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RESEARCH ARTICLE

Physiological and biochemical changes associated with flower development and senescence in *Consolida ajacis* Nieuwl cv. Violet blue

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Abstract **Flower development of *Consolida ajacis* cv. Violet blue growing in the Kashmir University Botanic Garden (KUBG) was divided into six stages (I–VI) from the tight bud stage to the senescent stage. The average life span of an individual flower after it is fully open is about 4 days. Membrane permeability of sepal tissues estimated as electrical conductivity of leachates increased during senescence. The content of sugars and soluble proteins in the sepal tissues increased during flower opening and declined thereafter during senescence. The α-amino acid content registered an increase as the flowers opened and senesced. The protease activity increased as the flower progressed toward senescence. Flower opening was closely correlated to the sugar status of sepals, while the increase in the protease activity was commensurate with the decrease in the tissue content of soluble protein levels. The results suggested that the reduction in sugar status and the elevation in protease activity are among the important changes associated with the sepal senescence of *Consolida ajacis* flowers. SDS-PAGE of protein extracts from sepal tissues suggested a general decrease in the expression of some high molecular weight proteins and an increase in low molecular weight proteins during the flower development and senescence. At this stage, it is not known whether these polypeptides play an important role in the senescence of *C. ajacis* flowers. Understanding the nature of these proteins can provide new insights into the pathways to execute the senescence and the post-transcriptional regulation of senescence in this flower system.**

Keywords α-amino acids, flower senescence, *Consolida ajacis*, membrane permeability, protease activity, proteins

Introduction

The physiology of flower senescence has received much attention during the past few years as the floricultural industry experienced a rapid growth due to globalization and its effect on income generation in different parts of the world. A great deal of research in this area has led to review and re-evaluate senescence and cell death in plant tissues (Rubinstein, 2000; van Doorn, 2004; Hoeberichts et al., 2005; Zhou et al., 2005; Rogers, 2006; Tripathi and Tuteja, 2007; van Doorn and Woltering, 2008, Ichimura et al., 2009; Trobacher, 2009; Bai et al., 2010). Flower senescence, characterized as an example of programmed cell death (PCD) in plants, is a genetically programmed event under tight developmental control that culminates in the death of floral organs (Rubinstein, 2000; Rogers, 2006). Flower senescence is manifested as petal wilting or color change in several species, whereas in others, the perianth abscises with little or no loss of fresh weight (van Doorn, 2001). The commercial life of a flower is typically determined by its perianth (sepals or petals) as such most of the studies related to flower senescence have focused on the perianth. Flower petals/sepals provide an excellent model system for studies of senescence as they have finite life span, relatively homogenous tissue and that chemical manipulation can be applied without substantial wounding. In addition, a number of morphological and physiologic changes are evident allowing the process to be readily documented (Wagstaff et al., 2002). Petal senescence in many species including *Consolida ajacis* is regulated by ethylene (Woltering and van Doorn, 1988; van Doorn, 2001; Finger et al., 2004; Shahri et al., unpublished results). Petal/sepal

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senescence is a distinct factor affecting vase life, which is an important determinant of the quality of cut flowers. In this perspective, the present investigation has been undertaken on isolated flowers of *Consolida ajacis* Nieuwl cv. Violet blue to understand the changes occurring during flower development and senescence with the ultimate aim to improve the postharvest performance of this flower and to follow changes in the protein patterns to study their qualitative expression during different stages of flower development.

Consolida ajacis (Ranunculaceae) commonly called as "Doubtful Knight's spur or Rocket larkspur" blooms from May to July in Kashmir. The plants possess blue to violet flowers borne on long erect spikes (40–50 cm) in racemes. An average flower is about 3.5 cm in diameter, consisting of 5 petaloid sepals, 4 petals, a single pistil and some stamens with golden yellow anthers. The upper sepal forms a hood in front and an upward-curving spur in back about 2–3 cm long. The middle and lower sepals are well rounded and spreading. The 2 upper petals form a protective inner hood of the reproductive organs. The 2 lower petals form a V-shaped landing pad for visiting insects. The outer sepals are larger in size than the inner petals (Fig. 1).

