



CHARACTERIZATION OF THE SENESCENCE-ASSOCIATED PROTEASES OF THE “TIGER FLOWER” (*Tigridia pavonia*) L.F. DC †

[CARACTERIZACIÓN DE LAS PROTEASAS ASOCIADAS A LA
SENESCENCIA DE LA “FLOR DE TIGRE” (*Tigridia pavonia*) L.F. DC]

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SUMMARY

Background: *Tigridia pavonia* is a Mexican native ornamental plant that displays great beauty and color of its flower. However, its quick senescence limits its use as cut flower, since these open in the morning and close in the afternoon of the same day exhibiting wilting of tepals. **Objective:** In this study flower senescence process of *T. pavonia* was analyzed. **Methodology:** The expression of several proteases during senescence of tepals, ovaries and anthers was measured by SDS-PAGE and their identification was through mass spectrometry. **Results:** Five proteases of apparent molecular weight of 130, 90, 65, 35 and 30 kDa were expressed during flower senescence process. The 65 kDa protease was identified as cysteine-protease and had a good homology score with early responsive to dehydration protein (ERD) besides their expression coincided with the onset of visible symptoms of tepals senescence. **Implications:** The preliminar sequence found belonging to ERD gen indicates that it could act as the main transcription factor integrating signaling pathways that trigger flower senescence in *T. pavonia*. **Conclusion:** Flower senescence of *T. pavonia* is mainly associated with the expression of cysteine-proteases. One of them is possibly associated with programmed cell death in tepals.

Keywords: Cysteine-protease; flower senescence; *Tigridia pavonia*; tepal wilting.

RESUMEN

Antecedentes: *Tigridia pavonia* es una planta ornamental nativa de México con gran belleza y colorido de sus flores. Sin embargo, su rápida senescencia limita su uso como flor de corte, pues estas abren por la mañana y cierran en la tarde del mismo día mostrando un marchitamiento de los tépalos. **Objetivo:** En este estudio se analizó el proceso de senescencia floral de *T. pavonia*. **Metodología:** La expresión de varias proteasas durante la senescencia de tépalos, ovarios y anteras se midió mediante SDS-PAGE y su identificación se realizó mediante espectrometría de masas. **Resultados:** Durante el proceso de senescencia floral se expresaron cinco proteasas con aparente peso molecular de 130, 90, 65, 35 y 30 kDa. La proteasa de 65 kDa fue identificada como cisteína-proteasa y tuvo una alta homología con la proteína de respuesta temprana a la deshidratación (ERD) además de que su expresión coincidió con la aparición de síntomas visibles de senescencia de tépalos. **Implicaciones:** La secuencia preliminar encontrada perteneciente al gen ERD indica que podría actuar como principal factor de transcripción integrando las vías de señalización que desencadenan la senescencia de las flores en *T. pavonia*. **Conclusión:** La senescencia floral de *T. pavonia* se asocia principalmente con la expresión de cisteína-proteasas. Una de ellas posiblemente está asociada con la muerte celular programada en tépalos.

Palabras clave: Cisteína-proteasa; senescencia floral; *Tigridia pavonia*; marchitamiento de tépalos.

INTRODUCTION

Petal senescence is considered a natural process of programmed cell death (PCD) of developmental (van Doorn and Woltering, 2008; Rogers, 2013; Mochizuki-Kawai *et al.*, 2015). Depending on the species, petal senescence is visibly shown by wilting or withering. Petal wilting is due to loss of turgor whereas withering

is a color change and slow dehydration (van Doorn and Woltering, 2008).

Patterns of flower senescence can be classified based on differences in how ethylene is involved: ethylene dependent, ethylene independent and intermediate or mixed patterns of senescence (Shibuya *et al.*, 2016; Shibuya, 2018).

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Although sufficient carbohydrate reserves may prevent sugar starvation and delay petal senescence (Woltering, 2019), this process is characterized by transient upregulation of numerous genes related to the remobilization of macromolecules in the senescent cell which is mainly due to autophagic processes in the vacuole, protein degradation in mitochondria, nuclei, cytoplasm; fatty acid breakdown in peroxisomes and nucleic acid degradation in nuclei (Shahri *et al.*, 2011).

