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Short Communication

Polyols Regulate the Flower Senescence by Delaying Programmed Cell Death in *Gladiolus*

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Programmed cell death (PCD) is associated with petal senescence, but little is known about the triggering or execution of the process of cell death in petals. In the present study, membrane disruption and DNA fragmentation, events characteristic of PCD, were found to be present in the advanced stage of petal senescence studied with ethylene-insensitive flowers of gladiolus, indicating that plant and animal cell death phenomena share one of the molecular events in the execution phase. When the gladiolus florets were treated with inositol both wilting and DNA fragmentation of petals were suppressed/ delayed. The present study has provided the initial evidence that inositol has an inhibitory/suppressive effect on apoptotic cell death.

Key words: ethylene-insensitive, flower senescence, *Gladiolus*, programmed cell death.

Senescence represents the sequence of metabolic events occurring in the final stage of development and ultimately culminating in the programmed death of whole plants, organs, tissues or cells. It is an actively ordered process that involves the synthesis of new RNAs and proteins and results in highly coordinated changes in the metabolism and the programmed disassembly of cells. In recent years molecular biological approaches have been utilized to identify genes that may be involved in the initiation and regulation of the senescence programme. The identification and characterization of these senescence related genes have begun to provide us with an understanding of the process of senescence.

An understanding of senescence, a process that limits yield, nutritional value, and marketability of many crops, will lead to ways of manipulating senescence for agricultural applications. The execution of senescence appears to require upregulated transcription of a plethora of genes, many encoding hydrolytic enzymes, like cysteine proteases (1). However, there is also evidence for constitutive proteolytic activity in plants that might be involved in programmed cell death (PCD).

Gladiolus flowers are not usually regarded as being ethylene-sensitive (2). Tepals from this species therefore were used in the present study as a system to investigate events associated with programmed cell death during ethylene-independent floral senescence. The typical vase-life of individual florets is just 4-6 days and senescent florets remain at the bottom of the spikes after the opening of the upper florets. Exogenous ethylene and ethylene inhibitors have no effect on petal senescence of gladiolus. However, several saccharides, such as sucrose and trehalose, do affect vase-life of gladiolus. Sucrose has been shown to extend the vase-life of rose, sweet pea and gladiolus. Trehalose is a disaccharide consisting of two glucose units that is involved with induction of drought resistance in plants (3). Otsubo and Iwaya-Inoue (4) have reported that treatment with trehalose prolongs vase-life of cut gladiolus spikes. From these observations, sucrose and trehalose appear to have an inhibitory effect on the process of cell death leading to petal senescence. In the present study we intended to test some of the potential polyol compounds for the regulation of flower senescence vis-à-vis PCD in ethylene-insensitive gladiolus. Although several mechanistic studies on senescence of gladiolus flowers have been reported, the molecular biology of this process remains to be clarified. We started a program to study the molecular biology of development and senescence of gladiolus flowers, and found that a novel gene encoding cysteine protease (*GgCyP*) homologue is induced during senescence (1).

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Abbreviations: AOA, amino oxyacetic acid; LOX, lipoxygenase; MII, membrane injury index; PCD, programmed cell death; STS, silver thiosulphate.

The gladioli (*Gladiolus grandiflora* var Snow Princess) were grown in the field of Indian Agricultural Research Institute, New Delhi, India, during 1st week of October, adopting standard cultural practices. The spikes were harvested when the lowermost floret started showing colour or unfolding petals. The spikes were cut to a uniform length of 15 cm and all leaves were removed to observe the actual potential of polyol compounds, except one bract like leaf below the florets.

After recording the fresh weight, each spike was placed in 25 mm diameter test tube containing water or aqueous solutions of various compounds at optimum concentrations: Inositol (75 mM); trehalose (100 mM); mannitol (100 mM) and sorbitol (100 mM). We changed the vase solution as and when required. The optimum concentrations of various polyols were arrived at from observations of a preliminary experiment involving a range of concentrations. Each treatment unit had seven spikes with each spike a replication i.e., total number of replication were seven. The spikes opened under normal laboratory conditions of room temperature (20 ± 1 °C) and humidity (70 ± 5%), and under continuous illumination (range 400 – 700 nm) of 20 µE m^{-2} s^{-1}.