Figure 1 Flowers of *Consolida ajacis* cv. Violet blue in full bloom

Materials and methods

Plant materials

Flowers of *C. ajacis* growing in the Kashmir University Botanic Garden (KUBG) were used. Flower development and senescence was divided into six stages (Fig. 2). These stages were defined as tight bud stage (I), Loose bud stage (II), half-open stage (III), open flower stage (IV), partially senescent stage (V) and senescent stage (VI). Visible changes were recorded throughout flower development and senescence.

Floral diameter, fresh mass, dry mass and membrane permeability

Diameter and fresh and dry mass of flowers were determined at each stage. Dry mass was determined by drying the material in an oven at 70°C for 48 h. Water content was determined as the difference between fresh and dry mass. Changes in membrane permeability were estimated by measuring the electrical conductivity (μS) of ion leachates of 5 sepal discs per flower (5 mm in diameter) punched from outer regions of sepals of five different flowers incubated in 15-mL glass distilled water for 15 h at 20°C.

Estimation of sugars, amino acids and phenols

At each stage, 1-g chopped material of the sepal tissue was fixed in hot 80% ethanol. The material was macerated and centrifuged thrice. The supernatants were pooled and used for the measurement of the amount of sugars, amino acids and total phenols. Reducing sugars were determined 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 and Hillis (1959) using gallic acid as the standard.

Estimation of protein levels and protease activity

Proteins were extracted from 1-g fresh sepal tissue drawn separately from different flowers. The tissue was homogenized in 5 mL 5% sodium sulphite (w/v) adding 0.1 g 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 $1000\times g$ for 15 min 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, 1 g pre-chilled sepal tissue was homogenized in 15-mL chilled 0.1 M 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 min at $5000\times g$ 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 and Qamar (1992), with modification. The reaction mixture comprised 1 mL 0.1% BSA dissolved in 0.1 M

phosphate buffer (pH 6.5). The reaction was stopped by adding 2 mL 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 μg tyrosine equivalents liberated protein per mg in the tissue extract.

SDS-PAGE

At each stage, 1-g sepal tissue was homogenized in 1-mL extraction buffer (0.1 M, pH 7.2) and 100-mg PVP. The mixture was centrifuged at 5000×*g* at −5°C in a refrigerated centrifuge (Remi K- 24) for 15 min. The supernatant was collected and used for SDS-PAGE. The extracted protein mixture was denatured by mixing equal volumes of protein mixture and 2X sample loading buffer (0.5 M Tris, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). The mixture was incubated in boiling water for 5 min. The concentration of the protein was determined in both the original extracts and the TCA precipitated samples by the method of Lowry et al. (1951) using BSA as the standard.

One-dimensional vertical gel electrophoresis was carried out according to the method as described by Ausubel et al. (1989). Slab gels 0.7 mm thick containing 12% gel {(Acrylamide + bisacrylamide), (1.5 M Tris, pH 8.8), 10% SDS, TEMED and 10% Ammonium persulphate (APX)}. 80 μL of the SDS-denatured protein extract was loaded into each lane. Electrophoresis was carried out at room temperature with a constant voltage of 50 V during stacking and 150 V during running. GENEI molecular weight standards were used for determining approximate molecular weights (Myosin, Rabbit muscle 205000; phosphorylase b 97400; Bovine serum albumin 66000; Ovalbumin 43000; Carbonic anhydrase 29000; Aprotinin 6500; Insulin (α and β chains) 3000). Following electrophoresis, the gels were stained overnight in 0.25% Coomassie brilliant blue in 45% methanol: 10% acetic acid. Gels were destained in 45% methanol : 10% acetic acid, then in 7% methanol : 5% acetic acid.

Statistical analysis

Each value represented in the tables corresponds to the mean ±*SE* of five to ten independent replicates.

