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Exogenous Putrescine Delays Senescence of Lisianthus Cut Flowers

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Abstract

The anti-senescence biogenic polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) are involved in plant growth processes and responses to stress. The physiological mechanism of exogenous Put to affect the senescence of cut lisianthus flowers during vase life was investigated. Fresh cut lisianthus flowers were treated with distilled water (control), 0.5, 1 and 2 mM Put and then held at 25 °C up to 12 days. Exogenous Put supply at 2 mM extended vase life, which was associated with reduced electrolyte leakage and MDA content. Put treatment also reduced activity of lipoxygenase (LOX), is responsible for membrane lipid peroxidation. Put treatment also enhanced activities of catalase (CAT) and ascorbate peroxidase (APX) and decreased H_2O_2 accumulation during vase life. Thus, exogenous Put supply could maintain membrane integrity by increasing antioxidant system activity, thereby retarding the senescence of cut lisianthus flower during vase life.

Keywords: Antioxidant enzyme, Lipoxygenase, Lisianthus, Putrescine, Vase life.

INTRODUCTION

Due to the ephemeral nature of the different tissues, the high respiratory activity and the low carbohydrate content of reserves flowers are classified as highly perishable products (Nowak and Rudnicki., 1990). Cut Eustoma flowers have many cultivars with variations in flower colour, size and shape. There are several opened flowers and buds in Eustoma inflorescence and for the extending the vase life of the inflorescence the longevity of each flower and the rate of bud opening are important (Shimizu-Yumoto and Ichimura., 2005). Thus, for the extending the vase life of cut Eustoma flowers preservatives containing sugars are effective (Cho *et al*., 2001). Increasing ethylene production during petal senescence indicating the Eustoma flower is ethylene-sensitive and ethylene involvement in flower senescence (Ichimura *et al.,* 1998). Some physiological and biochemical changes occurred during senescence such as loss of water from the senescing tissue, leakage of ions generation of reactive oxygen species (ROS), increase in membrane fluidity and peroxidation, hydrolysis of proteins, nucleic acids, lipids and carbohydrates (Tripathi and Tuteja, 2007). Polyamines (PAs) are ubiquitous and biogenic amines that have been implicated in cellular functions in living organisms. In plants they have been implicated in a wide range of biological processes including cell division, cell elongation, senescence, embryogenesis, root formation, floral initiation and development, fruit development and ripening, pollen tube growth and plant responses to biotic and abiotic stress (Hussain *et al.*, 2011). In plant tissues, the main PAs are putrescine (Put), spermidine (Spd) and spermine (Spm). Exogenous application of putrescine improvement of physiological status of *Helianthus annuus* and stimulated catalase activity (Rubinowska and Michałek., 2009). Pre- and post-harvest putrescine applications extend the vase life of cut alstroemeria flowers (Soleimany-Fard *et al.*, 2014). Conditioning of cut stems of rose 'Red Berlin' in the solution of putrescine at a concentration of 2 mmol resulted in the highest vase life (Rubinowska *et al.,* 2012). Exogenous spermidine delays senescence and extend vase life of cut *Dianthus caryophyllus* flowers (Tassoni *et al.,* 2006). In the present study, we investigated the effects of pulse treatment with Put on the vase life of cut lisianthus flowers, as well as physiological and biochemical changes during its petal senescence.

MATERIAL AND METHODS

Flowers and treatment

Cut flowers of lisianthus (*Eustoma grandiflorum*) 'Miarichi Grand White' were obtained from a commercial greenhouse and were re-cut under tap water to have uniform length of 30 cm. Flowers were then placed in a holding solution containing distilled water (control), 0.5, 1 and 2 mM Put. All treatments were kept at 25 ± 1°C under a 16:8 h light/dark cycle and 60 ± 5% RH for 24 hours. Subsequently, flowers were transferred to flasks containing only 200 mL distilled water and were kept at 25 ± 1°C under a 16:8 h light/dark cycle and 60 ± 5% RH for 12 days. The end of vase life was evaluated as the time which 50% of the open flowers had wilted (Cho *et al.,* 2001).

