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POST-HARVEST PHYSIOLOGY AND VASE LIFE OF CUT *CHRYSANTHEMUM* FLOWERS WITH SUCROSE, PUTRESCINE AND SPERMIDINE AS HOLDING SOLUTIONS

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Selecting sucrose as energy source and putrescine (Putr) and spermidine (Spmd) as senescence retarding phytohormone, an investigation was carried out to find out how they influence the longevity of cut flowers. Scapes of *Chrysanthemum dendranthema grandiflorum* var. Chandrima were taken in vase solutions like sucrose (0.1M), Putr (1×10^{-4}M), Spmd (1×10^{-4}M), Putr (1×10^{-4}M)+ sucrose (0.1M)and spermidine (1×10^{-4}M)+sucrose (0.1M). A combination of sucrose + Spmd as vase solution was most effective in checking the shrinkage of flower diameter and the decline in fresh weight. Maximum vase life of 14 days was recorded with sucrose + Spmd and Putr while that of sucrose alone was 9 days. Both polyamines (PAs) were highly effective in maintaining moisture content and MSI value. They were also able to reduce not only the decline in the amount of starch, protein and proline but also the rise in lipid peroxidation, α-amylase activity and total sugars. Further, Putr was more effective when used alone while Spmd was greater effective in combination with sucrose than alone in minimizing senescence effects. Overall comparisons indicated that Spmd was slightly better in performance than Putr in suppressing senescence.

Keywords : Cut flower, longevity, putrescine, senescence, spermidine, sucrose.

Between the maturity and death of a flower or flower part lies the phase of flower senescence that allows the removal of a metabolically costly tissue (i.e. petal), after it has attracted pollinators for sexual development, and signals the initiation of ovule development and seed production (Arora 2008). Senescence in plant parts is accompanied by organized disassembly of polysaccharides, proteins, lipids and nucleic acids (Winkenbach 1970a, b; Matile 1997). This disassembly results in production of sugars and amides, which are transported to other plant parts (Bieliski and Reid 1992, Buchanan-Wollaston and Morris 2000, Wagstaff *et al*. 2002). It also includes an increase in hydrolytic enzyme activity, degradation of macromolecules, increased respiratory activity and loss of membrane integrity and cellular compartmentation. Various organic compounds are oxidized in presence of free radicals (Thompson *et al.* 1987, Allen 1995, Arora *et al.* 2002). Generally, increment in petal or tepal ROS has been associated with floral senescence but it is yet to be proved whether-ROS provides an early signal or a direct cause of cellular death during petal senescence (van Doorn and Woltering *et al*. 2008, Rogers 2012). Lipid peroxidation is commonly used as an indicator of prevalence of free radicals in tissues (Smirn off 1993). This process affects not only membranes and membranous proteins but also produces a variety of toxic aldehydes and ketones (Valentine *et al.* 1998, Wilhelmova *et al.* 2006).

Cut flowers need the supply of sugars in the vase solution to fulfill the energy and carbon requirement to overcome stresses (Koizuka *et al.* 1995) as the synthesis of sugars has been hampered due to detachment from the plant. Besides sugars, phytohormones play crucial role in controlling petal senescence (Leopold and Nooden 1984). Polyamines (PAs) are low molecular weight polycationic, biogenic amines that are universal to all living organisms and involved in the regulation of growth, development and senescence probably by binding to negatively charged macromolecules (Bagini and Torrigiani 1992). Although, some of the studies have demonstrated senescence-retarding effects of PAs (Dantuluri *et al.* 2013) more investigations are needed to assess the effectiveness of putrescine (Putr), spermidine (Spmd) and spermine (Spm) in the regulation of petal senescence. Selecting

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Chrysanthemum dendranthema grandiflorum var. Chandrima, known to be a non-climacteric flower and also several species of the genus considered as less sensitive to ethylene (Woltering andvan Doorn 1988), attempt has been made to assess to what extent Putr and Spmd are able to minimize various harmful physiological and biochemical changes during petal senescence.

MATERIAL AND METHODS

Plantlets of *Chrysanthemum dendranthema grandiflorum* var. Chandrima collected from Floriculture Department of Indian Agricultural Research Institute, New Delhi were transplanted in uniformly maintained experimental plots of University Botanical Garden, Kurukshetra during the month of August-September. Experiments were carried out during January-February. Flowers of fully opened stage with maximum diameter were used for experimentation. The scapes (flowers without leaves) harvested in the morning hours were cut under water to prevent cavitations and were immediately brought to laboratory. The basal few centimeters were recut under double distilled water (DDW) to obtain an uniform length of 14 cm. Healthy scapes were transferred to different test solutions in conical flasks (Borosil-make) of 100 ml capacity. For each concentration, 10 conical flasks were used, each one having 30 ml test solution. One scape was kept in each flask (see Plate 1).

