

EFFECT OF CYCLOHEXIMIDE ON SENESCENCE AND POST-HARVEST PERFORMANCE OF *RANUNCULUS ASIATICUS* L. FLOWERS

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Abstract

The effect of pretreatment with different concentrations of cycloheximide (CHI) on senescence and postharvest performance of isolated flowers of *Ranunculus asiaticus* was examined. At a particular threshold concentration CHI delays senescence and above which it prevents flower opening and promotes senescence. The fact that cycloheximide delays petal/sepal senescence demonstrates the synthesis of particular proteins probably enzymes, responsible for degradation of cellular constituents, executes the cell death programme in flower petals. Pretreatment of flowers with CHI at 0.01 and 0.05 mM concentrations was found to delay visible signs of senescence, maintain high fresh and dry mass of flowers and lower electrical conductivity of ion leachates. An increase in soluble protein content was observed with a concomitant decrease in specific protease activity and α -amino acid content, besides improving postharvest performance. Pretreatment of flowers with 0.05 mM CHI for 1h can be used as an effective treatment to improve postharvest longevity in this flower system.

Introduction

Senescence is an integral part of the normal developmental cycle of plants and can be viewed on a cell, tissue, organ or organization level. It is the final event in the life of many plant tissues and is highly regulated process that involves structural, biochemical and molecular changes that in many cases bear the hallmarks of programmed cell death, PCD (Buchanan-wollaston & Morris, 2000; Rubinstein, 2000; Wagstaff *et al.*, 2003; Rogers, 2006; van Doorn & Woltering, 2008; Yamada *et al.*, 2009). The functional life of a flower is terminated by senescence. Multiple processes contribute to produce the visible signs of petal senescence, but one of the most important is that of protein degradation and remobilization (Wagstaff *et al.*, 2002). Treatment of flowers with compounds that inhibit protein synthesis, have been found to delay the visible symptoms of petal senescence, revealing that active protein synthesis is required for the execution of cell death in petals (Lay-yee *et al.*, 1992; Celikel & van Doorn, 1995; Sultan & Farooq, 1997; Wagstaff *et al.*, 2002; Xu *et al.*, 2007). The ultrastructural data indicates that floral abscission too requires high protein synthesis and secretory activity of material towards cell walls of the abscission zone cells (van Doorn & Stead, 1997). Protein turnover is mediated through proteasomes and various classes of proteasome-independent proteases whose transcripts have been found to accumulate in senescing floral tissues (Cervantes *et al.*, 1994; Jones, 2004; Jones *et al.*, 2005). The interaction between protease and their inhibitor proteins have been linked to modulation of cell death processes in plants and in certain cut flowers, chemical inhibition of protease action delays the onset of senescence (Eason *et al.*, 2002; Sin & Chy, 2004; Pak & van Doorn, 2005). The extension of vase life in cut flowers can therefore be achieved by the use of specific protein synthesis inhibitors. Cycloheximide (a protein synthesis inhibitor at the translational level) has been

implicated to effectively delay senescence in flowers such as *Consolida*, *Dianthus*, *Gladiolus*, *Hemerocallis*, *Ipomoea*, *Iris* and *Narcissus* (Wulster *et al.*, 1982; Lukaszewski & Reid, 1989; Courtney *et al.*, 1994; Jones *et al.*, 1994; Celikel & van Doorn, 1995; van Doorn *et al.*, 1995; Gulzar *et al.*, 2005; Shahri & Tahir, 2010).

Ranunculus asiaticus L., commonly known as 'butter cup' blooms from April to June in Kashmir. It possesses dark red terminal flowers with a cluster of brownish anthers at centre surrounding the carpel. It is widely grown as a garden plant, cut flower and flowering potted plants. Only scanty information is available on the studies related to senescence and improvement of *Ranunculus asiaticus* as a cut flower crop (Kenza *et al.*, 2000; Dole *et al.*, 2005). The present study was undertaken to investigate the effect of pretreatment with different concentrations of Cycloheximide (CHI) before transfer to distilled water (DW) on senescence with the ultimate aim to gain an insight into the mechanism of senescence and develop strategies to improve its postharvest performance.