The end of vase life was defined as when the fifth floret from the bottom started wilting (5). The number of days taken for this was recorded by the daily observation of the spikes. The pH of the vase-solution measured daily during the experiment by a pH meter.

Leakage of ions from the gladioli was measured according to Bailly *et al* (6) and the conductivity of the solution was measured using Conductivity Bridge (CM 180, Conductivity Meter, Elico Pvt Ltd, Hyderabad, India).

Lipoxygenase (LOX) enzyme assay was done according to the method of Doderer *et al* (7). For the measurement of this enzyme activity petal tissue from the third floret on the fifth day of vase life was used for preparation of the enzyme. The substrate solution was prepared by adding 35 µl linoleic acid to 5.0 ml distilled water containing 50 µl Tween 20. The solution was kept at pH 9.0 by adding 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by 0.2 M HCl, 0.1 M phosphate buffer (pH 6.5) was added to a total volume of 100 ml. LOX activity was determined spectrophotometrically by adding 50 µl of the enzyme sample to 2.95 ml substrate solution. Absorbance was read at 234 nm and the activity was expressed as changes in A_{234} per minute per mg protein in the flower petals. Fragmentation of the DNA was measured as described by Yamada *et al* (8).

Polyols may serve a range of functions in plants. They may serve as carbon storage and translocation compounds. Acyclic polyols are often as important as sucrose in phloem translocation from source leaves. Smirnoff and Cumbes (9) tested various compatible solutes for hydroxyl radical scavenging activity *in vitro*. Of the compound tested, myo-inositol was the most effective, closely followed by sorbitol and mannitol. Proline was less effective and glycine betain showed hardly any scavenging activity. In similar tests conducted with isolated cyclitols, pinnitol, ononitol, and querbrachitol proved to be even better hydroxyl radical scavengers than myo-inositol (10).

The senescence of *Gladiolus* flowers were delayed following treatments with various polyol compounds like trehalose, mannitol, inositol and sorbitol. The best treatment for extending the vase life and quality was treatment of flower spike with 75 mM of inositol followed by trehalose (100 mM), mannitol (100 mM), and sorbitol (100 mM). The vase life of cut flower spikes were significantly increased by the polyol treatments ranging from 14% with sorbitol and 38% with inositol against control. The flower quality was improved in terms of percentage flowering, fresh weight and water uptake. Treated flowers also exhibited delayed and reduced LOX activity as well as ion leakage (Table 1). The vase pH was also found to be lowest in the treatment of flower with inositol (75 mM).

Electrolyte leakage was delayed or reduced by the use of various polyol compounds. The flowers kept in inositol (75 mM) showed the least leakage while flowers kept in sorbitol and control water showed the highest leakage at senescence (Table 1). The changes in the rate of ion leakage from petals are supposed to demonstrate changes in membrane permeability or membrane protein composition. The loss of membrane integrity measured by the leakage of electrolytes/ions is increased continuously in flower spikes kept in water. While flower spikes were kept in vase solutions containing polyols, the efflux of electrolytes is delayed. Similar observations regarding leakage of electrolytes were made in *Gerbera* (11) and in cut carnation (12) with 60-fM cycloheximide in vase solution. These results support the view that peroxidation of membrane lipids and proteins leads to modification in

Table 1. Lipoxygenase activity, membrane injury index, vase life and vase pH of gladiolus florets during post harvest life (five days after treatments) as influenced by various polyols compounds

Treatments	MII (%)	LOX ($\triangle$ A_{234} min^{-1} mg^{-1} protein)	Vase life (days)	Vase pH
Control (DW)	64.30 ± 4.50	78.00 ± 3.9	5.2 ± 0.56	4.85 ± 0.44
Inositol (75 mM)	52.57 ± 3.25	66.27 ± 2.50	7.1 ± 0.88	3.92 ± 0.80
Trehalose (100 mM)	58.81 ± 3.87	69.85 ± 2.97	6.3 ± 0.35	4.25 ± 0.37
Mannitol (100 mM)	60.10 ± 3.08	72.39 ± 3.37	6.1 ± 0.67	4.10 ± 0.40
Sorbitol (100 mM)	61.00 ± 4.71	74.09 ± 3.89	5.9 ± 0.24	4.61 ± 0.29

membrane fluidity. Hence, there may be the involvement of free radicals in oxidative change to membrane proteins and lipids.