Vase solutions used for keeping these scapes were sucrose (0.1M), putrescine (1×10^{-4}M), putrescine (1×10^{-4}M) + sucrose (0.1M), spermidine (1×10^{-4}M) and spermidine (1×10^{-4}M) + sucrose (0.1M). Scapes kept in sucrose solution were considered as control.

Petal samples were collected at three day intervals until the flower senesces completely. Flower petals were plucked with the help of forceps from different treatments, washed and dried in the folds of filter paper and samples were made in such a manner that 3 replicates were available for each biochemical estimation and analysis. After recording the fresh weight, the samples were put in the deep freezer till their analyses.

Flower head diameter, fresh weight of each scape and vase life of flowers were recorded. Moisture content and membrane stability index (MSI) of petals were also determined. Dry mass was determined by keeping the petals in oven at 70 ºC for 24 h. Moisture content was determined as the difference between fresh and dry mass. MSI of petals was determined by recording the electrical conductivity of leachates in double distilled water at 40 and 100 °C (Deshmukh *et al*. 1991).

Lipid peroxidation : The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content following the method of Heath and Packer (1968). Two hundred milligram 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} TBA in 200 g L^{-1} 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 $mM^{-1}cm^{-1}$.

Protein estimation : Protein was determined by the method of Bradford (1976). To 0.2 ml of petal extract 0.8ml of DDW was added to make it 1 ml. Then 5 ml of dye was added in each test tube. Absorbance was read at 595 nm in a uv-vis spectrophotometer (Specord-205, Analytic Jena, Germany). Blank was prepared without petal extract; 1 ml of DDW was taken instead of enzyme along with 5 ml of dye.

Proline estimation : Proline content was measured by the method of Bates *et al.* (1973). Two hundred mg of petal sample was

homogenized in 2 ml of 3 % sulfosalicylic acid. The homogenate was centrifuged for 15 min at 10,000 rpm in a Remi centrifuge (R-8C). Free proline in the supernatant was treated with 2 ml of 3 % GAA and 2 ml of acid ninhydrin. Reaction mixture in the test tubes was kept in boiling water bath for 1 hour at 95°C Reaction was terminated in the ice bath and colour complex was extracted in 4 ml of pure toluene. The absorbance was recorded at 520 nm in a uv-vis spectrophotometer (Specord 205, Analytic Jena, Germany).

Estimation of starch and sugars : The amount of starch and sugars were determined by Anthrone method of Hart and Fischer (1971). One hundred milligram sample was extracted in 10 ml of DDW and centrifuged at 5000 rpm for about 10 min in a Remi centrifuge. The residue left after the separation of aqueous extract was later used for the determination of starch. It was mixed with 10 ml of DDW and stirred. This mixture was cooled in ice water bath for 3-4 min and 13 ml of 52 % perchloric acid was added followed by constant stirring for 5-min. The final volume was raised upto 100 ml with DDW. Three ml of diluted extract was take in triplicate for determination of starch in terms of glucose by anthrone method. The absorbance was read at 630 nm. The amount of starch was calculated by multiplying the starch value in terms of glucose with 0.90.

For the estimation of sugars, after separating the residue, the aqueous extract was collected and final volume was raised to 50 ml with DDW. The pH was also noted. 3 ml of extract was taken in triplicate and kept in water bath at 100 ºC for 5 min. 6 ml of anthrone reagent was added slowly after test tubes were placed in boiling water bath. Contents were mixed by gently shaking so that blue green colour is formed devoid of any turbidity. All test tubes were cooled under ice water, blank set was also prepared by adding 3 ml of DDW and 6 ml of anthrone reagent which is also kept in water bath with reaction set. Then absorbance was noted at 600 nm for total sugars in UV-Vis spectrophotometer.

The remaining extract was hydrolysed with 10 ml of 50 % HCl. The process of hydrolysis was carried out at room temperature for a day and next day same pH was set as it was on previous day with 6 N NaOH. This was followed by raising the volume to 100 ml with DDW. The same procedure was followed as above to prepare reaction and blank sets. The absorbance was recorded at 625 nm to observe reducing sugar. Difference between total sugars and reducing sugars indicated the amount of non-reducing sugars.

