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Effect of salicylic acid, malic acid, citric acid and sucrose on antioxidant activity, membrane stability and ACC-Oxidase activity in relation to vase life of carnation cut flowers

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Combinations of malic acid, salicylic acid, citric acid and sucrose were used as preservative mixture for cut carnations and their effect on regulation of senescence was examined. The study was conducted in a factorial arrangement, carried out in a complete randomized design. The factors were malic acid (0,100 and 150 mg l^{-1}), salicylic acid (0, 1.5 and 3 mM), citric acid (0 and 150 mg l^{-1}) and sucrose (0 and 3% w/v) .The effects of treatments and their interaction on the total chlorophyll content, ACC-Oxidase activity, anthocyanin leakage, membrane stability and malondialdehyde content of cut flowers of carnations (*Dianthus caryophyllus L*. cv. *White.)* were investigated. 150 mg l^{-1} MA and 1.5 mM SA both caused significant decrease in anthocyanin leakage, ACO activity and MDA content compared to other levels ($p \leq 0.05$). Flower stems that were kept in water containing either 150 mg l^{-1} MA or 1.5 mM SA or their combinations; all had significantly increased vase life relative to the control treatment. MA application increased water uptake and decreased microbial growth as well.

Key Words: carnation, Membrane stability, vase life, malondialdehyde, ACC-Oxidase activity

Abbreviations: MA, malic acid; SA, Salicylic acid; Suc, Sucrose; MDA, malondialdehyde; ROS, reactive oxygene species; ACO, ACC-Oxidase; ACS, ACC Synthase

Introduction

Carnation is a climacteric flower that is highly sensitive to ethylene (Pun *et al*., 1999). The gas ethylene enhances senescence and shortens the vase life of many flowers (Reid and Wu, 1992; Teixeira da Silva, 2003). Ethylene is produced autocatalytically during carnation petal senescence. During the climacteric respiration, there is a coordinated increase in the activities of ACS and ACO (Woodson *et al*., 1992), which convert S-adenosylmethionine (SAM) to 1- aminocyclopropane -carboxylic acid (ACC) and ACC to ethylene,

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respectively (Yang and Hoffman, 1984). Expression of the ACS and ACO genes in carnation petals depends on the presence of ethylene (Savin *et al*., 1995). Senescence of flower petals is characterized by nonreversible cellular processes leading to death. Ethylene production increases sharply with senescence. Ethylene enhanced flower senescence and wilting, increased permeability of petal cells and accelerated the decrease in the cell membrane fluidity (Kazemi *et al*., 2010). The other consequences include an increase in the cell membrane permeability and solute uptake capacity, degradation of membrane lipids and MDA production (Teixeira da Silva, 2003). Ethylene production causes a marked increase in production of oxygen free radicals which is responsible for stress dependent peroxidation of membrane lipids (Reezi *et al*., 2009). One effect of ROS accumulation in plant cells under stress is lipid peroxidation via oxidation of unsaturated fatty acids leading to membrane damage and electrolyte leakage (Liu *et al*., 1987; Allen, 1995; Mittler, 2002). MDA, a decomposition product of polyunsaturated fatty acids, have been utilized as a biomarker for lipid peroxidation. MDA content can serve as an indicator of the rate of oxidative processes in cell (Shakirova, 2007). During senescence, MDA accumulates rapidly as the product of peroxidation of membrane lipids, (Hernandez *et al*., 1993; Lutts *et al*., 1996; Fadzilla *et al*., 1997), which results in an increase in permeability of plasma membranes. The peroxidation process may be retarded by using ethylene inhibitors, which could delay flower senescence in many sensitive cut flowers (Serek and Sisler, 2001). The senescence effects can be reduced by inhibitors of ethylene biosynthesis and increased antioxidant enzymes activity (Khan *et al*., 2003; El-tayeb *et al*., 2006; Shi and Zhu, 2008; Joseph *et al*., 2010). SA is a well-known phenol that can prevent ACC-oxidase activity and decrease ROS with increasing antioxidant enzyme activity (Ansari *et al*., 2007; Mba *et al*., 2007; Mahdavian *et al*., 2007;Canakci, 2008; Kalidage, 2009). Mei-hua *et al*. (2008) showed that SA could extend the vase life of cut flowers by decreasing ROS and ethylene. SA cause delay in the onset of hydrolysis of structural cell components, decrease ROS production, ACC-oxidase activity and sensitivity (Li *et al*., 1992; Srivastava and Dwivedi, 2000). CA seems to act by reducing the water pH and, consequently, the bacterial growth, which block the xylem vessels in the cut region of stem (Nowak and Rudnicki, 1990). Suc is another organic molecule known to delay senescence and prevents up-regulation of senescence-associated genes in carnation petals (Hoeberichts *et al*., 2007). Previous work had revealed that MA sprays during the growth period increased chlorophyll content of cut flowers while CA spray caused extended post harvest vase life (Darandeh *et al*., 2010). Based on these results and use of CA in many floral preservative formulas as a water conductance aid, we