Membrane integrity evaluation

Membrane permeability, expressed by relative electrolyte leakage rate, was measured by the method of Jiang and Chen (1995). Thirty petal discs were immersed in 20 mL of 0.3 M mannitol solution at 25 ◦C, followed by shaking for 30 min. Electrolyte leakage was determined with a conductivity meter. Total electrolyte leakage was determined after boiling the samples for 10 min and cooling to 25 ◦C. Relative electrolyte leakage rate was expressed as a percentage of total electrolyte leakage. MDA content was measured according to the method of Heath and Parker (1968). Frozen petal tissues (1 g) from 10 flowers were ground finely in liquid nitrogen, then homogenized in 15 mL of 10% trichloroacetic acid (TCA) and finally centrifuged at 5000 × g for 10 min. The supernatant phase was then collected. MDA content was determined by adding 5 mL of 0.5% thiobarbituric acid (dissolved in 10% TCA) to 0.5 mL supernatant. The solution was heated at 95 ◦C for

20 min, quickly cooled, and centrifuged at 10,000 × g for 10 min to clarify precipitation. Absorbance at 532 nm was measured and subtracted from the non-specific absorbance at 600 nm. The concentration of MDA was calculated with an extinction coefficient of 1.55 n mol $L^{-1}m^{-1}$. MDA content was expressed as n mol g^{-1} fresh weight (FW).

According to method of Doderer *et al.*,(1992), for analysis of LOX activity, frozen petal tissues (1 g) from 10 flowers were ground finely in liquid nitrogen and then homogenized in 15 mL of 50 mM phosphate buffer (pH 7.0). After centrifugation at 10,000 × g and 4 ˚C for 20 min, the supernatant was collected and then used as the crude enzyme extract. LOX activity was assayed at 25 ◦C by monitoring the formation of conjugated dienes from linoleic acid at 234 nm according to the method of Axelrod *et al.*, (1981). The reaction mixture (3 mL) contained 2.8 mL of 50 mM sodium phosphate buffer (pH 7.0), 0.1 mL of 10 mM sodium linoleic acid solution and 0.1 mL of the crude enzyme solution. One unit of LOX activity was defined as a change of 0.01 in absorbance per minute at 25 ˚C. The specific LOX activity was expressed as U mg^{-1} protein.

Antioxidant system activity evaluation

Frozen petal tissues (2 g) from 10 flowers were ground finely in liquid nitrogen and then homogenized in 15 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) PVP. The homogenate was centrifuged at 10,000 × g for 15 min at 4 ˚C and then the supernatant was used to determine activities of CAT and APX. CAT activity was assayed by measuring the disappearance of hydrogen peroxide (H_2O_2) according to the method of Oracz *et al.*, (2009). The assay mixture (3 mL) contained 2.95 mL of 44.25 M H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 0.05 mL of enzyme extract. CAT activity was calculated by a decrease in absorbance at 240 nm for 3 min at 25 ˚C. One unit of CAT activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute and the specific activity was expressed as U mg^{-1} protein. APX activity was determined by the method of Nakano and Asada (1981). The reaction mixture (3 mL) consisted of 1.5 M ascorbic acid, 0.3 M EDTA and 0.3 M H_2O_2 solution in 50 mM phosphate buffer (pH 7.0) and 0.1 mL of enzyme extract. Ascorbate concentration was followed by the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}). One unit of APX activity was defined as 1 M ascorbate oxidized per minute at 290 nm and the specific activity was expressed as U mg^{-1} protein. The protein concentration of petal extracts was estimated using the method of Bradford (1976) by BSA as standard. The H_2O_2 content measured according to Patterson *et al.,* (1984). Frozen petal tissues (1 g) from 10 flowers were homogenized with 10 ml of acetone at 0 °C. After centrifugation for 15 min at 6000 × g at 4 °C, the supernatant was collected. The supernatant (1 ml) was mixed with 0.1 ml of 5% titanium sulphate and 0.2 ml ammonia, and then centrifuged for 10 min at 6000 × g and 4 °C. The pellets were dissolved in 3 ml of 10% (v/v) H_2SO_4 and centrifuged for 10 min at 5000 × g. Absorbance of the supernatant phase was measured at 410 nm. H_2O_2 content was calculated using H_2O_2 as a standard and then expressed as µmol g^{-1} fresh weight (FW).