The action of proteases during senescence has been suggested to be involved in senescence-related protein degradation which is one of the first cellular responses to senescence induction (Griffiths *et al.*, 2015; Gul *et al.*, 2015; Rabiza-Świder *et al.*, 2019). In plant genomes, serine-proteases and cysteine-proteases have been described as the most abundant enzymes associated with senescence in different plant species, whereas aspartic-, threonine- and metallo-proteases also participate in this physiological process but their role has been less documented (Diaz-Mendoza *et al.*, 2016).

Among all, cysteine proteases are the most frequent (Batelli *et al.*, 2014). Many of them are involved with proteolytic physiological functions in plants, such as plant defense senescence, seed reserves mobilization besides senescence and flowering time (Martínez *et al.*, 2012). In ephemeral flowers as *Ipomoea nil* or *Hibiscus rosa-sinensis* flower senescence is regulated by proteases expression and ethylene plays no significant role (Shibuya *et al.*, 2009; Trivellini *et al.*, 2016).

Tigridia pavonia (L.f) DC also known as “Oceloxochitl” or “Tiger flower” is a Mexican native ornamental plant that displays great variability in color and beauty of its ephemeral flowers (Piña-Escutia *et al.*, 2013; Arzate-Fernández *et al.*, 2015; Arroyo-Martínez *et al.*, 2017). Although their flower longevity consists of one day, flower production continues for up eight weeks, being an excellent outdoor plant.

In addition to its historical value for Mexico, this species is used as food and medicinal besides being a source of genetic richness so ecological studies are important to know the current situation of their populations and to establish actions of conservation and rational use. Nowadays it is part of the program of conservation and use of native ornamental species of Mexico in which the study of flower senescence is a priority (Gámez-Montiel *et al.*, 2017). So, breeding efforts focused to delay flower senescence could increase its exploitation as landscape plant or cut flower even. Previously it was reported that *T. pavonia* flower senescence was not affected by exposure to ethylene (van Doorn, 2001). In this context, the objective of the research was to analyze the process of floral senescence of *T. pavonia*.

MATERIAL AND METHODS

Tigridia plants (var. Dulce) were grown in a mixture of mountain ground, sand and bovine manure (proportion 1:1:1), with a temperature of 26 °C in average and under sprinkler irrigation in a rustic greenhouse at the Faculty of Agricultural Sciences of the Autonomous University of the State of Mexico. According to timing of flower opening, four different senescence stages were defined: opening start (I) (tepal unfolding visible); fully opening (II) (tepals fully extended); partially senescence (III) (first tepal folding visible); and wilted flowers (IV) (tepals are fully wilted) (Figure 1). Individual flowers in these stages were cut with a sharp blade and placed in water.

Protein extraction and protease assay

Samples of ovaries, anthers and tepals (400 mg tissue) from ten flowers of different senescent stages were ground in liquid nitrogen. Protein extraction and total protease assay were done according to the methodology proposed by Azeez *et al.* (2007) and were repeated twice. Namely: the ground tissue was suspended in 2 ml buffer and centrifuged at 12,000 g for 5 min. The supernatant was used for total protease assay using azocasein as a synthetic substrate and also for in-gel assays. Briefly: 20 µl extract was mixed with 300 µl 100 mM sodium phosphate buffer (pH 7.5) containing 50 µl azocasein, supplemented with 100 µl 0.1% Triton X-100, and the mixture was incubated at 37 °C for 3 h. The reaction was terminated by adding 200 µl 10% TCA, incubated at 4 °C for 30 min, centrifuged at 10,000 g for 10 min and the absorbance of the supernatant was determined at 366 nm.