Results

The average life of an individual flower on the spike after it had opened fully was about 4 days. The initial symptom of flower senescence was drying and withering of anthers on stamens and protrusion of the pistil out of the protective hood formed by upper petals. This was followed by the slight loss of turgor in the petaloid sepals and petals. The sepals became papery and developed curvy margins immediately before they abscise. The sepals and petals finally abscised leaving behind a single carpel and a multitude of stamens with withered anthers. The single carpel registered a sharp increase in its dimensions as the other floral parts abscise, and finally developed into fruit (follicle) (Fig. 2). Flower diameter increased as the development progressed up to stage V and thereafter could not be recorded due to the abscission of sepals and petals, whereas fresh mass, dry mass and water content of flowers increased with flower development up to Stage V and declined thereafter (Figs. 3, 4). Membrane permeability estimated as electrical conductivity of ion leachates from sepal discs generally remained constant during bud stages (Stages I and II) and registered a gradual increase during flower opening and senescence (Stages III–VI) (Fig. 5). The tissue content of total and reducing sugars increased through Stages I to V and then registered a sharp decline thereafter (Stage VI). The concentration of non-reducing sugars increased gradually as the development progressed through Stages I to IV but registered a sharp increase as senescence progressed through Stages V and VI. The content of non-reducing sugars was much lower

Figure 2 Stages of flower development and senescence in *Consolida ajacis* cv. Violet blue

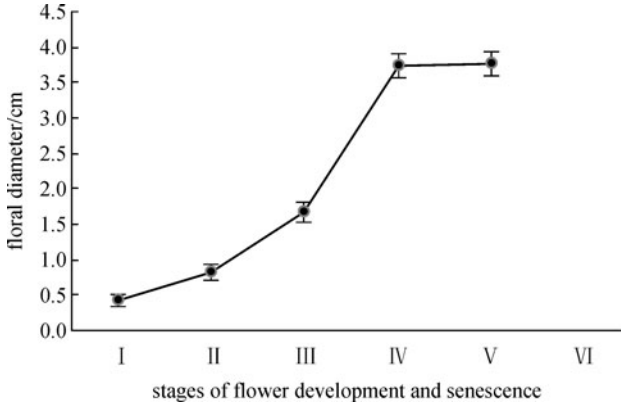

Figure 3 Changes in flower diameter (cm) during various stages of flower development and senescence

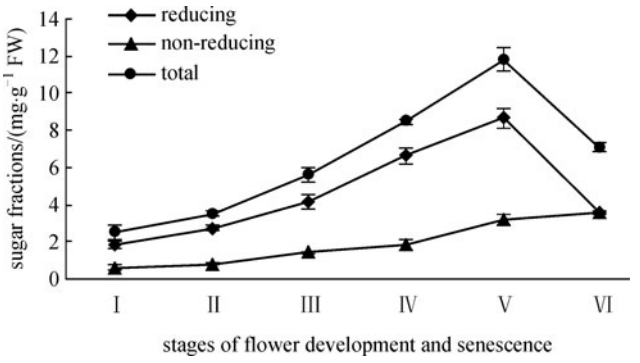

Figure 6 Changes in the content of sugar fractions (expressed as $mg \cdot g^{-1}$) during various stages of flower development and senescence

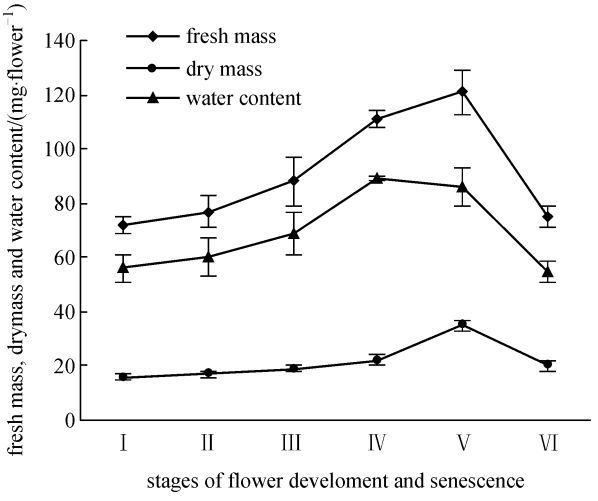

Figure 4 Changes in fresh mass, dry mass and water content of flowers ($mg \cdot flower^{-1}$) during various stages of flower development and senescence