Materials and Methods

Plant material: Isolated flowers of *Ranunculus asiaticus* growing in open in the University Botanic Garden were used for the present study. The flowers were harvested at 0800 h at half-open stage. The harvested flowers were immediately brought to the laboratory, cut to a uniform size of 15 cm and pulse treated for 1 h separately in different concentrations of cycloheximide (0.01, 0.05, 0.1, 0.25 and 0.5 mM). After pulse treatment the pedicle ends were washed with distilled water thrice. In each case two flowers were transferred to 100 ml Erhlenmeyer flasks containing 75 ml of distilled water (DW). A separate set of five flasks each containing untreated spikes represented control. Overall there were 6 treatments including control. Treatment effects were evaluated by keeping the flowers in the laboratory at a temperature of $25 \pm 2^\circ\text{C}$ under cool white fluorescent light with a mix of diffused natural light (10 W m^{-2}) 12 h a day and RH of $60 \pm 10 \%$. The day of harvest was designated as day zero.

Assessment of vase life and solution uptake: The average vase life of the flowers was counted from the day of transfer of spikes to holding solutions and was assessed to be terminated when flowers lost their ornamental/display value (underwent color change; wilt and loose turgidity). The volume of holding solution absorbed by the buds was calculated by measuring the volume of solution on a particular day and subtracting it from the initial quantity of the vase solution kept in the flasks; taking into account the volume of solutions evaporated by using blank flasks in triplicate (containing particular vase solutions without buds) alongside the flasks with buds.

Conductivity of leachates, floral diameter, fresh and dry mass: Conductivity of leachates from petal samples, diameter, fresh and dry mass of the flowers was determined on 3rd day of harvest (transfer of buds to distilled water). Dry mass was determined by drying the material in an oven for 48 h at 70°C . The changes in membrane permeability were estimated by measuring the electrical conductivity of ion leachates ($\mu\text{S/cm}$) of petal discs (5mm in diameter) incubated in dark in 15 ml glass distilled water for 15 h at 20°C .

Estimation of sugars, amino acids and phenols: At each stage 0.5 g chopped material of petal tissue was fixed in triplicate in hot 80% ethanol. The material was macerated and centrifuged three times. The supernatants were pooled and used for the estimation of

sugars, α -amino acids and total phenols. Reducing sugars were estimated by the method of Nelson (1944) using glucose as the standard. Total soluble sugars were estimated after enzymatic conversion of non reducing sugars into reducing sugars with invertase (BDH). Non reducing sugars were calculated as the difference between total and reducing sugars. α -amino acids were estimated by the method of Rosen (1957) using glycine as the standard. Total phenols were estimated by the method of Swain & Hillis (1959) using Gallic acid as standard.

Estimation of soluble proteins and protease activity: Proteins were extracted from 1g petal tissue drawn separately from different flowers. The tissue was homogenized in 5 ml of 5% Sodium sulphite (w/v) adding 0.1g of Polyvinylpyrrolidone (PVP) and centrifuged. Proteins were precipitated from a suitable volume of the cleared supernatant with equal volume of 20% trichloroacetic acid (TCA), centrifuged at 1000x g for 15 minutes and the pellet redissolved in 0.1 N NaOH. Proteins were estimated from a suitable aliquot by the method of Lowry *et al.*, (1951) using Bovine serum albumin (BSA) as the standard.

At each stage 1g pre-chilled petal tissue was homogenized in 15 ml chilled 0.1M phosphate buffer (pH 6.5) in a pre-cooled glass pestle and mortar. The contents were squeezed through four layers of muslin cloth and centrifuged for 15 minutes at 5000x g in a (Remi K- 24) refrigerated centrifuge at -5°C. The supernatant was used for the assay of protease activity by the method of Tayyab & Qamar (1992), with modification. The reaction mixture comprised 1 ml of 0.1% BSA dissolved in 0.1M phosphate buffer (pH 6.5). The reaction was stopped by adding 2 ml of 20% cold TCA. Blanks in which TCA was added prior to the addition of the enzyme extract were run along with each sample. The contents were centrifuged and supernatants collected. Free amino acids were estimated (as tyrosine equivalents) in a suitable aliquot of the supernatant by the method of Lowry *et al.*, (1951) using tyrosine as the standard. The specific enzyme activity has been expressed as μ g tyrosine equivalents liberated per mg protein in the tissue extract.