To evaluate the expression of each class of proteases, different protease inhibitors were selected namely: 50 mM EDTA (metallo protease specific); 1 mM AEBFSF (serine protease specific); 100 µM APMSF (specific for serine proteases with Lys/Arg cleavage specificity) and 2 µM E-64 (cysteine protease specific). The enzyme aliquot (20 µg protein) was incubated with the inhibitor (without substrate) for 30 min and then assayed according to these authors. One unit of protease activity was defined as the enzyme amount that gives 0.01 absorbance increase per min.

Electrophoretic detection of protease

Staining for protease activity was according to Azeez *et al.* (2007) with a few changes. Briefly, 20 µg crude extracts of protein were loaded in a 10% SDS polyacrylamide gel. Proteases (20 µl) were mixed with non-reducing buffer, incubated at 37 °C for 30 min and electrophoresed at room temperature. When electrophoresis finished, the gel was washed in renaturing buffer for 30 min. Protease activities were monitored by incubating the gel in Ca⁺² (10 mM), Mg⁺²

(10 mM), Tris-HCl (50 mM), pH 7.5, at 37 °C overnight. After incubation, the gel was stained with Coomassie Blue-R250 and faded with a solution of methanol/acetic acid (5:1). Clear bands against a dark-blue background were considered as protease activity.

Proteases observed on the gel were identified with the addition of protease inhibitors above mentioned which were added to the protein samples (20 µg protein) before electrophoresis and incubated for 30 min at 37 °C following the protocol reported by Azeez *et al.* (2007).

Proteases identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

From the SDS-PAGE three bands with cysteine protease activity were subjected to LC-MS/MS identification using a mass spectrometer LTQ-Orbitrap XL (Thermo Fisher Scientific™). For peptide fragmentation collision-induced dissociation and high-energy collision dissociation methods were used. Data obtained were contrasted with the NCBI data base using the program Protein Prospector (<https://prospector.ucsf.edu/prospector/mshome.htm>). The analysis was realized in the proteomic unit of the Institute of Biotechnology of the National Autonomous University of Mexico.

RESULTS

Total protein levels and protease activity

The level of water-soluble protein in ovary tissue increased between the two first stages, fell in the third stage and then increase in stage IV when the flower is completely wilted (Figure 2A). Meanwhile anther and external tepal showed a steady increase protein level until stage III then this fell in the final stage (Figures 2B y 2D). Only internal tepal showed a continuous increase in the four stages (Figure 2C).

When protease activity was measured, it was observed a strong decrease in ovary and anther tissue (Figures 2A y 2B). By contrast internal tepal showed a dramatically amount in total protease activity with the onset of senescence (Figure 2C) then this fell in the last stage. Likewise, external tepal maintained a continuous and linear increase in the first three stages, and later a slight increase was observed in the last senescence stage (Figure 2D).

Characterization of protease activity by zymography

Zymography revealed protease activity in five distinct bands; the molecular masses of which were 130, 90, 65, 35 and 30 kDa, respectively (Figure 3). The 90 kDa protease was the major one at all times and all organs



Figure 1. Stages of flower development and senescence in *Tigridia pavonia* var. *Dulce*. A. Opening start (stage I). B. Fully opening (stage II). C. Partially senescence (stage III); arrow indicates the fold of the external tepal as the first visible signal of senescence. D. Wilted flowers (stage IV).