α-Amylase activity : The specific activity of α-Amylase was measured by the method of Bernfeld (1951). One hundred mg of petal sample was homogenized in 10 ml of DDW. It was centrifuged at 5000 rpm for about 10 min. in R-8C centrifuge. The supernatant was collected and used for the determination of α-amylase activity. Now 1 ml of extract was incubated for 3 min. at 20 °C with 1 ml of substrate (starch) solution. The enzyme reaction was interrupted by the addition of 2 ml of 3, 5-dinitrosalicylic acid reagent. Then test tubes were placed in boiling water bath at 100 °C for 5 min., they were cooled in running tap water. After adding 10 ml DDW, the absorbance of the solution containing brown reduction product was determined at 540 nm. Blank was prepared in the same manner without petal extract; 1 ml DDW was taken instead of it. Specific activity was expressed as α-amylase activity per mg protein. Protein content was estimated in terms of BSA by the method of Bradford (1976) using Coomassie Brilliant blue G-250.

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 carried out 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

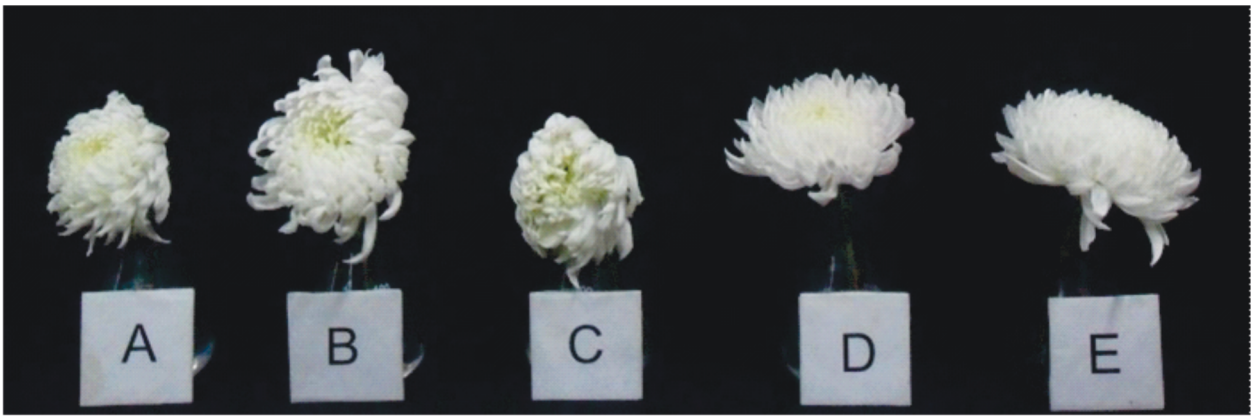
The longevity of the *Chrysanthemum* flowers

used in this study was recorded as shown in Table 1. Flowers picked on initial day were highly fresh and flowers kept in control (sucrose) have vase life of 9-day. Maximum freshness was noticed till 14-day in flowers treated with combination of spermidine (Spmd) + sucrose (Suc) (Plate 1). Onset of flower senescence showed discoloration of petals which turned brown later on. Control sets of flower petals exhibited shrinkage due to wilting. Spmd + Suc combination was most effective in checking shrinkage. Flower diameter was maximum when the experiment was started. Applied concentrations of polyamines were unique in checking shrinkage in flower diameters in cut chrysanthemums, the best result was obtained with Spmd + sucrose followed by Spmd, Putr,Putr + Sucand Suc. Similarly, a constant loss of fresh weight was also noticed which is a characteristic symptom of flower senescence, finally leading to visible wilting (Halevy and Mayak 1979, Borochov and Woodson 1989). Water stress is the most common reason for reduced vase life of cut flowers (Halevy 1976, Joyce and Jones 1992). Treatments of Putr, Putr + Suc, Spmd, Spmd + Suc were effective in reducing the loss in flower diameter, fresh weight (fr. wt.) and percent declinein fresh weight as compared to flowers kept under control (sucrose). Putrescine and spermidine have been reported earlier to enhance longevity by increasing fresh weight and cumulative uptake in *Narcissus* cut flowers (Sardoei *et al.* 2013) while increased vase life by spermidine has been reported in cut spikes of *Gladiolus* (Dantuluri *et al.* 2008). The trend of effectiveness among treatments revealed spermidine + sucrose as most effective having maximum flower diameter (10.0 ±0.12 cm), fresh weight (14.5 ±0.3 gm) and least percent decline in fr. wt. (26.40 %).Moisture content of fresh cut flowers was highest at initial day (84 %) and decreased subsequently in control (sucrose) at 3rd (76 %), 6th (64 %), 9th(51 %)-day with the progress of days. Again, the retention of moisture using above concentrations of Spmd and Putr was higher than control. Combination of spermidine + sucrose was able to retain maximum moisture content of 84, 80 and 76.8 percent at 3rd, 6th and 9th day (Table 2).

