



Original Contribution

ANTIOXIDANT ENZYMATIC PROTECTION DURING *PELARGONIUM* PLANT LEAF SENESCENCE IS MEDIATED BY THIDIAZURON

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ABSTRACT

Pelargonium (*Pelargonium zonale*) is grown as potted plants for their colorful, showy flowers and scented foliage. Leaf senescence is a common problem in *Pelargonium* and leads to high post harvest losses during transport and storage. Dark stress has been implicated in promoting senescence, while cytokines retard it. The effects of post-harvest treatments with Thidiazuron (TDZ) in four level (0, 25, 50, 75, 100 μM) during simulated transport (5 days, 20 ± 2 °C and 95% RH) were investigated in two cultivars 'Anthony' and 'Blue wonder'. We examined the possible use of this compound for delaying leaf yellowing in potted pelargonium during dark storage. Petal abscission increased after dark stress in both cultivars. Furthermore, leaf yellowing extended without any treatments. Plants treated with TDZ for 3 days had higher leaf chlorophyll contents than untreated especially in (75 μM). In addition, they showed more antioxidant enzyme activity (APX, SOD) than control during dark stress. So, thidiazuron could counteracted the deleterious effects of darkness by delaying the onset of leaf yellowing in *Pelargonium* pot plants.

Key words: Antioxidant enzyme, Ageing, *Pelargonium zonale*, Cytokinins, Darkness

INTRODUCTION

Pelargonium is one of the world's most important bedding and pot plants. Leaf yellowing, senescence, and abscission are significant problems that reduce the marketability and longevity of many cut flowers and potted flowering plants, including alstroemeria, lilies, chrysanthemums and pelargonium (1). In addition to the visual quality implications of leaf yellowing, it may have a physiological effect in reducing the photosynthesis that is crucial for normal flower development and longevity (2).

Senescence in ornamental plants appears as leaf yellowing due to chlorophyll loss. This is a significant problem in Pelargonium (3). Dark

induced senescence occurs during storage and shipment of agricultural products, including Pelargonium cuttings (4) and manifested in chlorophyll breakdown, and an increase in reactive oxygen species (ROS) levels. Their generation is one of the earliest responses of plant cells under a biotic stresses and senescence. And also many antioxidant enzymes reduce their activity (5).

Plant cells need to have protective mechanisms by which they respond to oxidative stress: (a) non-enzymatic antioxidants such as ascorbate and glutathione and (b) enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) (6, 7).

It was previously reported that activities of antioxidant enzymes reach their maxima at the beginning of the leaf development (8). In general. The antioxidant enzyme activities

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decrease in the course of aging and reach the lowest values in senescent leaves (7).

It is recognized that exogenous application of cytokinin delays degradation of Chl and photosynthetic proteins (9). Cytokinins are potent anti-senescence hormones and they play an important role in delaying the onset of leaf senescence. Thidiazuron (TDZ), a substituted phenyl urea with powerful cytokinin-like activity has been shown to be very effective in preventing leaf yellowing and retarding chlorophyll degradation, (3). They can also participate in removal of ROS from the cell. The extent of senescence of the leaves was characterized by determinations of the contents of photosynthetic pigments (chlorophyll a,b) and carotenoids. The activities of antioxidant enzymes SOD, APX in leaves of both cultivar in all levels of TDZ during darkness were measured with the aim to improve the postproduction quality of pelargonium pot flowers by TDZ application and also to determine the influence of cytokinin deficiency on antioxidative enzymatic protection during leaf senescence.

MATERIALS AND METHODS

1. Plant material

Stock plants of Pelargonium cv. 'Blue wonder', and cv. 'Anthony' were cultivated in the greenhouse in a peat substrate under natural light (air temperature 17–25 °C). Water and fertilizers were supplied manually, aiming at the following concentrations of nutrients in the substrate: Nitrate-N: 150 mg l⁻¹, P: 100 mg l⁻¹, K: 400 mg l⁻¹. Terminal cuttings were removed with a sterilized knife, leaving the first two leaves of the axillary shoot on the stock plant. The cuttings were maximally 4 cm in length, having three leaves, from which at least one was fully developed.

2. TDZ treatment

Thidiazuron (Sigma-Aldrich, Germany), were dissolved in 1 M KOH to prepare separate stock solutions. Deionised water was used to prepare 25, 50, 75 and 100 µM TDZ solutions then sprayed on pot plants.