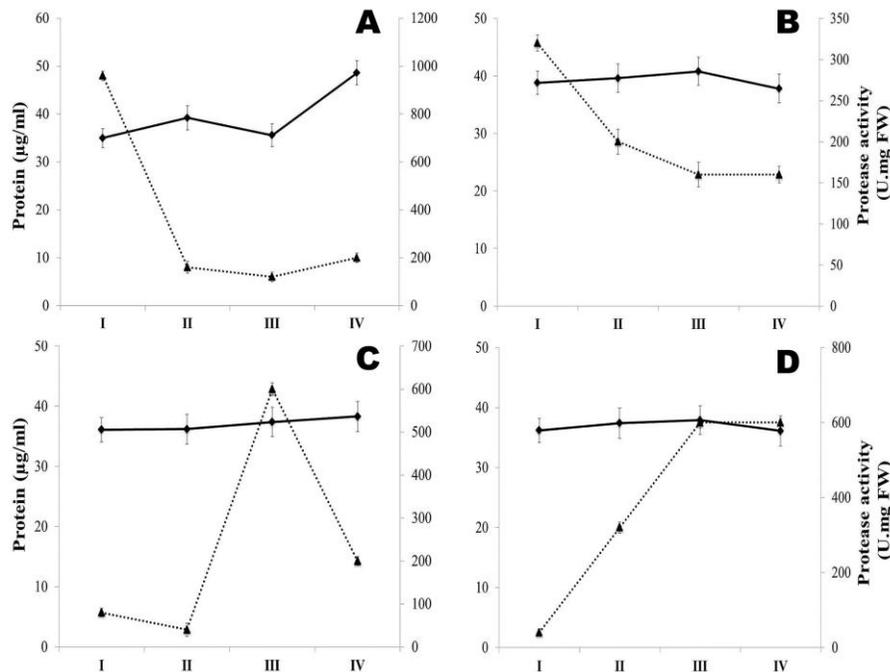


Figure 2. Total protein content and total protease activity in the ovary (A), anther (B), internal tepal (C), and external tepal (D) of *Tigridia pavonia* var. *Dulce* at four different stages of flower senescence; (I) opening start, (II) fully open stage, (III) partially senescence stage, (IV) wilted flowers. Square boxes: protein content, the diamond with broken lines: protease activity.

during senescence, whereas the 35 and 30 kDa proteases expression were specific of the anther. On the other hand, the expression of 130 kDa protease was observed in the first three stages of senescence in anther however, on internal tepal the same protease was present in the two last stages whereas on external tepal it was observed at II and IV stages when the flower senescence was already very advanced.

Interestingly, the 65 kDa protease expression observed at the third stage of senescence in anther, was also present at the third stage of internal tepal and on the second stage of external tepal (Figure 3). Moreover, the expression of this protease coincided with visible symptoms of flower senescence (Figure 1C). These results would indicate that this protease is the one that triggers senescence signals not only in anther but also at internal and external tepals which senesce earlier. Finally, the 35 and 30 kDa proteases were expressed only in anther on all senescence stages studied.

In order to study the changes of proteases at four different senescence stages, several specific inhibitors were used for protease activity determination using

azocasein as substrate. According to Figure 4BC, no inhibition was observed with AEBSF and APMSF so these proteases would not belong to the serine proteases. However, after treatment with EDTA (Figure 4A), the activities of 130 and 90 kDa bands were diminished as compared with the other inhibitors, indicating that metalloproteases might participate in all stages of *Tigridia* senescence.

Likewise, the 65, 35 and 30 kDa bands were inhibited by EDTA but interestingly, E-64 treatment also inhibited their expression (Figures 4A y 4D). Therefore, it is concluded that these could be cysteine proteases regulated by metals.

Protein Prospector analysis for 65, 35 and 30 kDa bands gives identity with several proteases (unpublished data). Among them the partial sequence of 65 kDa protease had a good homology score with early responsive to dehydration protein (ERD-2) which it has been identified to be rapidly induced in response to the dehydration, main visible symptom in tepals of flower senescence of *T. pavonia*.