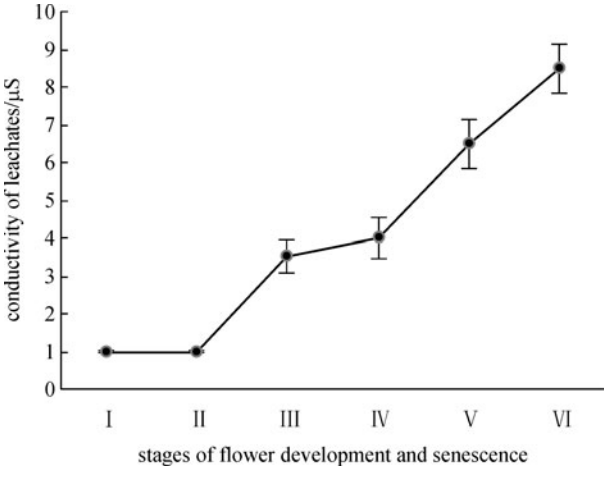

Figure 5 Changes in the electrical conductivity of ion leachates (μS) during various stages of flower development and senescence

compared to that of reducing sugars at each of the stages (Fig. 6). The concentration of soluble proteins registered a gradual decrease as the development progressed from Stages I to VI. There was about 48% decline in protein levels as the sepals senesced. The decrease was concomitant with the increase in specific protease activity and α-amino acid content in sepals. The specific protease activity registered about 2-fold increase in the senescing sepals, whereas about 5-fold increase was registered in the tissue content of the α-amino acids (Fig. 7). The concentration of total phenols initially registered a gradual increase as the flowers opened and senesced (Fig. 8). A highly positive correlation was observed between electrical conductivity of ion leachates and the content of total phenols (Fig. 9a) and between flower opening and the sugar content (Fig. 9b), while between soluble proteins and specific protease activity, it was found to be negative (Fig. 9c). The SDS-PAGE of sepal proteins at

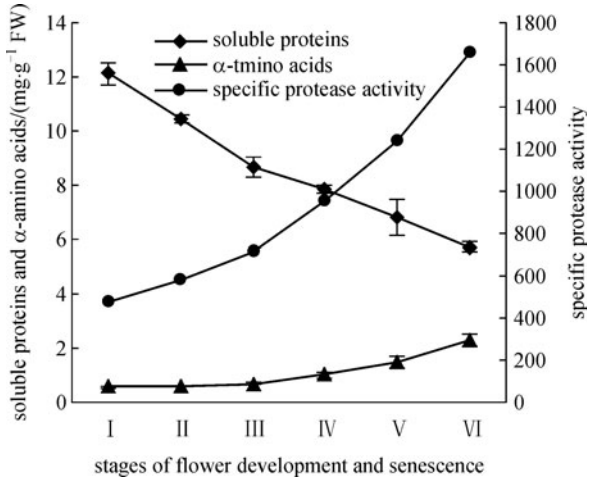

Figure 7 Changes in the content of soluble proteins (expressed as $mg \cdot g^{-1}$), specific protease activity (expressed as μg tyrosine equivalents liberated per mg protein) and α- amino acids (expressed as $mg \cdot g^{-1}$) during various stages of flower development and senescence