Values of MSI using Putr and Spmd alone and also in combination with sucrose have been shown in Fig 1 A. MSI values after polyamine treatments were higher in comparison to control and they enhanced vase life. Maximum petal MSI (99.4 %) was observed in cut *Chrysanthemum* flowers having Spmd + Suc in vase solution while the control was showing lowest value (62 %) at 9th –day. An increase in membrane permeability is a characteristic attribute of senescing plant tissues (Ferguson and Simon, 1973; Suttle and Kende, 1980). Significant increase in lipid peroxidation was observed after 3-day. Maximum MDA content (0.0626n mol g^{-1} fr. wt.) was recorded in control cut *Chrysanthemum* flowers while, the lowest value (0.0482 n mol g^{-1} fr. wt.) was observed in Spmd + sucrose treated cut flowers (Fig 1 B). 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). Various studies have indicated an increment in MDA content during senescence as in tulips (Jones and Mc Conchie 1995), roses (Fukuchi-Mizutani *et al.* 2000) and gladiolus (Ezhilmathi *et al.* 2007). Investigation with cut flowers carried out in our laboratory also indicated increment in the levels of MDA with the progress of senescence in inflorescences of *Salvia officinalis* L. (Kaur and Mukherjee 2010), *Matricaria parthenium* L. (Kaur and Mukherjee 2012), *Chrysanthemum* (Khokhar et al 2013), and *Calendula officinalis* L. (Kaur and Mukherjee 2013).

Fig 2 A, revealed a significant decline in starch content in flower petals of *Chrysanthemum* with the passage of time. The breakdown of starch on successive days during senescence was checked by the application of Putr and Spmd as holding solutions. The combination of spermidine with sucrose played a significant role in minimizing the degree of senescence and was

Initial Day



12-Day **Plate 1 (A-E)**

Cut flowers of *Chrysanthemum* treated with : **A.** Sucrose (0.1 M) **B.** Putrescine (1×10^{-4} M) **C.** Putr (1×10^{-4} M) + sucrose (0.1 M) **D.** spermidine (1×10^{-4} M) **E.** Spmd (1×10^{-4} M) + sucrose (0.1 M).

Table 1 : Flower diameter, fresh weight, percent decline, volume of holding solution and vase life in cut flowers of *Chrysanthemum* treated with sucrose (0.1M), putrescine (1×10^{-4}) and spermidine (1×10^{-4}).

Treatments	Flower diameter (cm)		Fresh weight of scape (gm)		Percent decline in fresh weight	Cumulative uptake of holding solutions (ml)† I_D = 30 ml	Vase Life (Day)
	I_D	9_D	I_D	9_D			
Suc	±0.5	6.06 ±0.12	19.2 ±0.1	6.11 ±0.5	68.17	5.2 ± 0.44	9
Putr	12.8 ±0.3	8.23 ±0.09	20.4 ±0.5	11.5 ±0.8	43.63	10.8 ±0.72	14
Putr + Suc	13.1 ±0.5	7.23 ±0.15	19.8 ±0.3	7.5 ±0.7	62.12	6.2 ±0.60	10
Spmd	12.9 ±0.3	9.23 ±0.12	20.0 ±0.1	11.1 ±0.4	44.50	9.8 ±1.10	11
Spmd + Suc	13.0 ±0.2	10.0 ±0.12	19.7 ±0.2	14.5 ±0.3	26.40	11.3 ±0.88	14

Table 2 : Moisture content in cut *C. dendranthema grandiflorum* flowers treated with sucrose (0.1 M), putrescine (1×10^{-4} M) and spermidine (1×10^{-4} M)