Statistical analysis: Each value represents the mean of six independent replicates. The data has been analyzed statistically and LSD computed at $p=0.05$.

Results

Vase life and solution uptake: The average life of an individual flower after it opens fully was about 4–5 days. Flower senescence was characterized by loss of turgor in petals and change in petal color from dark red to brick red. Finally the petals wilt and drop when slightly touched. Pretreatment of flowers with higher concentrations of CHI resulted in pedicel bending and the extent of bending increased with the increase in CHI concentration (Fig. 1). The flowers pretreated with 0.01 and 0.05 mM CHI before transfer to (DW) registered an increase in longevity by an increment of about 2-3 days as compared to untreated flowers. Pretreatment with higher concentrations of CHI (0.1, 0.25 and 0.5 mM) registered a decrease in vase life as compared to distilled water (control) or sucrose (Fig. 1). Pulse treatment with higher concentrations of cycloheximide (0.1 and 0.25 mM) delayed flower opening and promoted premature senescence but delayed the abscission of petals from flowers. Pretreatment of flowers with higher concentrations (0.25 and 0.5 mM) of CHI resulted in a decrease in the volume of holding solution absorbed as compared to untreated flowers as also flowers pretreated with CHI at 0.01, 0.05 and 0.1 mM concentrations (Fig. 2).