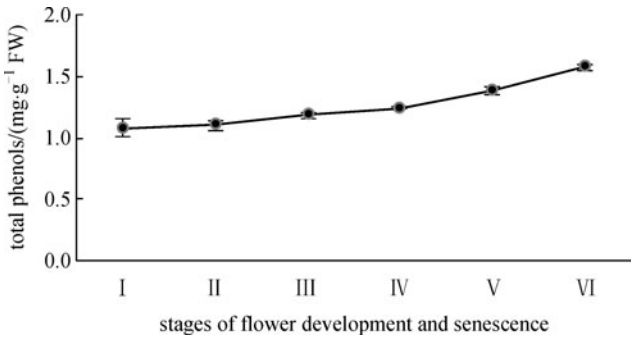

Figure 8 Changes in the content of total phenols (expressed as $mg \cdot g^{-1}$) during various stages of flower development and senescence

various stages of flower development and senescence showed that the levels of some of the polypeptides (15.8 and 45.7 kDa) were maintained throughout flower development and senescence. The decrease was particularly observed in case of polypeptides with molecular weight of 87.1, 42.6, 32.3 kDa. Polypeptide with a molecular weight of 28.9 kDa showed an increase as the flower development progressed through opening (Stages III and IV) and decreased thereafter during senescence. A pronounced increase was particularly observed during senescence (Stages V and VI) in the polypeptide having a molecular mass of approx. 70.8, 32.3 and 4.3 kDa (Fig. 10).

Discussion

The impact of characterizing senescence in sepals/petals allows an understanding of a process crucial to the survival of the plants and other plant parts as well as providing economic benefits. In view of the available literature and the results of the present study, it is suggested that flowers of *C. ajacis* exhibited the 3rd category of flower life cessation (van Doorn, 2001) characterized by ethylene-sensitive sepal/petal abscission. Flower opening was closely related to the increase in fresh and dry mass. Similar relationship between opening and fresh mass has been reported in cut rose and *Iris* flowers (Evans and Reid, 1988; van Doorn et al., 1991). Water content of flowers was found to increase as the flowers developed and opened, and then declined as they senesced. The increase in water content is suggested due to the fact that flower opening requires a continuous influx of water required for the expansion of the cells (Yamada et al., 2007). The decline in fresh mass, dry mass and water content of flowers during senescence is attributed to the fact that flowers (sepal/petal tissues) switch from being a sink to a source during senescence, and these changes are often linked to PCD in ornamental plants (Zhou et al., 2005). A sharp increase in the dimensions of the pistil in flowers of *C. ajacis* during senescence confirms the fact that the developing pistil becomes a strong sink during senescence as observed in