TREATMENTS	Moisture Content (%) I_D = 84 ±0.61		
	3_D	6_D	9_D
Suc	76 ± 0.41	64 ± 0.21	51 ± 0.38
Putr	82 ± 0.86	79.2 ± 0.8	75.8 ± 0.88
Putr + Suc	80 ± 0.31	74.4 ± 0.64	61 ± 0.52
Spmd	84.5 ± 0.58	78.8 ± 0.92	74.5 ± 0.64
Spmd + Suc	84 ± 0.54	80 ± 0.84	76.8 ± 0.56

showing best result. From an initial value of 14 ±0.04 mg100 mg^{-1} dry wt., the starch content was only 3.26 ±0.001 mg100 mg^{-1} dry wt. on the 9th day. The values of starch contents in Spmd + Suc treated flowers were 9.82 ±0.013, 6.79 ±0.0012 and 6.18 ±0.004 on 3, 6 and 9-day respectively A comparison of percent decline in starch content from 'initial' to 9th day between control and Spmd + Suc treated samples revealed about 14-21 per cent retention in the latter (Fig 2 A). The loss in fresh weight and moisture content affected starch content in petal tissue as such decline was observed during progressive days (Table 1 and 2). Such changes like decline in fresh mass and soluble carbohydrates are often linked with PCD (Zhou *et al.* 2005). Data exhibit active degradation of starch content in senescing and stressed tissues with the enhanced induction of α-amylase activity. Reports of Koizuka *et al.* (1995) and Yakimova (1997) revealed that under stressed situations a cell require more sugars to fulfill energy and carbon needs, which leads to active degradation of sucrose/starch by enhanced induction of invertase and α-amylase. α-Amylase enzyme is capable of releasing glucans from starch granules (Steup *et al.* 1983; Sun *et al.* 1995; Scheildig *et al.* 2002).

A clear increment has been shown in specific activity of α- amylase at 3, 6 and 9-day stage of flower petals in *Chrysanthemum*. Fig 2 B shows much lower values of α-amylase activity of 0.00917 mg^{-1} protein in *Chrysanthemum* petals at 'initial' stage. Among all the treatments Spmd with Suc most effectively reduced the increase in α-amylase activity on progressive days. Spermidine, spermidine + sucrose and putrescine have 8.35, 8.22 and 10.90 fold increments in α-amylase activity from initial to 9-day. Control and putrescine + sucrose treated cut flowers have much higher values of α-amylase activity at 6th and 9th-day.

Fig 3 A-C clearly showed that at initial-day, among the total sugars the amount of reducing sugars was significantly higher than non-reducing sugars. Further, a sharp increase in reducing, non-reducing and total sugars was noticed in cut flowers of *Chrysanthemum* petals. At initial-day, the amount of reducing sugars was 8.07 mg 100 mg^{-1} dry wt. The percent increments in reducing sugars at 3, 6 and 9-day in control were 143.5, 166.4 and 273.7 respectively; likewise in non-reducing sugars percent increments were 100.2, 126.2 and 136.1. According to Hammond (1982) and Tirosh and Mayak (1988), the activity of α-amylase plays an important role in the mechanism of petal opening and regulate the appearance of senescence syndrome. Some earlier studies in roses (Nichols 1973; Ho and Nichols 1977) indicate that reducing sugars rather than sucrose was the main constituents of sugar pool of mature petals. Amount of both reducing and non-non-reducing sugars has been found to decline at senescent phase of petals in cut flowers of carnation (Nichols 1975). However, report by Trivellini *et al.* (2011) showed a decline in reducing sugars and increase in sucrose in petals of *Hibiscus rosa sinenesis*.

In our laboratory, the specific activity of α-amylase increased sharply with a gradual decline in starch content (1.62 mg to 0.26 mg/100 mg dry wt.) in *Matricaria parthenium* cut flowers during 8days (Kaur and Mukherjee 2012). At initial stage, the amount of non-reducing sugar was significantly higher than reducing sugars but during subsequent 8 days the increment in latter was