For physiological parameters, results were expressed as mean ± SE from 3 replications. Statistical significance between mean values was assessed using one way analysis of variance with SAS (Version 9.1) statistical software. Means were compared using the LSD test.

RESULTS AND DISCUSSION

Vase life

Putrescine treatment increased significantly the vase life cut lisianthus flowers, as compared to control treatment during vase life. As shown in Fig. 1, treatment with postharvest Put at 2 mM resulted in a higher lisianthus cut flowers vase life ($P<0.01$). Based on these results, 2 mM Put for postharvest treatment was chosen for further analyses. Exogenous putrescine treatment increase vase life of cut alstroemeria and chrysanthemum flowers (Soleimany-Fard *et al.*, 2014; Kandil *et al.,* 2011). Effect of putrescine on the extending vase life of cut lisianthus flower is might be due to suppressed water loss, inhibiting ethylene action and decrease in transpiration rate (Pandey *et al*., 2000).

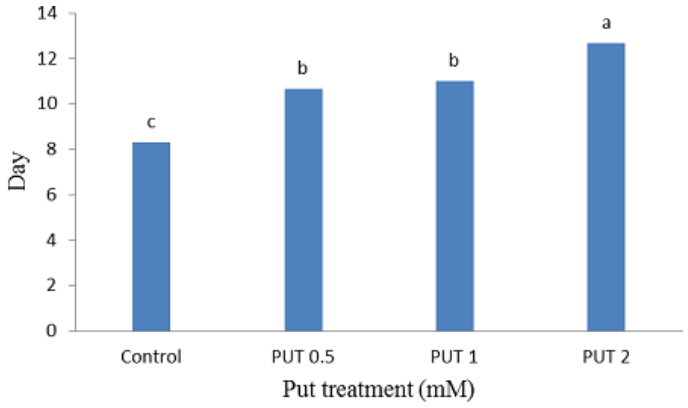

Fig.1. Effects of Put treatment at 0.05, 1 and 2 mM on vase life of lisianthus cut flowers.

Putrescine treatment and membrane integrity

Electrolyte leakage of the lisianthus cut flowers increased during vase life (Table. 1). The electrolyte leakage of lisianthus cut flowers treated with 2 mM Put at postharvest stage remained lower than that in untreated control flowers ($P<0.01$; Table. 1). As well, during vase life, the MDA content in the lisianthus cut flowers increased (Table. 1). Compared to the controls, a lower content of MDA was found in the lisianthus cut flowers treated with postharvest 2 mM Put ($P<0.01$; Table. 1). There was a significant increase in the activity of LOX in lisianthus cut flowers during vase life (Table. 1). The treatment with Put at 2 mM caused reduction in LOX activity in comparison to the control for the whole 12 days of vase life ($P<0.01$; Table. 1).

Increased the electrolyte leakage is one of the first symptom of correlated with advancing senescence (Shahri and Tahir., 2011). Exogenous application of putrescine decrease electrolyte leakage during leaf senescence of *Helianthus annuus* potted plants (Rubinowska and Michałek., 2009). Polyamines increase of plant's cellular membranes resistance by inhibition of lipid peroxidation process (Roy *et al.,* 2005). Application of polyamines decrease electrolyte leakage during the vase life of cut rose 'Red Berlin' (Rubinowska *et al.,* 2012). Inhibited protease and RNA-se activities by exogenous spermidine was reported, which probably supported the membrane stability and protected plant cell against injuries due to a stress (Kubiś., 2006). The increment in MDA has been described as a biomarker of lipid peroxidation (Bailly *et al*., 1996) and thus decreased its level in lisianthus cut flowers treated with Put indicates reduced lipid peroxidation. Reduced lipid

Table. 1. Effect of postharvest Put treatment at 2 mM on electrolyte leakage, MDA content and LOX enzyme activity of lisianthus cut flowers for 12 days.