considered testing MA in floral preservative mixture to investigate any potential positive effect(s). As MA is readily metabolized by plants, but not by many microorganisms, so we considered using it as a possible substitute for Suc. Use of Suc necessitates the addition of biocidal agents, which is not considered an environment friendly method due to the side effects like facilitating the emergence of resistant strains of microorganisms to frequently used biocides. Therefore, In this study, the preservative effects of MA, SA, CA, Suc and their interaction on the vase life of cut carnation flowers were studied.

Materials and methods

Plant material and storage conditions

Cut flowers (Dianthus caryophyllus L. cv. White) were harvested in open stage in the morning from a local commercial greenhouse (Pakdasht, Tehran, Iran), and transported with appropriate covers immediately to Laboratory (horticulture laboratory of agriculture faculty of Islamic Azad university, Karaj Branch). Stems were recut to 40 cm length. In this study, three levels of MA (0,100 and 150 mg l^{-1}), two levels of Suc (0 and 3% w/v), three levels of SA (0, 1.5 and 3 mM) and two levels of CA (0,150 mg l^{-1}) were applied on 144 carnation cut flowers cv. White. After recording the fresh weight, each flower was placed in a 250-ml bottle containing preservative solutions. The flowers were held at ambient temperature (19 ±5 ℃). The experiment was started on February 15 2010 and chlorophyll content, membrane stability, MDA content and ACC Oxidase activity were measured on the last day of vase life for each flower.

Vase life

Vase life was determined as the number of days to wilting of flowers. The flowers were checked once a day for signs of deterioration.

Chlorophyll index

Chlorophyll index was measured by chlorophyll meter (SPAD-502, Minolta Co. Japan), which is presented by SPAD value. Average of 3 measurements from different spots of a single leave was considered.

Determination of anthocyanin leakage

Anthocyanin leakage was measured based on the method of Poovaiah (1979). Petal samples were cut into 1*1 cm segments and placed in individual tubes containing 25 ml of deionized water. After two washes with distilled water to remove surface contamination, 10 ml of distilled water was added to samples. After 12 h incubation at 25 ℃ the anthocyanin leakage to liquid was determined at 525 nm using spectrophotometer (Perkin-Elmer- EZ-201).

Determination of Acc-oxidase activity

The extraction and quantification was conducted based on the method described by of Moya-Leòn *et al*. (2004) with slight modifications. 1 g petal tissue was homogenized by a mortar and pestle with 3 ml extraction buffer consisting of 1% (w/v) polyvinyl polypyrrolidone, 0.1 mM Tricine with (pH adjusted to 7.5), 10% glycerol, 5mM DTT and 30 mM sodium ascorbate for 2 minutes . The homogenate was centrifuged at 20000×g for 20 min, and the supernatant was collected for enzyme assays. All procedures were conducted at 4°C.