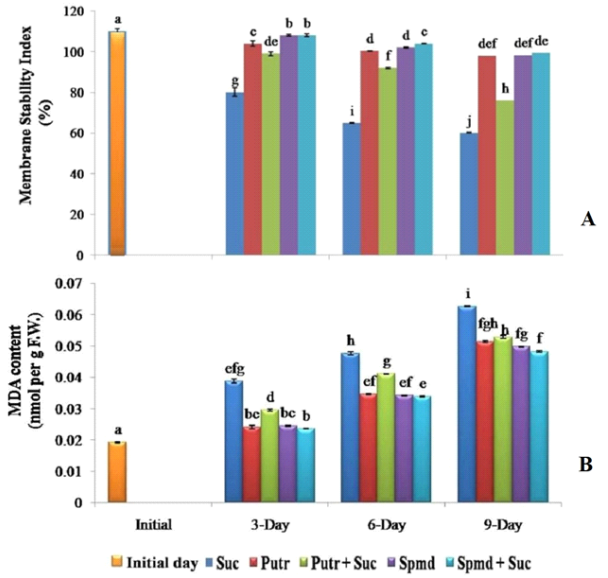


Figure 1 : (A) MSI and (B) MDA content in petals of *Chrysanthemum dendranthema grandiflorum* flowers treated with various vase solutions. Different letters in the figure represent values significantly different at 0.5 % level, using Duncan Multiple Range Test (DMRT).

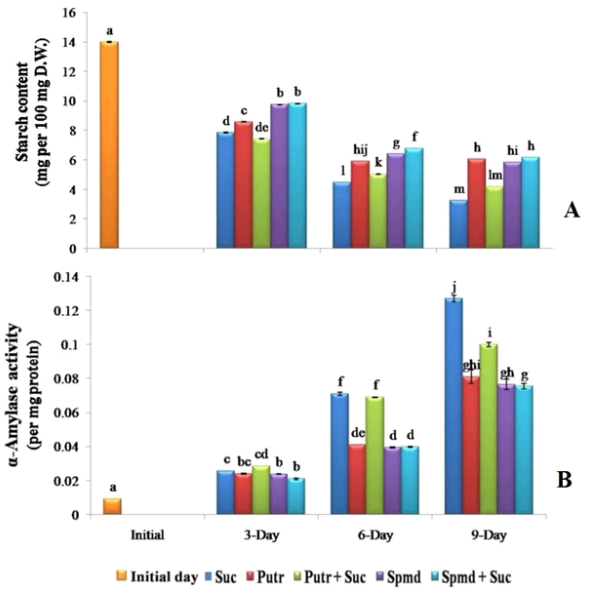


Figure 2 : (A) Starch content and (B) α-amylase activity in petals of *C.dendranthema grandiflorum* flowers treated with various vase solutions. Different letters in the figure represent values significantly different at 0.5 % level, using Duncan Multiple Range Test (DMRT).

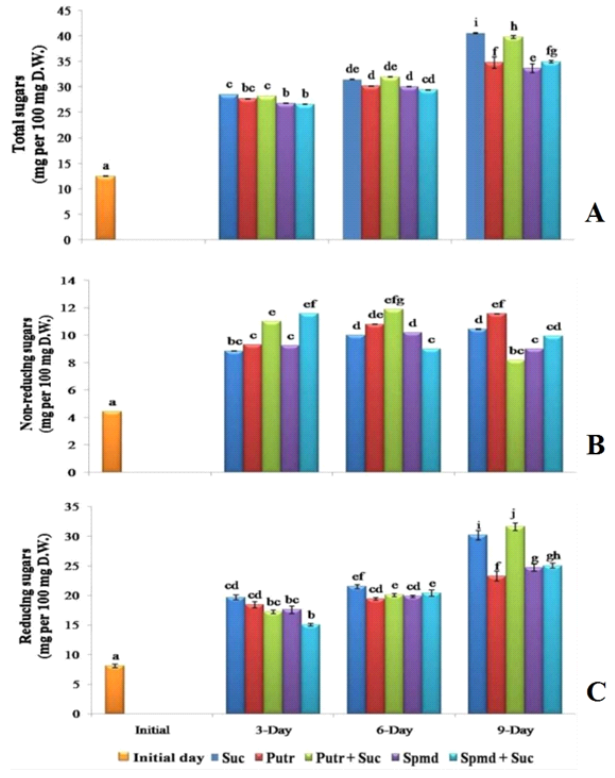


Figure 3 : (A) Total, (B) non-reducing and (C) reducing sugars in petals of *C. dendranthema grandiflorum* flowers treated with various vase solutions. Different letters in the figure represent values significantly different at 0.5 % level, using Duncan Multiple Range Test (DMRT).