Time (day)	Treatment	Membrane integrity		
	Put (mM)	EL (%)	MDA (n mol g^{-1} FW)	LOX (U mg^{-1} protein)
3	0	18.55 ± 1.231 ^{c}	2.125 ± 0.751 ^{d}	0.908 ± 1.531 ^{d}
	2	19.63 ± 0.559 ^{c}	1.450 ± 0.164 ^{e}	0.701 ± 0.659 ^{e}
6	0	22.15 ± 0.342 ^{b}	3.172 ± 0.282 ^{c}	1.876 ± 0.382 ^{c}
	2	19.41 ± 0.787 ^{c}	1.481 ± 0.425 ^{de}	0.671 ± 0.707 ^{e}
9	0	26.48 ± 0.472 ^{ab}	5.353 ± 0.354 ^{b}	2.650 ± 0.452 ^{b}
	2	20.43 ± 1.481 ^{c}	2.628 ± 0.185 ^{cd}	1.275 ± 1.182 ^{c}
12	0	37.43 ± 1.652 ^{a}	7.785 ± 0.425 ^{a}	3.589 ± 1.128 ^{a}
	2	23.45 ± 0.772 ^{b}	5.225 ± 0.896 ^{b}	1.459 ± 0.275 ^{b}
Significant	df			
Treatment	1	**	**	**
Time	3	**	**	*
T × T	3	*	*	*

Table. 2. Effect of postharvest Put treatment at 2 mM on antioxidant enzymes CAT and APX activity and H_2O_2 accumulation of lisianthus cut flowers for 12 days.

Time (day)	Treatment	Antioxidant system activity		
	Put (mM)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)	H2O2 (µ mol g^{-1} FW)
3	0	0.921 ± 0.151 [a]	2.43 ± 0.234 [a]	59.44 ± 7.235 [a]
	2	1.382 ± 0.330 [a]	2.97 ± 0.688 [a]	51.38 ± 5.526 [a]
6	0	0.865 ± 0.381 [b]	1.36 ± 0.564 [b]	65.46 ± 3.875 [b]
	2	0.998 ± 0.391 [c]	2.69 ± 0.342 [a]	54.82 ± 3.977 [a]
9	0	0.785 ± 0.175 [c]	1.22 ± 0.457 [c]	70.63 ± 7.789 [c]
	2	0.826 ± 0.123 [cd]	1.83 ± 0.218 [b]	62.50 ± 9.789 [ab]
12	0	0.687 ± 0.156 [d]	0.88 ± 0.456 [d]	92.66 ± 5.724 [d]
	2	0.965 ± 0.524 [c]	1.63 ± 0.178 [b]	84.68 ± 15.452 [cd]
Significant	df			
Time	3	**	**	*
Treatment	1	**	**	**
T × T	3	*	**	*

peroxidation participates to decreased electrolyte leakage in response to Put treatment. Levels of malondialdehyde (MDA) in Lilium measured during senescence in inner and outer tepals, and cut flowers and flowers on the plant (Arrom and Munné-Bosch., 2010). Levels of MDA in outer tepals of cut flowers fell throughout development and senescence. In contrast, levels of MDA in inner tepals rose in both flowers still attached to the plant and in cut flowers during development. Ramezanian and Rahemi (2011) reported that the combination of calcium chloride and spermidine treatment reduce the MDA content in pomegranate fruit. In a number of flowers including carnation (Lynch and Thompson., 1984), day lily (Panavas and Rubinstein., 1998) and rose (Fukuchi-Mizutani *et al.*, 2000), lipid peroxidation and ion leakage precedes by increase in LOX activity. lipid peroxidation levels in day lily increased even before flower opening and were associated with an increase in LOX (Panavas and Rubinstein., 1998). LOX activity and lipid peroxidation may be consequences of senescence in some species (Rogers., 2012). Polyamines due to its antioxidant property mainly prevent lipid peroxidation, and thus protect the membrane lipid from being conversion in physical state (Mirdehghan *et al.*, 2007). According to our results, Put might extend vase life through improving membrane permeability and decreasing of lipid peroxidation. Since lipid peroxidation is mediated by ROS (Kellogg., 1975), therefore Put may either be directly scavenging ROS and thus decreasing lipid peroxidation, or it may be modulating the activity of antioxidant enzymes. Senescing plant tissue also experiences an increase in LOX activity, which also promotes the process of membrane polyunsaturated fatty acid peroxidation (Lynch and Thompson., 1984). Similar to lipid peroxidation (MDA content), Put caused a decrease in LOX activity during vase life (Table. 1). An increase in LOX activity has been correlated with an increase in cell membrane permeability and senescence in Daylily and Rose (Panavas and Rubinstein 1998; Fukuchi-Mizutani et al. 2000). ROS accumulation may cause oxidative damage to lipids, forming toxic products, such as MDA, a secondary end product of polyunsaturated fatty acid oxidation. Accumulation of MDA is often taken into consideration as an indicator of senescence, because degradation of polyunsaturated fatty acids produces peroxide ions and MDA production (MacRae and Ferguson., 1985). Thus, MDA build-up is usually considered as an indicator of plant oxidative stress and also as the degree of damage to the structural integrity of cell membranes of plants under postharvest senescence (Posmykt *et al.*, 2005).