LOX activity ($\triangle_{234}$ min^{-1} mg^{-1} protein) was low in all the treatments studied as compared to the control. The treatment of inositol (75 mM) reduced the activity of LOX by more than 15% in comparison to the control (Table 1). LOX has been shown to be involved as a fat-oxidizing factor in processes linked to aging, and an increase in LOX activity is generally correlated with senescence and an increase in cell membrane permeability (13). Current theories on the physiological roles of plant LOX suggest its involvement in such degradative processes as senescence, wounding and infection, all of which include membrane breakdown. Thus, it can be conceived that the free radicals produced through membrane may be scavenged by polyols fed from vase solution. It can also be concluded that, despite little ethylene production, petal wilting in gladiolus is associated with rapid increase in synthesis of LOX. This is in agreement with the findings of Jones and McConchie (14) that *de novo* synthesis of LOX associated with the wilting of tulips.

We detected DNA fragmentation in gladiolus petals by electrophoresis (Fig.1). Total DNA isolated from senescing petals 4-6 days after flower opening showed a ladder - like DNA banding pattern after staining with SYBR Gold. This result showed that DNA had been fragmented into multimers of nucleosomal base-pair size in the petals undergoing senescence. The ladder-like DNA banding pattern began developing in wilting petals 1-3 days after flower opening but was not detected in petals showing no symptoms of senescence (Fig.1).

Petal senescence is a distinct factor affecting vase-life, which is an important determinant of the quality of cut flowers. Programmed cell death is associated with petal senescence, but little is known about the triggering or

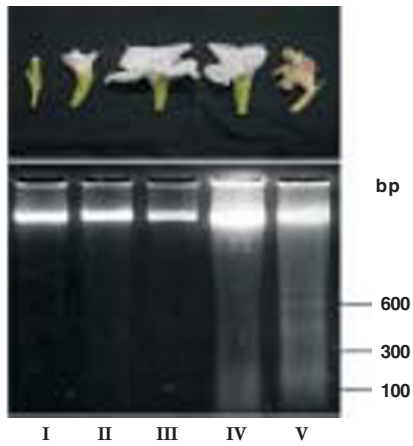

Fig.1. DNA fragmentation detected in tepals from each stage during different stages of floral development and senescence. The different developmental stages of flower studied are: Stage I: bud-stage; Stage-II: half-open; Stage-III: fully-open; Stage-IV: incipient senescent; Stage-V: senescent.

execution of the process of cell death in petals. In the present study, some features of apoptosis were detected in senescing petals taken from ethylene-insensitive flowers of gladiolus. Agarose gel analysis of DNA extracted from petals revealed that DNA fragmentation, an apoptotic process, occurred in cells of senescing petals. When the florets were treated with ethylene inhibitors (STS/AOA) or inositol, both wilting and DNA fragmentation of petals was suppressed only by inositol. From these observations, we conclude that the cell death leading to petal senescence of gladiolus florets is similar to apoptosis. Furthermore, the present study has provided the evidence that inositol has a suppressive effect on apoptotic cell death.

Physiological changes in senescing organs of plants have been considered to be part of the pathway of PCD (15). However, neither the essential features of PCD nor the complete process has been clarified in plants. Apoptosis is a type of PCD, which was originally distinguished from necrosis by morphological and

biochemical characterization in animal cells (16). Although an understanding of the molecular mechanisms of apoptosis is advanced in animal cells, studies of the specific mechanisms of plant cell death are only now beginning. In senescing petals, some physiological changes associated with PCD, such as loss of membrane permeability, an increase in reactive oxygen species (ROS), activation of proteases and a loss of nucleic acid, have been detected, and several genes that participate in these changes have also been isolated (15). In addition to the physiological changes occurring during PCD, characteristic processes of apoptosis, such as DNA fragmentation, chromatin condensation and nuclear fragmentation, have been detected in senescing petals of both ethylene-sensitive (17) and –insensitive (18) flower species. The present study has provided the initial evidence that inositol is involved in apoptotic cell death in plants similar to trehalose on apoptotic cell death leading to petal senescence in gladiolus (8). Further studies are required with more evidences like TUNEL assay, nuclear fragmentation and *in situ* hybridization to confirm that inositol regulates the flower senescence by delaying the PCD in gladiolus flowers.

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