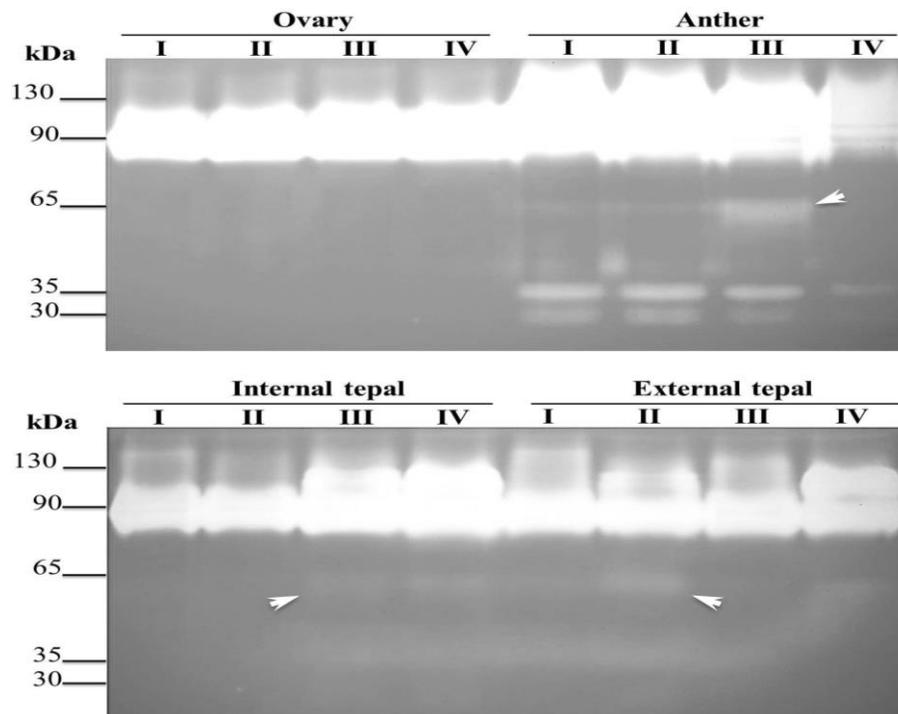


Figure 3. Protease in-gel activity at various stages (I to IV) of flower senescence and development from ovary, anther, internal tepal, and external tepal tissues of *Tigridia pavonia* var. *Dulce*. Samples were electrophoresed on a 10% polyacrylamide gel containing 0.1% gelatin. The molecular weight marker is indicated on the left. Arrows show a protease that is enhanced during senescence.

DISCUSSION

Protein degradation and recycling of nitrogen and nutrients, is probably the most important degradation process that occurs during senescence, which is characterized by upregulated expression of protease genes, raised enzymatic activity and a decline in soluble protein levels both in ethylene sensitive and ethylene-insensitive flowers (Shahri *et al.*, 2011; Roberts *et al.*, 2012). This allows the remobilization or translocation of nutrients from the petals to developing ovary in a pollinated flower, or to other sink tissues when the flower remain unpollinated (Jones, 2013). In species as *Gladiolus grandiflorus* (Azeez *et al.*, 2007), *Consolida ajacis* (Waseem *et al.*, 2011) and *Helleborus orientalis* (Shahri *et al.*, 2011) senescence is preceded by a decrease in protein level. On the contrary, in the present study, the symptoms of visible senescence showed a slight increase in the protein level in all organs (Figure 2). This can be explained because although proteins in water-soluble could be degraded, the new synthesis and possibly, transfer of insoluble proteins into the soluble fraction can compensate it, as was reported by Lerslerwong *et al.* (2009) in *Dendrobium*.

In species as *Tulipa gesneriana* protease activity increased conspicuously with the petal wilting (Azad *et al.*, 2008) but in others as *Lily*, petal protease activity

has shown rise before visible senescence (Mochizuki-Kawai *et al.*, 2015). In the present investigation, a dramatic increase in protease activity in tepals was observed as the flowers progressed towards senescence (Figures 2 and 3). More than 60 % of total protease activity was due to metalloprotease according to inhibitors used *in vitro*. Interestingly E-64 treatment also inhibited expression of 65, 35 and 30 kDa bands (Figures 4A y 4D) suggesting that these could be cysteine proteases regulated by metals.