Putrescine treatment and antioxidant system activity

Plants cells are usually protected against ROS by antioxidant system such as CAT and APX enzymes activity which scavenging ROS produced during oxidative stress. As shown in Table. 2,

lisianthus cut flowers treated with Put at 2 mM showed higher activities of CAT and APX associated with lower H_2O_2 accumulation during vase life ($P<0.01$; Table. 1). The activities of antioxidant enzymes are considered as a response against oxidative stress (Zhou *et al.,* 2014). Put treatments enhanced the activities of antioxidant enzymes which scavenge the ROS, as indicated by the decreased level of MDA (Table 1 and 2). Exogenous application of putrescine decrease catalase activity during leaf senescence of *Helianthus annuus* potted plants (Rubinowska and Michałek, 2009). Pomegranate fruit treated with combination of calcium chloride and spermidine had significantly higher activities in CAT and SOD and a lower activity in peroxidase (POX) (Ramezanian and Rahemi., 2011). in senescent petals of several species such as day lily (Panavas and Rubinstein., 1998), Iris (Bailly *et al.*, 2001), Gladiolus (Yamane *et al.*, 1999) and carnation (Zhang *et al*., 2007). CAT activity has also been measured., CAT activity increases during flower development up to flower opening in all these flowers and there is then a fall in CAT activity during senescence. However, in Iris and day lily, levels appear to rise even during senescence. APX activity was generally high in young flowers and fell during floral senescence in Gladiolus (Hossain *et al.*, 2006). As senescence progresses slightly earlier fall in the activity of APX compared to the other ROS-scavenging enzymes (Rogers., 2012). There is an increase in petal or tepal ROS levels during senescence. In Lilium both inner and outer tepals increase in H_2O_2 levels was much greater in detached flowers compared to flowers still attached to the plant (Arrom and Munné- Bosch., 2010). PAs have multifaceted nature, and working as an antioxidant, a free radical scavenger and a membrane stabilizer (Hussain $H_2O_2$2011).

Cellular membranes are highly prone to ROS such as H_2O_2 attack, and it is reasonable to propose that progressive decline in membrane stability assayed by MDA content is probably the consequence of enhanced ROS attack under decreasing antioxidant activity such as CAT and APX enzymes activity during vase life (Table. 2). In the present study, the decline in membrane integrity of lisianthus cut flowers was alleviated by treatment with Put, which was associated with an increase in CAT and APX activity in treated flower. Put has a role in the induction of antioxidant enzymes and/or might also be acting as a scavenger of ROS (Magda *et al.*, 2015) thus maintaining membrane integrity for extended period.

CONCLUSION

In conclusion, the study was an attempt to investigate the potential roles of Put in delaying the senescence of cut lisianthus flowers. Put was able to prolong the vase life and delay flower senescence by maintaining membrane integrity, which was result from decreasing LOX enzyme activity as responsible for membrane lipid peroxidation and increasing the antioxidant enzymes CAT and APX activities, which was led to diminishing H_2O_2 accumulation. The effects of Put treatment on retarding flower senescence was due to increased antioxidant enzyme activities and thus reduced lipid peroxidation and maintained membrane stability, assayed by electrolyte leakage and MDA content.

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