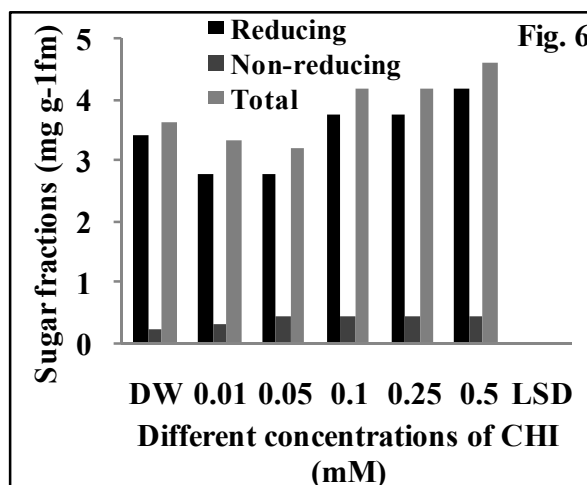
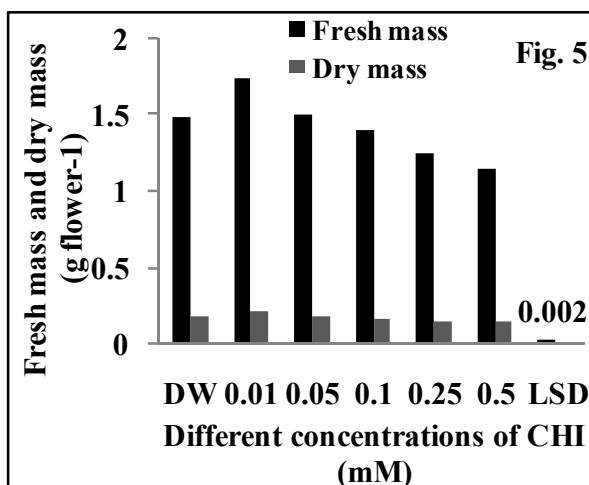
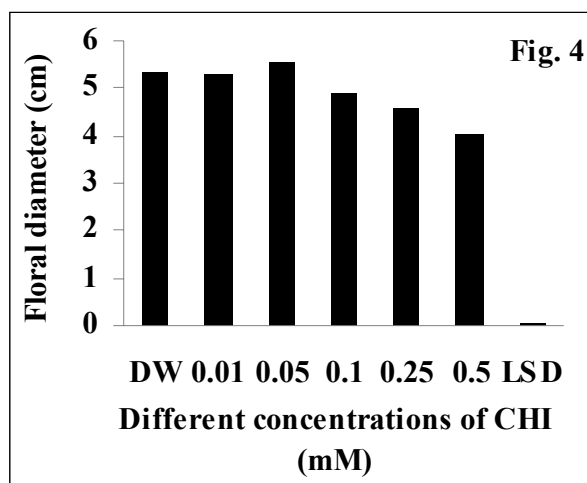
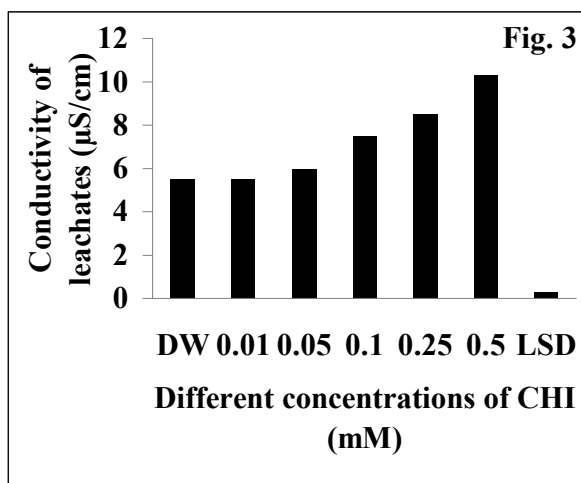
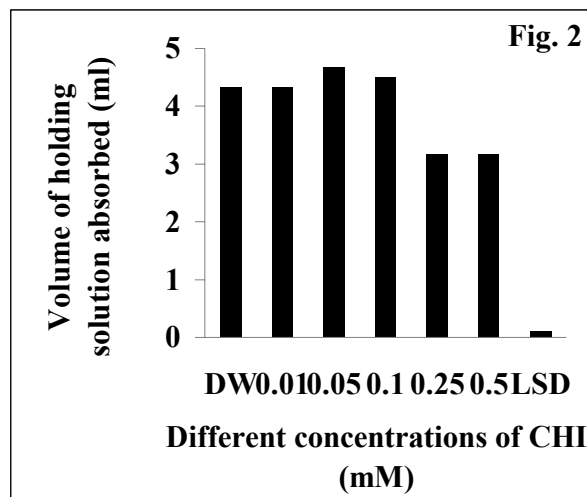
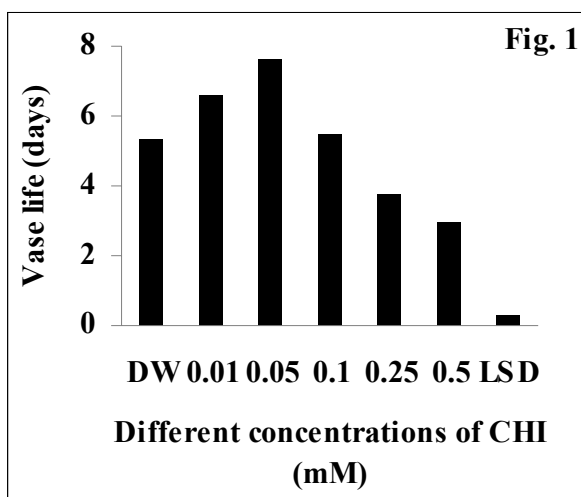


Fig. 1. Effect of pretreatment with different concentrations of cycloheximide (CHI) on vase life of isolated flowers of *Ranunculus asiaticus*.