Cysteine proteases have been related to senescence and cell death in several species (Tripathi *et al.*, 2009; Martínez *et al.*, 2012) being considered important in determining the time to visible senescence (Lerslerwong *et al.*, 2009; Mochizuki-Kawai *et al.*, 2015). Arora and Singh (2004) revealed a dramatic up-regulation in the expression of cysteine protease *GgCyP* at the incipient senescent stage of flower development indicating that this gene may encode an important enzyme for the proteolytic process in *Gladiolus*. In the present study the 65 kDa protease showed an incipient regulation on early senescent stages at external tepal, but at internal tepal and anther, their expression was observed in last senescence stages. These results are similar with those reported in others ephemeral flower as morning glory or Chinese hibiscus where fluctuation on cysteine protease

expression has been also reported (Shibuya *et al.*, 2009; Trivellini *et al.*, 2016).

Petal wilting is under strict control of signaling cascades, which trigger up-regulation in genes encoding specific classes of the transcription factors as NAC, MYB, MYC, MADS-box, WRKY, and zinc finger proteins (Aleksandrushkina and Vanyushin, 2009; Wagstaff *et al.*, 2010). NAC transcription factors (TFs) are wide-spread in plants and modulate transcription rate of target genes. Thus, the expression of many NAC genes is induced by abiotic and biotic stresses like cold, high salinity, and dehydration (Alves *et al.*, 2011; García-Morales *et al.*, 2013; Matallana-

Ramirez *et al.*, 2013; Shibuya *et al.*, 2014; Shao *et al.*, 2015). For example, a TF transcriptionally induced by abscisic acid (ABA), drought and high salinity, bind to the promoter of *EARLY RESPONSIVE TO DEHYDRATION (ERD)* (Thirumalaikumar *et al.*, 2018). In the present investigation *T. pavonia* exhibited rapid dehydration, and petal wilting was observed approximately six hours after flower opening, with petals shrink completely (Figure 1bd). Even more, partial sequence of 65 kDa protease had a good homology score with early responsive to dehydration protein (*ERD*) (unpublished data), one of the genes that has been identified by its capacity to be rapidly induced by dehydration (Alves *et al.*, 2011).