Determination of ACC oxidase activity: After a 20 minute incubation of the enzyme extract with the ACC containing enzyme activation complex [0.1M Tricine (pH 7.5), 30mM sodium ascorbate, 0.1mM ferrous sulfate,10% (v/v) glycerol, 1mM ACC, 2.5mM DTT and 30mM sodium bicarbonate] , the ACO activity was assayed as the amount of evolved ethylene, which was quantified on a GC apparatus.

Assays of MDA content (Lipid peroxidation)

Malondialdehyde content was measured based on the method of Heath and Packer (1978) with some changes. Fresh petals tissue was homogenized with a mortar and pestle in 5 ml solution of 1% trichloroacetic acid. The homogenate was centrifuged at 10000×g for 5 min. 4.5 ml TCA 20 % solution containing 0.5 percent TBA acid was added to 1 ml of the supernatant and incubated for 30 minutes at 95 °C water bath. The mixture was cooled immediately in ice and again centrifuged at 10000×g for 10 The absorption was measured with a spectrophotometer at 532 nm.

Microbe population

In day 11, samples were isolated from vase solutions of carnations in sterile containers. Aliquots of the vase solutions were diluted 100-times, and 25

µL aliquots of the diluted solution were spread on sterile nutrient agar in sterile petri plates. The plates were allowed to incubate for 48 hr at room temperature, and individual colonies of bacteria were counted.

Water absorption by cut flowers

The water uptake was calculated by subtracting the mean volume of water evaporated from three control bottle without cut flowers, from the amount of water decreased in bottles containing flowers in experimental course.

Experimental design and statistical analysis

Experiment was arranged in a factorial test with complete randomized design with four replications. Analysis of variance was performed on the data collected using the general linear model (GLM) procedure of the SPSS software (Version 16, IBM Inc.). The mean separation was conducted by tukey analysis in the same software (p= 0.05).

Results and discussions

The results indicated that 150 mg l^{-1} MA and 1.5 mM SA both caused a significant decrease in anthocyanin leakage, ACO activity and MDA content compared to other levels ($p \leq 0.05$). CA and Suc caused no positive effect or had a negative effect as increasing anthocyanin leakage and ACO activity (Table 1 and Figure 1). On the other side, highest means of ACO activity were found in cut flowers treated with 100 mg l^{-1} MA + 3 mM SA and 3 mM SA (Table 1). The interaction between MA and SA on ACO activity was significant, as well (Figure 3). These findings are similar to previous results (Jamali, 2011; Kazemi *et al*., 2011a,b). The results indicate that 150 mg l^{-1} MA and 1.5 mM SA and their combination caused a significant increase in SPAD value ($p \leq 0.05$).

Application of Suc caused a significant decrease in chlorophyll index while CA had no significant effect (Table1 and Figure 1). These results are in agreement with those of (Kazemi *et al.*, 2011b,c) who found that adding SA, MA and GLU in vase water increased chlorophyll content cut flowers. similarity, Canakci (2008) reported that treatment with salicylic acid significantly extends the vase life with increases chlorophyll content. Holding carnation cut flowers in vase solutions containing 150 mg l^{-1} MA significantly increased their vase life and delayed flower senescence compared to flowers either held in 100 mg l^{-1} MA or distilled water (Table 1). MA was found to be significantly and positively correlated with vase life of the carnation cut

flowers as well. Flower stems which were kept in water containing either 150 mg l^{-1} MA or 1.5 mM SA or their combinations all had significantly increased vase life compared to the control treatment ($p \leq 0.05$, Table 1 and Figure 2).