Fig. 2. Effect of pretreatment with different concentrations of cycloheximide (CHI) on volume of holding solution absorbed in isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 3. Effect of pretreatment with different concentrations of cycloheximide (CHI) on conductivity of leachates (μS) in isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 4. Effect of pretreatment with different concentrations of cycloheximide (CHI) on floral diameter in isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 5. Effect of pretreatment with different concentrations of cycloheximide (CHI) on fresh and dry mass of flowers in isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 6. Effect of pretreatment with different concentrations of cycloheximide (CHI) on sugar fractions (reducing, non-reducing and total) in samples from isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

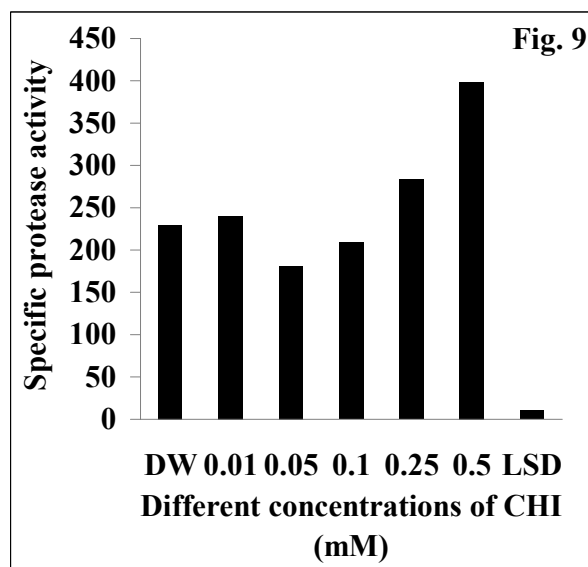
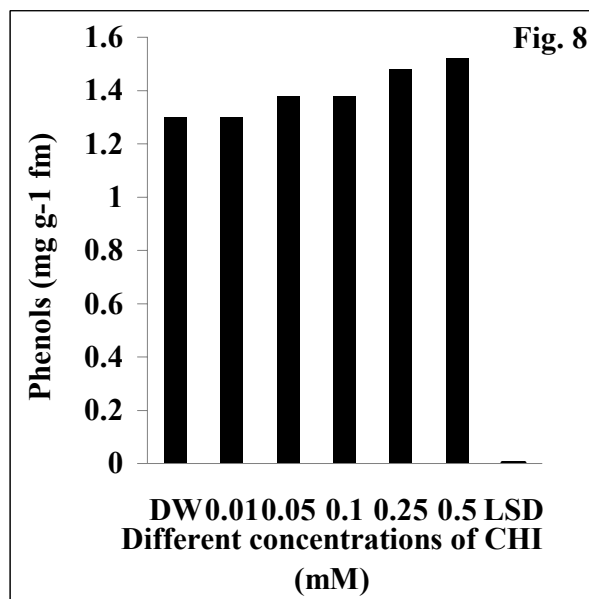
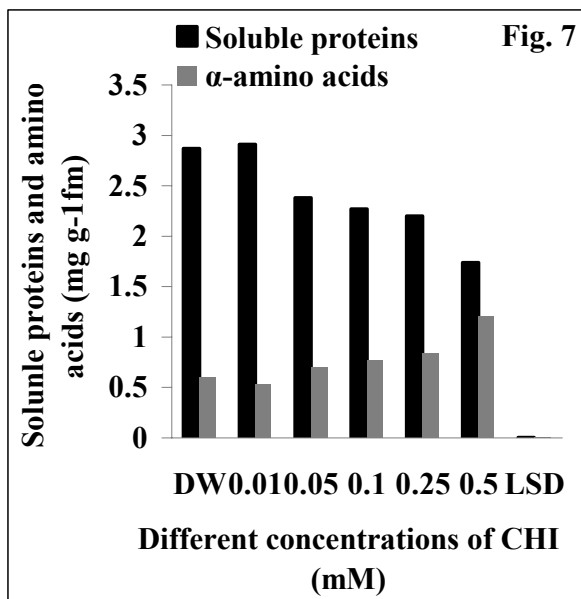


Fig. 7. Effect of pretreatment with different concentrations of cycloheximide (CHI) on soluble proteins and α -amino acids in samples from isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 8. Effect of pretreatment with different concentrations of cycloheximide (CHI) on the content of phenols in samples from isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Fig. 9. Effect of pretreatment with different concentrations of cycloheximide (CHI) on specific protease activity (expressed as μ g tyrosine equivalents liberated per mg protein) in samples from isolated flowers of *Ranunculus asiaticus* at day 3 of transfer to distilled water.