Whole plants were sprayed with equal amounts of 50 ml aqueous solution of TDZ. Both sides of the leaves were sprayed. Control plants were treated only with deionised water.

Leaves were sprayed in the morning; eight leaves of two different plants of the same age were pooled.

3. Dark storage

Two cultivars of pelargonium transferred to growth chamber for simulated transport (darkness) for 5 days, 20±2 °C and 95% RH.

4. Measurements of ion leakage

The relative intactness of the plasma membrane was measured as the leakage percentage of electrolytes, as described by(10). Fresh leaves (0.1 g) were cut into pieces of 0.5 cm length, washed with distilled water (DW) and placed in glass vials containing 10 mL of DW. The vials were incubated in a water bath at 30 °C for 30 min and the initial electrical conductivity (C₁) in the medium was measured. The samples were boiled at 100 °C for 15 min to release all electrolytes, cooled and the final electrical conductivity was measured (C₂). Three readings per leaf of each of five leaves per treatment were averaged (n = 5)

5. Pigment measurements

Leaf material (0.5 g) was homogenized in 0.5 ml acetone, and the samples were shaken vigorously for 15 second at room temperature. After centrifugation at 14 000g for 20 min at room temperature, total chlorophyll content of the supernatant was measured and calculated following the method described. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids were measured in 80% acetone extracts as described by (11). Determinations were performed on twenty leaves per treatment (n = 20)

6. Ascorbate peroxidase activity (APX EC 1.11.1.11)

Leaf tissue (0.5 g) was ground with liquid N₂. Soluble protein was extracted by homogenizing the powder in 2 mL of 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H₂O₂ and enzyme extract equivalent to 50 µl of protein. Insoluble materials were removed by centrifugation at 11 000g for 15 min at 4 °C. The activity of APX was determined by the method of (12). The activity was recorded as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

7. Superoxide dismutase (SOD, E.C. 1.15.1.1)

Superoxide dismutase activity was determined by the method of (13). The required cocktail for the estimation of SOD activity was prepared by mixing 27 ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg ml^{-1}), 1 ml of NBT ($14.4 \text{ mg } 10 \text{ ml}^{-1}$), and 1.5 ml of 2 mM EDTA. To 1 ml of this cocktail, 10 μl of riboflavin ($4.4 \text{ mg } 100 \text{ ml}^{-1}$) and enzyme extract containing 50 μl of protein were added. The reaction mixture taken in the cuvette was illuminated for 8 min using three comptalux bulbs (100 W, Philips India Ltd.).

A tube with enzyme extract kept in dark would serve as blank, while the tube without extract but kept in light would serve as control. Activity of SOD is the difference in NBT reduction monitored at 560 nm in light with and without enzyme extract. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

8. Statistical analysis

Experiments were conducted in a Factorial design, replicated three times with five pots per replication. Data were subjected to analysis of variance (ANOVA) using the general linear model (Proc GLM) of the Statistical Analysis System (SAS, 2002) computer program. Multiple comparisons among treatment means was done using the protected Tukey's Honest Significant Difference (HSD) at $P = 0.05$

RESULTS AND DISCUSSION

1. Electrolyte leakage

The oxidation of lipids and proteins during membrane injury by ROS was evidenced by membrane electrolyte leakage (Fig. 1). Senescing tissue (control) showed a marked increase of ion leakage, 33.1% in “anthuny” and 58.9% in “bluewonder”, indicating membrane damage. In contrast TDZ treated leaves maintained low electrolyte leakage percentages (23% and 34.33%) in both cultivars at $75 \mu\text{ml}^{-1}$, respectively, evidence of the protective role of the TDZ against membrane damage.