Table 1. Effect of malic acid, salicylic acid, citric acid and sucrose combinations in preservative mixture on cut carnations, shaded row is the control combination. Data were recorded in the last day of vase life. The citric acid, which was not effective on most variables, was omitted from first part to avoid an unnecessary complicated table

MA (mg l^{-1})	SA (mg l^{-1})	Suc (% w/v)	vase life (day)	total chlorophyll (SPAD reading)	ACC Oxidase activity (nmol h^{-1} ml^{-1})	antocyanin leakage (absorption at 525 nm)	MDA (µmol/mg protein)	water uptake (ml per flower)	colony count (cfu ml^{-1})
0	0	0	6.5	2.2	19.5	234.0	159.9	105.0	42.5
		30	5.0	0.7	35.2	334.5	199.4	75.0	68.5
	1.5	0	9.0	5.5	10.6	128.0	131.3	95.0	17.5
		30	8.5	4.3	12.7	173.0	143.6	97.5	32.0
	3	0	3.5	0.8	39.1	475.5	234.3	77.5	22.5
		30	4.5	1.1	35.2	407.5	197.4	75.0	32.5
100	0	0	6.0	1.4	24.4	252.5	191.0	102.5	18.5
		30	7.0	1.6	20.0	199.0	159.6	97.5	31.5
	1.5	0	7.0	2.1	15.9	191.0	140.6	97.5	17.0
		30	7.0	2.0	15.9	216.5	142.4	95.0	30.5
	3	0	4.5	0.9	35.6	456.0	221.2	82.5	14.5
		30	5.5	1.0	31.9	432.5	189.7	77.5	28.0
150	0	0	9.0	6.4	9.5	146.0	121.3	127.5	13.0
		30	8.5	3.9	12.1	179.0	117.6	115.0	20.5
	1.5	0	10.1	6.0	8.5	115.5	117.0	145.0	10.5
		30	10.5	5.5	8.8	116.5	116.2	127.5	19.0
	3	0	5.5	1.3	24.1	344.5	200.7	97.5	13.0
		30	6.0	0.8	27.1	290.5	180.9	95.0	20.0
	F-test probabilities								
		MA	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		SA	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		Suc	0.414	0.040	0.354	0.970	0.183	0.036	0.000
		CA	0.053	0.282	0.424	0.078	0.242	0.007	0.064