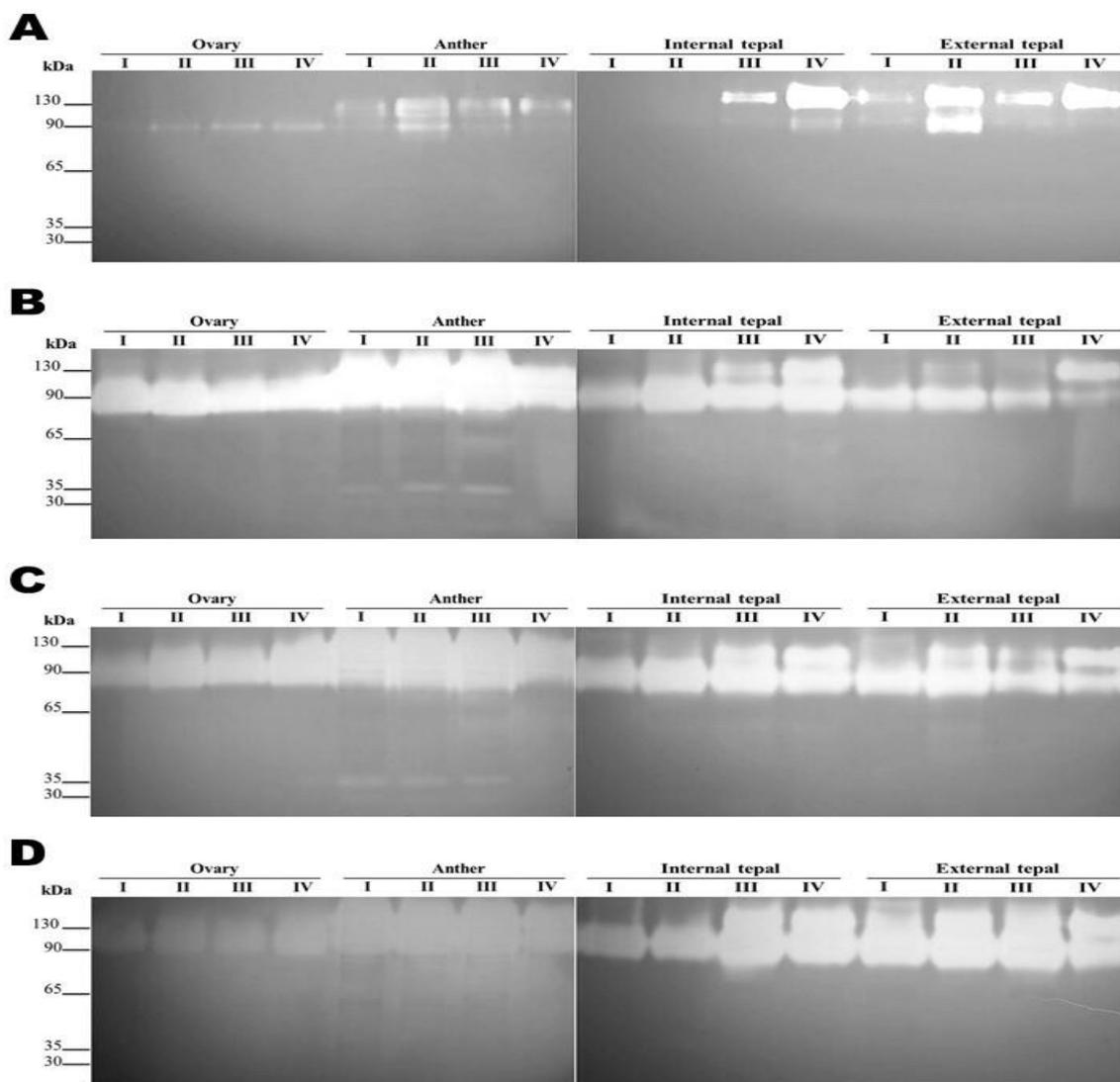


Figure 4. Effect of specific protease inhibitors on in-gel activity of senescence-associated proteases at various stages (I to IV) of flower senescence and development from ovary, anther, internal tepal, and external tepal tissues of *Tigridia pavonia* var. *Dulce*. EDTA (A), AEBSF (B), APMSF (C), and E64 (D).

Similar to *T. pavonia*, morning glory has ephemeral flowers that open in the morning and generally show visible symptoms of petal senescence within the same day. In this species Shibuya *et al.* (2014) identified the gene *EPHEMERAL 1 (EPHI)* as a key regulator of PCD in petal senescence, and phylogenetic analysis of Arabidopsis NAC proteins revealed that *EPHI* clusters with *ORESAR1 (ORE1)*, another gene that not only plays a key role in the regulation of leaf senescence, but also has a function in programmed cell death (PCD) (Matallana-Ramirez *et al.*, 2013). Interestingly, Lindemose *et al.* (2014) reported that *ORE1* and *ERD1* contain at least one copy of the NAC-BS core CGT motif that has been identified as the core binding site of stress-inducible NAC TFs. Therefore, different variations as palindromic versions, of the NAC-BS can be used to identify direct NAC target genes from co-expressed senescence associated genes (Podzimska-Sroka *et al.*, 2015). Considering the above mentioned, and the results found in this research, new studies are required to confirm if the partial sequence found truly belong to *ERD* gene, as main TF that integrates signaling pathways that trigger flower senescence in *T. pavonia*, or if the senescence process involves the action of a large number of TFs.

CONCLUSIONS

It was shown that senescence of *T. pavonia* is mainly associated with the expression of cysteine-proteases. The onset of visible senescence symptoms involves the enhanced expression of a 65 kDa protease which is possibly associated with programmed cell death in tepals.

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Conflict of interest. The authors confirm that this is an original work that is not considered for publication in any other journal, which does not present any conflict of interest that may inappropriately influence the work of persons or institutions.

Compliance with ethical standards. The research was conducted according to the established procedures of the Autonomous University of the State of Mexico.

Data availability. Data are available with the corresponding author Dr. Amaury Martín Arzate Fernández at: (amaury1963@yahoo.com.mx) upon reasonable request.

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