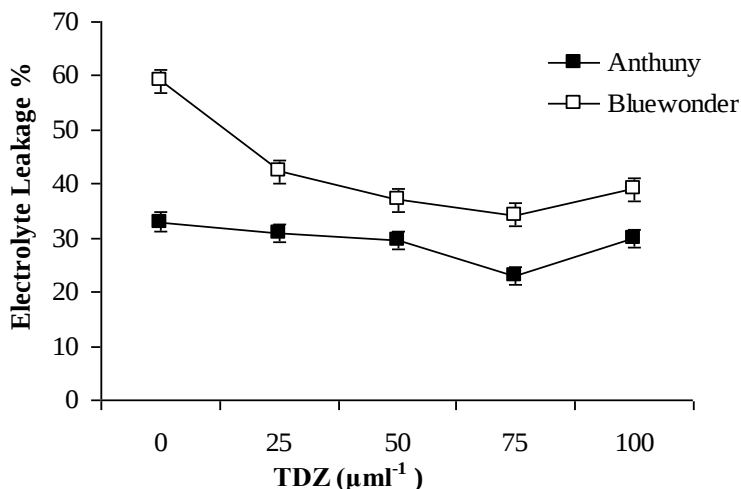


Fig. 1. Effect of TDZ on membrane electrolyte leakage percentage of two *pelargonium* species during 5 days of leaf senescence in the dark storage conditions.

2. Chlorophyll content

The retention of chlorophylls in the presence of TDZ reflects the delay of senescence. Control leaves started to lose chlorophyll and antioxidant enzyme, earlier than the TDZ treated leaves. “Anthuny” and “bluewonder” had retained 54 %

and 40% of Chl in both cultivars in $75 \mu\text{ml}^{-1}$ TDZ, respectively. These results indicated that “anthuny” was the better cultivar during darkness and retained pigments, (Table 1).

Table 1. Effect of TDZ on photosynthetic pigments of two *pelargonium* species during 5 days of leaf senescence in the dark storage conditions.

TDZ (μml^{-1})		Variety	
		Ant	Blu
0	Chl a	0.035 $\pm$ 0.0029	0.035 $\pm$ 0.0018
	Chl b	0.019 $\pm$ 0.0021	0.014 $\pm$ 0.0020
	Cartonoid	2.11 $\pm$ 0.073	1.16 $\pm$ 0.0093
25	Chl a	0.034 $\pm$ 0.0027	0.034 $\pm$ 0.0016
	Chl b	0.018 $\pm$ 0.0022	0.015 $\pm$ 0.0021
	Cartonoid	2.22 $\pm$ 0.018	1.18 $\pm$ 0.086
50	Chl a	0.035 $\pm$ 0.0026	0.035 $\pm$ 0.0019
	Chl b	0.019 $\pm$ 0.0023	0.015 $\pm$ 0.0022
	Cartonoid	2.44 $\pm$ 0.086	1.21 $\pm$ 0.014
75	Chl a	0.036 $\pm$ 0.0027	0.035 $\pm$ 0.0028
	Chl b	0.019 $\pm$ 0.0021	0.015 $\pm$ 0.0030
	Cartonoid	2.45 $\pm$ 0.018	1.20 $\pm$ 0.019
100	Chl a	0.036 $\pm$ 0.0076	0.036 $\pm$ 0.0042
	Chl b	0.019 $\pm$ 0.0023	0.015 $\pm$ 0.0032
	Cartonoid	2.28 $\pm$ 0.024	1.21 $\pm$ 0.025

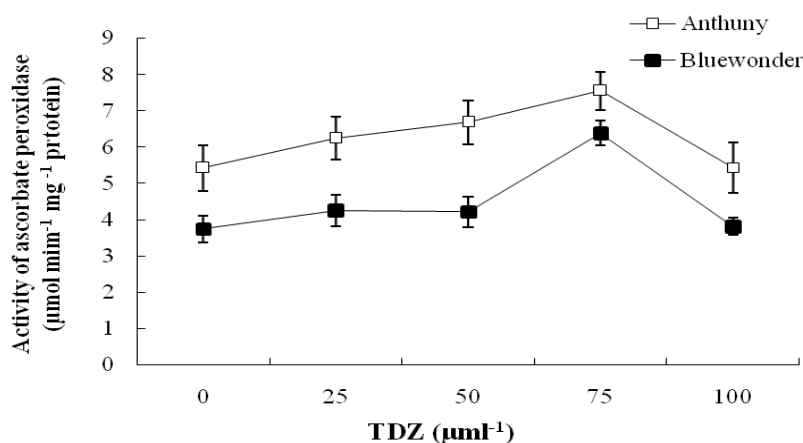
Leaves treated with TDZ (75 μml^{-1}) retained significantly more carotenoids (2.45 $\pm$ 0.018 mg . g FW $^{-1}$) than control leaves (2.11 $\pm$ 0.073 mg .g FW $^{-1}$) in anthuny and (1.20 $\pm$ 0.019 mg . g FW $^{-1}$) compare to control (1.16 $\pm$ 0.0093 mg .g FW $^{-1}$) in bluewonder cultivars.

