 4). The maximum APOX activity (83.09 mmol/mg protein/min) was recorded when cut flowers were sprayed with 100 ppm ascorbic acid at S_2 compared to control. Higher availability of ascorbic acid stimulated APOX activity in chrysanthemum petals. Exogenous ascorbic acid might have supplemented as substrate for APOX activity in reducing endogenously produced H_2O_2 (Davey *et al.* 2000). APOX activity is highly crucial in detoxifying H_2O_2 produced during flower senescence (Foyer *et al*, 1994). Reduced activity in control was attributed to triggered H_2O_2 level owing to inefficient APOX activity. It has been reported in gladiolus (Hossain *et al*, 2006) that down regulation of APOX activity indirectly up-regulates SOD activity due to higher accumulation of H_2O_2. APOX activity was surprisingly higher (84.78 mmol/mg protein/min) in T_3 at 21 days after treatment (S_4). This might be due to reduced activity of SOD at the end of vase life period. Therefore, the activity of SOD appears to be inversely proportional to APOX activity in petals during flower senescence.

Results depicted that highest CAT activity (5.98 mmol/mg protein/min) was observed in T_4 just after harvest (S_1) (Table 4). It was significantly higher after spraying with 100 ppm ascorbic acid at 7 days after harvest (S_2) compared to control. This might be due to increased scavenging of H_2O_2 produced after harvest. Similar findings were recorded by Bartoli *et al.* (1997) in chrysanthemum. Catalase in cooperation with peroxidases and other enzymes destroy the H_2O_2 produced by SOD and other reactions (Foyer *et al.* 1994). Ascorbic acid might have stimulated APOX enzyme to reduce H_2O_2 thereby delayed senescence.

POX activity progressively increased after harvest and reached maximum at S_4 (Table 4). Highest activity (44.54 mmol/mg protein/min) was recorded in control at the end of vase life (S_4). Ascorbic acid treatments reduced peroxidase activity which was observed in S_3 and S_4 compared to control. The present investigations are in close conformity with the findings of Bartoli *et al.* (1995) who reported that peroxidase activity increases during senescence in carnation. Lower POX activity at the end of vase life indicates reduced oxidative stress due to antioxidant action of ascorbic acid against reactive oxygen species.

Ascorbic acid plays a pivotal role in defense against the oxidative stress caused by hydrogen peroxide and cell membrane breakdown. Exogenous application of ascorbic acid treatments specifically enhanced APOX activity at 7 days after treatment, which is found to be very crucial in monitoring and quenching of H_2O_2 toxicity in leaves and petals. Chlorophyll levels were maintained in leaves to mobilize stored food to petals. Progress of flower senescence was delayed by the action of antioxidant action of SOD,

POX and CAT enzymes by maintaining their constant level at S_2 and S_3. Coordinated control of leaf and petal senescence by various antioxidant enzymes like SOD, CAT, POX and APOX might have influenced longevity of cut flowers. Among treatments, spraying 100 ppm ascorbic acid proved to be effective to delay flower senescence and can be utilized to reduce leaf yellowing in chrysanthemum. Ascorbic acid sprays will be helpful for consumers to keep the cut flowers fresh, turgid and attractive for longer time. However, more studies are needed to validate efficacy of ascorbic acid in other important ethylene insensitive flowers.

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