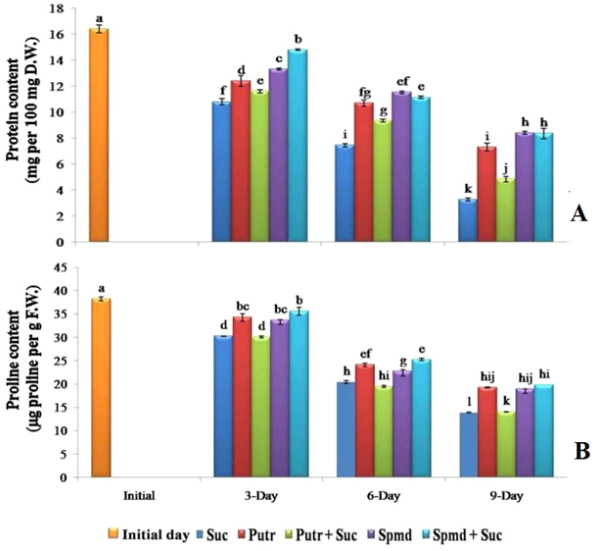


Figure 4 : (A) Protein and (B) proline content in petals of *C. dendranthema grandiflorum* flowers treated with various vase solutions. Different letters in the figure represent values significantly different at 0.5 % level, using Duncan Multiple Range Test (DMRT).

much higher than the former although the amount of former was still slightly higher than the latter. Starch content and α-amylase activity in *Calendula officinalis* L. exhibited similar trend as found in *M. parthenium.* However, reducing sugars was much lesser in concentration than non-reducing sugars initially in cut flowers of *C. officinalis*, both of which increased sharply at later stages (Kaur and Mukherjee 2013). While working on the cut flowers of *C. officinalis* and *Arctotis grandis*, Khokhar and Mukherjee (2010) could notice accumulation of both

reducing and non-reducing sugars in senescing petals, the concentration of former was slightly higher than latter. In the present investigation on *Chrysanthemum*, petal senescence was characterized by increment in both types of sugars during 9-day that was partly lowered by Putr and Spmd by regulating starch breakdown and α-amylase activity.

Changes in protein and proline content were also studied at intervals during treatment of *Chrysanthemum* cut flowers. The protein and proline content was high at initial stage (16.4 ±0.31 mg 100 mg^{-1} dry wt. and 38.26 n g g^{-1} fr. wt. respectively) and decreased with the progressive days as shown in Fig4 A-B. Maximum amount of protein (8.42 ±0.11 mg 100 mg^{-1} dry wt.) and proline content (19.8 ±0.016 n g g^{-1} fr. wt.) in petals at 9-day were recorded in cut flowers placed in vase solution enriched with spermidine and spermidine + sucrose respectively. Control samples recorded minimum amount of protein and proline content (3.26 ±0.12 mg 100 mg^{-1} dry wt. and 13.88 n g g^{-1} fr. wt. respectively) at 9-day . The decline inprotein and proline during 0-9 day was effectively controlled by Spmd with sucrose and Putr. Changes in the levels of proteins have been associated in part as a result of synthesis of "new" specific protein during senescence (Woodson and Handa 1987) such as proteases degrade proteins by hydrolyzing internal peptide bond and is one of the best characterized cell death proteins in plants (Beers *et al.* 2000). From the amount of total protein plants conditions in the field and post-harvest can be evaluated. In *Hemerocallis* (Lay-Yee *et al.* 1992), *Sandersonia* (Eason *et al.* 2002) and *Dendrobium* cv. Khao Sanan peatls (Lerslerwong *et al.* 2009), protein content decreased during senescence. In addition to role of proline in protein synthesis and the plant cells' responses to environmental stress, evidence suggested that proline may also play significant role in flowering and development both as metabolite and as a signal molecule (Mattioli *et al.* 2009). Earlier report proved that under stress conditions many plant species accumulated proline as an adaptive response to adverse conditions (Verbruggen and Hermans 2008). Schwacke *et al.* (1999) observed that the content of free proline in tomato flowers was 60-fold higher than in any other organ analyzed. Our observations revealed that cut flowers treated with Putr and Spmdwere able to reduce stress having higher amounts of protein and proline (Fig 4 A-B).

In this work it has been hypothesized that exogenous application of spermidine and putrescine could improve the water uptake, fresh weight, membrane stability index of cut *Chrysanthemum* flowers during their vase life. These treatments were able to reduce the increments in MDA content, sugars (non-reducing and reducing); decline in starch, protein and proline content. On comparison between various treatments; spermidine with sucrose was most effective in extending vase life.

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