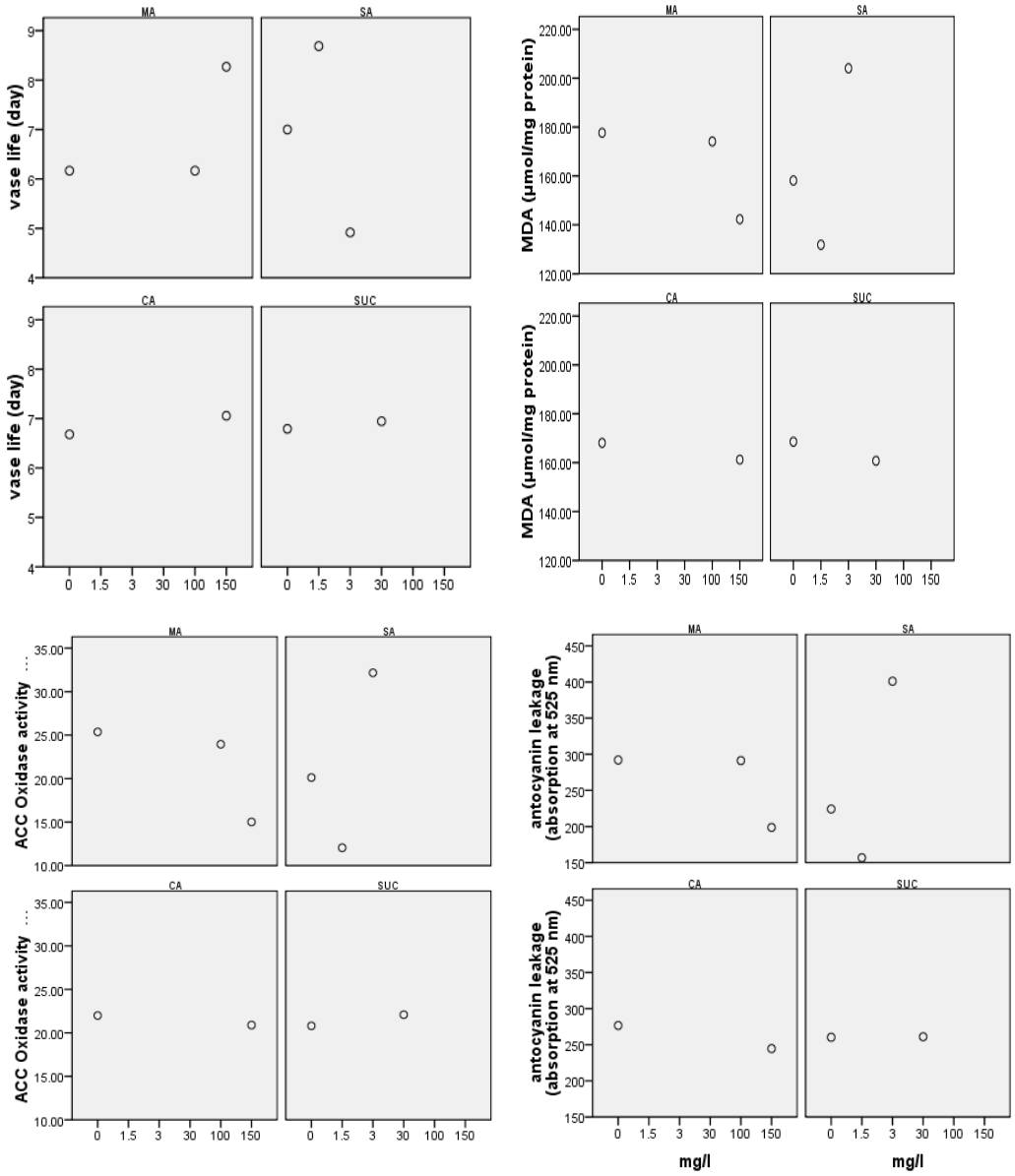

Fig. 1. Effect of experimental factors on vase life, MDA content, ACO activity and anthocyanin leakage of cut flowers. The similar pattern among recorded variables is notable

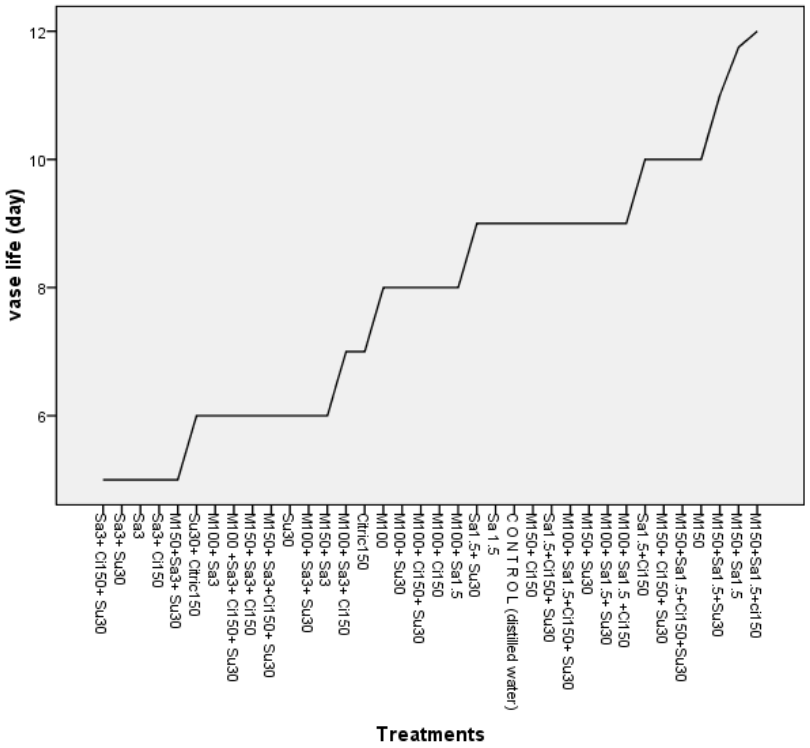

Fig. 2. Effect of factor-level combinations (treatments) on vase life of cut carnations