Conductivity of leachates, floral diameter, fresh and dry mass: The electrical conductivity of ion leachates in samples from flowers pretreated with 0.01 and 0.05 mM CHI was comparable to that of samples from untreated flowers. However an increase in the conductivity of leachates was registered in samples from the flowers pretreated with 0.1, 0.25 and 0.5 mM CHI before transfer to distilled water (Fig. 3). Flowers pretreated with higher concentrations of CHI (0.1, 0.25 and 0.5 mM) registered a decrease in floral diameter as compared to untreated flowers. However, it was comparable to that of untreated flowers on pretreatment with CHI at 0.01 and 0.05 mM concentrations (Fig. 4). Pretreatment of flowers with higher concentrations of CHI (0.1, 0.25 and 0.5 mM) resulted in a general decrease in fresh and dry mass of flowers as compared to untreated flowers. An increase in fresh and dry mass of flowers was observed when pretreated with CHI at 0.01 and 0.05 mM concentrations (Fig. 5).

Sugars, α -amino acids and phenols: The content of reducing and total sugars was maintained with reference to controls in the samples from flowers pretreated with CHI at higher concentrations (0.1, 0.25 and 0.5 mM) before transfer to distilled water; however a decrease in the content of the sugar fractions was recorded in samples from flowers pretreated with 0.01 and 0.05 mM CHI (Fig. 6). The non reducing sugar content generally registered an increase by treatment with various concentrations of CHI (Fig. 6). The α -amino acid content registered a decrease in samples from flowers pretreated with 0.01 mM CHI before transfer to distilled water followed by increase with increase in CHI concentration (Fig. 7).

Pretreatment with CHI generally resulted in an increase in the content of total phenols, the increase was however pronounced in samples from flowers pretreated with CHI at higher (0.1, 0.25 and 0.5mM) concentrations (Fig. 8).

Soluble proteins and protease activity: The soluble protein content registered an increase in samples from flowers pretreated with 0.01 mM CHI before transfer to distilled water followed by decrease with increase in CHI concentration (Fig. 7). Pretreatment of flowers with CHI at 0.05 and 0.1 mM concentrations resulted in a decrease in specific protease activity as compared to that of samples from untreated flowers and flowers pretreated with 0.01 mM CHI. However a marked increase in the protease activity was observed in samples from flowers pretreated with CHI at 0.25 and 0.5 mM concentrations (Fig. 9).

Discussion

The results of our experiment suggest that pretreatment of flowers at a particular threshold level of CHI (0.01 and 0.05 mM) before transfer to distilled water enhanced vase life by an increment of 2-3 days. However at high concentrations of CHI (0.1, 0.25 and 0.5 mM) the opening of flowers was delayed. Cycloheximide has been shown to inhibit the flower opening and also delay senescence depending on the stage at which it is included in the experiment (Celikel & van Doorn, 1995; Gulzar *et al.*, 2005; Zhou *et al.*, 2005). The effect of cycloheximide in delaying the senescence does not seem to be due to improvement of water balance of isolated *Ranunculus* flowers as pretreatment of flowers with different concentrations of CHI did not result in a significant increase in the water uptake, instead a decrease was registered in the volume of holding solution absorbed by flowers pretreated with CHI at higher concentrations. Cycloheximide has been shown to rapidly reduce the rate of transpiration and water uptake in *Iris* tepals which apparently was not suggested to be the reason for the delay in tepal wilting, as CHI had little effect on the time until the water balance of the flowers became negative and CHI did not have an effect on water potential (van Doorn *et al.*, 1995). The present work suggests that pretreatment of flowers with CHI (0.01 and 0.05mM) resulted in a decrease in the ion leachates of petal tissues. The loss of membrane integrity has been shown to cause an increase in the permeability and leakage during senescence in various flowers such as *Arum*, *Ipomoea*, *Dianthus*, *Iris*, *Hemerocallis*, *Rosa* and *Gerbera* (van Meeteren, 1979; Halevy & Mayak, 1979; Lay-Yee *et al.*, 1992; Celikel & van Doorn, 1995; Gulzar *et al.*, 2005). The delay in leakiness of tepal cells due to the application of cycloheximide has been shown to indicate that one or more proteins synthesized *de novo* are responsible for the increase in leakiness. Maintenance of higher fresh and dry mass of flowers particularly at lower concentration of CHI (0.01 and 0.05 mM) could be due to lower respiratory losses as CHI has been found to suppress respiration in certain plant tissues; besides in *Hemerocallis* it has been shown to abolish the peak in respiration at the start of