Significant chlorophyll breakdown occurred concomitantly with increases in ROS levels. Application of dark storage accelerated senescence in the leaves of *Pelargonium*. Cytokinins activate NADH protochlorophyllide reductase, an enzyme involved in chlorophyll biosynthesis, and reduces chlorophyll degradation (14).

It has been demonstrated that cytokinin treatment reduced activity of chlorophyllase, Mg-dechelataase and peroxidase-linked chlorophyll bleaching in broccoli florets during postharvest (15).

3. Antioxidants enzymes

It was observed that all the antioxidant enzyme activity was higher in thidiazuron sprayed plants compared with the control. After 4 and 5 d of treatment, leaves in control showed significantly reduced APX and SOD activity in comparison with the initial stage. The APX and SOD activity in TDZ treated leaves was slightly but significantly higher at the end of the treatment compared with the control (Fig. 2, 3).

**Fig. 2.** Effect of TDZ on activity of ascorbate peroxidase (APX) of two *pelargonium* species during 5 days of leaf senescence in the dark storage conditions.

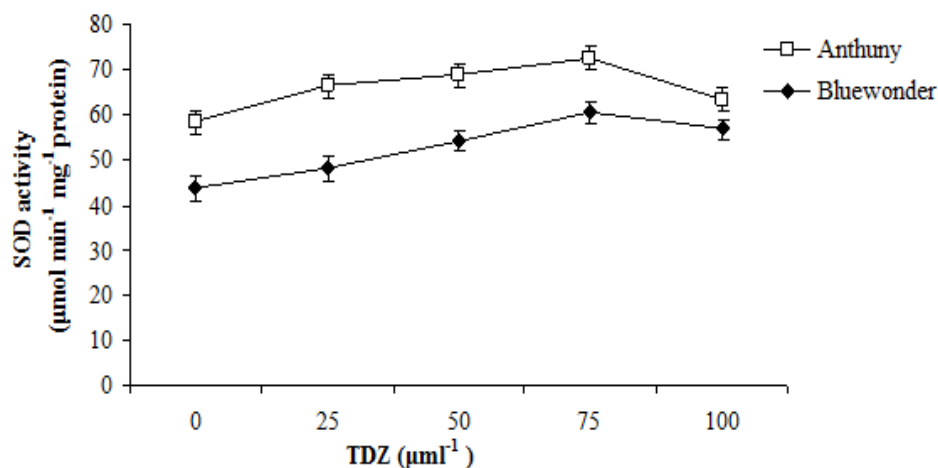


Fig. 3. Effect of TDZ on activity of superoxide dismutase (SOD) of two *pelargonium* species during 5 days of leaf senescence in the dark storage conditions.

It has been suggested that increase in ROS plays a role in dark-induced senescence (16). Temporary reduction in ROS levels by TDZ

occurred when TDZ was applied at the onset of ROS accumulation, during the dark period (**Fig. 4a, b**).



Fig. 4a. Effect of different TDZ levels on post production and leaf senescence of “Anthuny” pot pelargonium.



Fig. 4b. Effect of different TDZ levels on post production and leaf senescence of “Blue Wonder” pot pelargonium.

A hypothesis has been developed that ageing results from an accumulation of harmful free radicals and that the onset of senescence is mainly due to the uncontrolled strong enhancement in the generation of ROS, especially superoxide, singlet oxygen, hydroxyl radical and H_2O_2 (5). Oxidative stress arises with

more ROS (such as H_2O_2 , $\text{OH}^\cdot$ and $\text{O}_2^\cdot$) being produced than are metabolized (17).

CONCLUSION

Taken together, our results suggest that TDZ may act to inhibit leaf senescence of *Pelargonium* by alleviating the immediate effect of dark stress, via reduction of ROS levels.

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Abbreviations

ROS Reactive oxygen species

TDZ Thidiazuron

APX Ascorbate peroxidase

SOD Superoxide dismutase

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