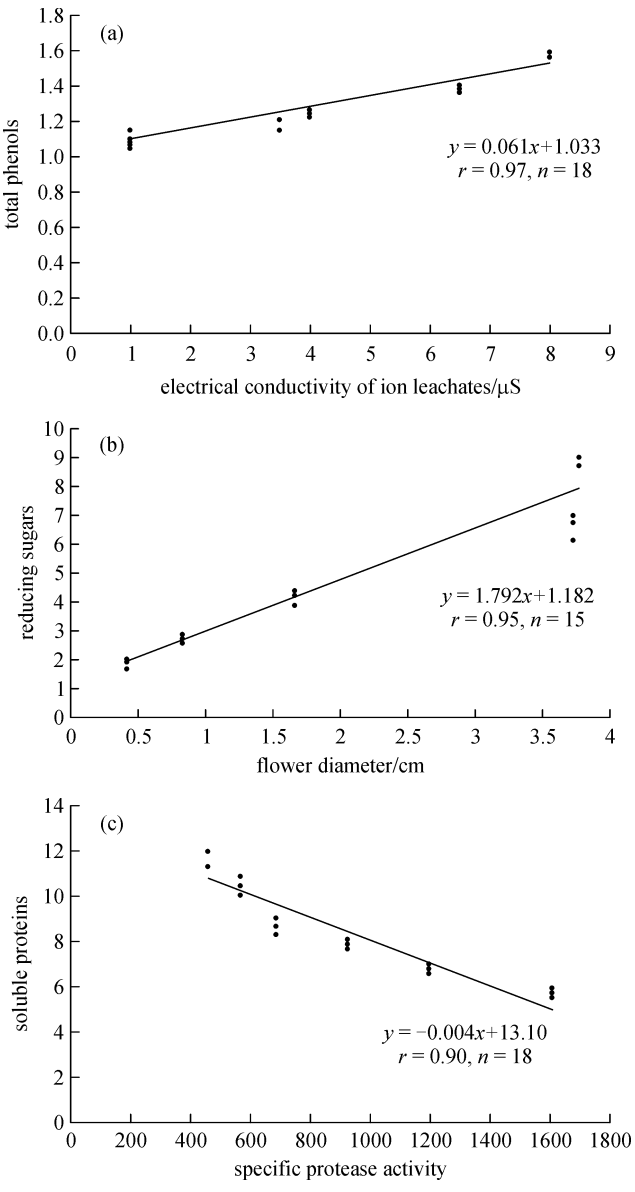

Figure 9 Relationship between electrical conductivity of ion leachates and total phenol content of sepal tissues (a), soluble protein content and specific protease activity (b), flower opening and reducing sugar content (c) during various stages of flower development and senescence

various other flower systems (Have and Woltering, 1997; Woltering et al., 2005).

The sepal discs of *C. ajacis* exhibited a gradual increase in ion leakage as the development progressed through various stages of senescence. The increase in ion leakage preceded the visible senescence symptoms, suggesting that the loss of membrane integrity occurred much earlier before the visible signs of senescence became apparent. It was in agreement with that of Hopkins et al. (2007). The ion leakage was shown to increase with age in various flowers; besides, it was suggested that the leakage may rather be an indicator of cell death, and its increase is a measure of dead cells (Celikel and

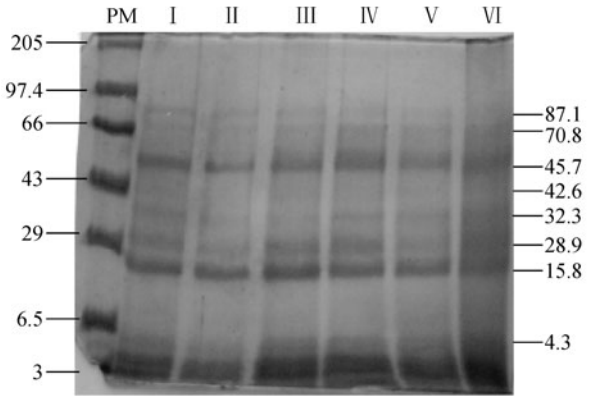

Figure 10 12% SDS-PAGE of equal amounts of extractable protein at various stages (I to VI) of flower development and senescence from sepal tissues of *Consolida ajacis*

Note: The gel was stained with Coomassie blue. Numbers above the lanes correspond to developmental stages. Molecular weight standards are indicated on the left (kDa) and ca molecular weights of major polypeptides to the right of the gel (kDa).

van Doorn, 1995; van Doorn, 2004). The tissue content of phenols followed a time course almost similar to that of the ion leakage. A highly positive correlation (r = 0.97 at $P_{0.05}$) was obtained between ion leakage and the content of total phenols. The increase in solute or ion leakage was suggested to be due to the increased permeability of plasmalemma which may be due to the decrease in the phospholipid content and loss of membrane proteins (Borochov et al., 1994; Celikel and van Doorn, 1995; Hopkins et al., 2007).

The present investigation revealed a highly positive correlation (r = 0.95 at $P_{0.05}$) between flower opening and sugar status of sepals. The tissue content of reducing and total sugars as well as flower diameter registered an increase as the development progressed through Stages I to V and then declined during senescence (Stage VI). However, non-reducing sugars registered a gradual increase during Stages I to V and then a sharp increase during senescence. Such a decrease in sugar levels during senescence was also reported in other flower systems (Lay-Yee et al., 1992; Eason et al., 1997; Yamada et al., 2007). It is suggested that sugars provide ATP for active transport of substances into the expanding corolla tissue, and also contribute to the decrease in osmotic potential to promote water uptake that drives cell expansion (van Doorn et al., 1991; Yamada et al., 2007). Azad et al. (2008) recently reported that it is the intracellular energy depletion that triggers PCD in tulip petal senescence. The increase in the content of non-reducing sugars during senescence may be attributed to the decline in invertase activity, responsible for conversion of sucrose to hexoses (glucose/fructose). Hexoses, produced by invertase and accumulated in the vacuole, were suggested to reduce the osmotic potential thereby promoting water influx in perianth tissues (Bieleski, 1993; Yamada et al., 2007). From the present study, it becomes evident that opening of flowers of *C. ajacis* depends on the presence of adequate levels of storage carbohydrates in the sepals.