senescence (Ellis & Macdonald, 1970; Bieleski & Reid, 1991). During the current investigation it has been shown that the content of reducing, non-reducing and total sugars was maintained in samples from flowers pretreated with CHI at higher (0.1, 0.25 and 0.5 mM) concentrations. They may be suggested to be accumulated due to reduced metabolic activity as CHI at higher concentrations promoted premature senescence of flowers. The reduced content of sugar fractions in samples from flowers pretreated at 0.01 and 0.05 mM CHI could be due to utilization of available sugar fractions as the flowers showed an improvement in vase life. Flower maturation and senescence has been shown to be accompanied by a decline in total soluble carbohydrate content in flowers such as Carnations (Nichols, 1973; Paulin & Jamain, 1982; Lukaszewski & Reid, 1989).

Pretreatment of flowers with CHI resulted in a decrease in the tissue content of phenols, particularly at (0.1 and 0.5 mM) CHI concentration. The higher content of phenols has been shown to be associated with longer vase life in cut rose petals and *Hemerocallis* (van Doorn & Stead, 1994; Mwangi *et al.*, 2003; Gulzar *et al.*, 2005). However in the present study, increased vase life was found associated with decrease in the phenolic content. Pretreatment of flowers with CHI (0.01 mM) resulted in an increase in the content of soluble proteins followed by a decrease with increase in CHI concentration. An overall decrease in cell protein levels has been found during both ethylene sensitive and insensitive flower senescence; besides in day lily tepals, a sharp decrease in protein levels preceded the visible symptoms of senescence and cycloheximide delayed the decrease in protein levels and increased the time to visible senescence (Lay-Yee *et al.*, 1992; Courtney *et al.*, 1994). This suggests that protein degradation or decreased synthesis may lead to senescence. Conversely pretreatment of flowers with CHI (0.01 mM) resulted in a decrease in the content of α - amino followed by an increase with increase in CHI concentration. CHI maintained a high protein content in the perianth tissue probably by inhibiting the synthesis of specific proteases responsible for protein degradation. The specific protease activity of samples from flowers pretreated with CHI at 0.05 and 0.1 mM concentrations was found to be lower as compared to that of controls as also flowers pretreated with higher (0.25 and 0.5 mM) concentrations. Regulating the senescence associated activity of proteases may be achieved with different molecular strategies e.g. the accumulation of cysteine protease mRNA's in senescing carnation flowers is associated with a decrease in protease inhibitor mRNA (Sugawara *et al.*, 2002), indicating that inhibitor proteins may play a role in senescence associated protease activity in flowers.

The present results suggest that the effects of Cycloheximide indicate a programme at the cellular level. The fact that Cycloheximide delays petal senescence demonstrates that the synthesis of particular suicide proteins orchestrates the cell death programme, however it is necessary to show that these proteins and their products actually play a causal role. Pretreatment of flowers (harvested at half open stage) with CHI (0.01 and 0.05 mM) before transfer to water resulted in an enhancement of vase life, maintenance of membrane integrity and improving postharvest performance of *Ranunculus asiaticus*.

Acknowledgements

The authors thank Head Department of Botany for providing facilities. Waseem Shahri thanks University Grants Commission, India for providing Junior Research fellowship. We also acknowledge Prof. A.Q. John Professor Emeritus, SKUAST for cultivar identification.

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(Received for publication 09 February 2010)