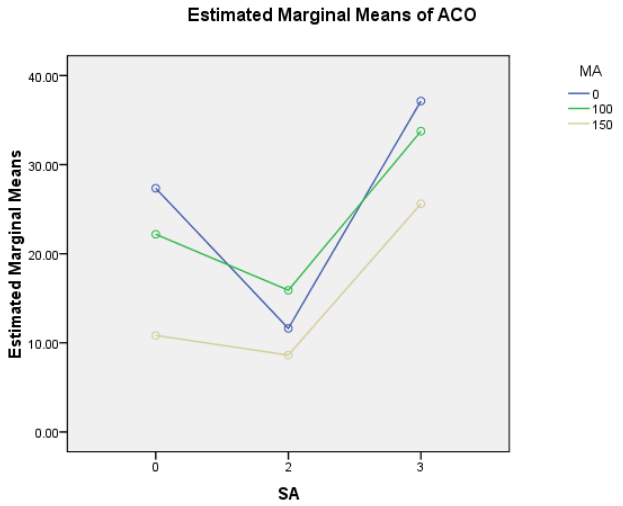

Fig. 3. Interaction between MA and SA on ACO activity.MA at 150 mg l-1 had reduced SA effect on ACO activity

Some factor-levels containing CA and Suc were among those with significantly higher vase life than control (Figure 2). Yuping (2009) reported

that treatment with salicylic acid significantly extends the vase life with increases the enzyme antioxidant activity and decreased ROS production. 150 mg l^{-1} MA caused an increase in water uptake by flowers while 1.5 mM SA was not effective significantly. MA caused a linear reduction in the colony count, from 36 cfu ml^{-1} in 0 levels to 23 and 16 cfu ml^{-1} in 100 and 150 mg l^{-1} MA respectively. Both 1.5 mM SA and 3 mM SA decreased the colony count significantly from 32 cfu ml^{-1} to around 21 cfu ml^{-1}. 150 mg l^{-1} CA significantly increased water uptake by individual flowers 8 ml per flower but had no effect on colony count. Suc application caused decreased water uptake by mean of 11 ml per flower and increased the colony count 12 cfu ml^{-1}($p \leq 0.05$, Table 1). SA is a well known phenol that can prevent ACC-oxidase activity that is the direct precursor of ethylene and decrease ROS with increase enzyme antioxidant activity(Ansari and Misra, 2007; Mba *et al*., 2007; Mahdavian *et al*., 2007; Canakci, 2008). These findings are in agreement with those reported by Lamikanra and Watson (2001), Mei-hua *et al*. (2008), Kazemi and Shokri (2011). The effect of SA on senescence and vase life extension of cut flowers was reported earlier which is confirmed here was anticipated, but the effect of MA on senescence indices, which is reported here for the first time, could be promising. 150 mg l^{-1} MA not only enhanced the senescence related variables like anthocyanin leakage, ACO activity, MDA content and chlorophyll index in par with SA, but also reduced the bacterial load of vessel like SA. In addition, MA increased water uptake by cut flowers that was not noted by applied SA levels. MA did the well-known duty of CA as a water uptake-increasing agent in a better manner together with a large effect on senescence related factors, which makes it a suitable substitute for CA in preservative mixtures with a broad-spectrum effect. Presumably, the mode of action for these broad effects by MA may be due to its role as both a carbon source, which could fuel many carbohydrate dependent metabolic pathways, and an important anion in cell vacuoles, which could help to sustain the turgor pressure and water uptake and balance. Being unusable for many of ordinary microorganisms growing in vase solutions makes it a possible candidate for substitution of Suc, as well.

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