During the present investigation, it was observed that there was a gradual loss of proteins during flower development and senescence. The decrease in protein content was commensurate with the increase in specific protease activity and to the tissue content of α-amino acids, which supports the earlier findings on other flower systems (Lay-Yee et al., 1992; Wagstaff et al., 2002; Jones et al., 2005; Lerslerwong et al., 2009; Tripathi et al., 2009). The degradation of proteins by proteolytic cleavage and the remobilization of degradation products (amino acids) to the developing tissues are thought to play a significant role in the senescence of flowers, as the expression of protease genes is one of the earliest senescence-related gene changes to be identified, and the free amino acids are transported via phloem to other tissues of the same flower as the developing pistil (Solomon et al., 1999; Eason et al., 2002). Beers et al. (2000) reported endopeptidases to be one of the most well-characterized cell death proteins in plants particularly those of cysteine proteases. These proteases have been identified and isolated for senescing ethylene-sensitive flowers such as carnations, *Petunia* and rose (Jones et al., 1995; Jones et al., 2005; Tripathi et al., 2009) as well as ethylene-insensitive flowers as *Alstroemeria*, *Hemerocallis*, *Sandersonia* and gladiolus (Guerrero et al., 1998; Eason et al., 2002; Wagstaff et al., 2002; Arora and Singh, 2004). In some wilting flower systems such as *Ipomoea* and *Petunia*, large protein losses as much as 3–8 folds were found to occur prior to senescence, but in *C. ajacis*, the protein content declined by about 1.8 fold prior to abscission supporting the hypothesis that protein loss occurs least in species showing petal/sepal abscission rather than wilting (Wagstaff et al., 2002). In the present study, the proteolytic activity was found to precede loss in fresh weight suggesting that the sepal abscission is driven by increased protease activity. Although the quantitative determination of soluble proteins suggested an overall decline in the protein synthesis during flower senescence, SDS-PAGE revealed a differential expression of proteins during various stages of development. The high molecular weight proteins (ca. mol. Mass 40–90 kDa) registered a general decrease during various stages with the exception of a polypeptide of ca. mol mass 45.7 kDa which was found to be upregulated during senescence. A general increase in the expression of low molecular weight proteins was observed. Polypeptide having ca. mol. mass of 28.9 and 4.3 kDa was found to show an increase as the flowers opened (Stages IV and V). It corroborates with the earlier findings on flowers such as *Hibiscus*, *Hemerocallis* and *Petunia* (Woodson and Handa, 1987; Courtney et al., 1994; Bai et al., 2010). The changes in protein profiles were partly the result of *de novo* protein synthesis and, in part, the degradation by different classes of proteases. Recently, a detailed proteomic analysis of *Petunia* corolla revealed the upregulation of 46 proteins

and downregulation of 27 proteins during senescence. The proteins identified are implicated in a wide range of biologic processes with the majority having a putative role in stress and defense responses or catabolism and remobilization (Bai et al., 2010). At this stage of investigation, it is not possible to know whether the polypeptides with the approximate mol. mass of 45.7, 32.3 and 15.8 kDa play an important role in senescence of *C. ajacis* flowers. The increase in these polypeptides during flower senescence is of particular interest because they may be linked to flower longevity. Understanding the nature of these proteins can provide new insights into the pathways that execute senescence and the post-transcriptional regulation of senescence in this flower system.

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