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Oxidative stress, antioxidant capacity and ethylene production during ageing of cut carnation (*Dianthus caryophyllus*) petals

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Abstract

The objective of this work was to study the role of free radicals in relation to ethylene production during ageing of cut carnation petals. Ethylene production by freshly cut flowers was negligible, but 8 d after cutting ethylene production began to increase and reached a peak by day 9, before beginning to decline again. The efflux of electrolytes (membrane damage index) increased 101% and 2′,7′-dichlorofluorescein oxidation rate (oxidative stress index) increased 53% from day 8 to day 11 after detachment. Ethylene peak was either (a) not affected significantly by the supplementation of exogenous ethylene on the day of cutting, or (b) expanded between days 7–9 after ethylene supplementation on day 6 of cutting, or (c) was inhibited by amino-oxyacetic acid and paraquat treatments. After ethylene supplementation, conductivity and 2′,7′-dichlorofluorescein oxidation increased significantly as compared to control petals, and the activity of antioxidant enzymes was not affected. However, both α-tocopherol and glutathione content decreased significantly after ethylene supplementation on day 6 after detachment. Amino-oxyacetic acid treatment prevented the increases in conductivity and 2′,7′-dichlorofluorescein oxidation, did not alter the activities of antioxidant enzymes and significantly increased the content of α-tocopherol and glutathione as compared to control carnation petals. Paraquat treatment paralleled qualitatively ethylene supplementation after 6 d of cutting.

Taken as a whole, the data presented here may be understood as experimental evidence of a close association between ethylene production and oxidative stress in ageing of cut carnations.

Key words: Carnation petals, oxidative stress, ethylene, antioxidants.

Introduction

Ageing of petals is accompanied by morphological, biochemical and biophysical deterioration. Senescing carnation flowers exhibited a climacteric-like rise in ethylene production (Beyer, 1977; Mayak and Dilley, 1976; Trippi and Paulin, 1984) and exposure of carnation flowers to exogenous ethylene induced inrolling of the petals, triggered ethylene synthesis (Nichols, 1968), and induced chemical and physical changes in microsomal membrane lipids of senescing petals (Thompson *et al.*, 1982). Both ethylene biosynthesis and membrane breakdown, which appear to be closely linked, seem to involve free radicals, although the sequence of events generating these free radicals is still poorly understood. Paulin *et al.* (1986) suggested that transformation of lipids leads to membrane breakdown, free radicals are then produced by peroxidation and these free radicals promote the burst of ethylene. This hypothesis is in agreement with previous work (Mayak and Adam, 1984) who suggested that ethylene synthesis requires membrane deterioration so that aminocyclopropane carboxylic acid (ACC), a polar molecule,

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may approach the ethylene-forming enzyme, the membrane enzyme that transforms ACC into ethylene.

Sylvestre *et al.* (1989) showed that, during petal development in cut carnations, ethylene content increased when peroxidation began and that the activities of superoxide dismutase and catalase decreased from the initial stage to blooming (Droillard *et al.*, 1989).

Conditions inhibiting the action of (i.e. by the supply of silver salts) or the synthesis of ethylene (i.e. by the supplying of α-amino-oxyacetic acid) prolonged the vase-life of carnations. However, there was no correlation between the time of appearance of ethylene and the time of onset of lipid breakdown (Sylvestre and Paulin, 1987). Baker *et al.* (1977) reported that the vase-life of carnations was increased by 70% by the use of sodium benzoate (a free radical scavenger) that also inhibited the outburst of ethylene. Moreover, when the ethylene burst was inhibited by addition of silver thiosulphate, peroxidation was delayed and slowed, but enzyme activities varied very little under these conditions (Sylvestre *et al.*, 1989). However, it was not clear if silver salts acted by inhibiting the ethylene peak or through an indirect action on enzymatic activities.

Paraquat is a potent herbicide that produces marked changes in cell ultrastructure including disruption of membranes (Birchem *et al.*, 1979). Its phytotoxicity is dependent on the formation of the highly reactive superoxide radical that arises when the partially reduced dipyridyl cation is re-oxidized by molecular oxygen (Moustafa Hassan *et al.*, 1978). It was proposed that much of the membrane deterioration occurred during senescence could be closely simulated by paraquat treatment (Sar Chia *et al.*, 1981).

In the present study, the loss of membrane integrity, oxidative stress, and the content of enzymatic and non-enzymatic antioxidants were followed in relation to ethylene production during the normal ageing of cut carnation petals and in petals treated with exogenous ethylene, amino-oxyacetic acid and paraquat.

Materials and methods

Plant material

Cut carnation flowers (*Dianthus caryophyllus* L. cv. White Sim) were obtained directly from a local greenhouse. Flowers were maintained in a culture room at 22 °C and at a photosynthetic photon flux of 40 μmol photon $m^{-2} s^{-1}$ from a source of six cool white fluorescent light. The flowers were cut to 45 cm and were supplied with a basal solution containing a substance ordinarily used to conserve cut flowers (ampicillin 100 ppm, Sigmá Chemical Co.). Where indicated, one of the following treatments was performed on carnation flowers: (a) cut flowers were sprayed with 1 mM paraquat (PQ) on the harvest day, (b) carnations were supplied continuously through cut stems with 1 mM amino-oxyacetic acid (AOA), or (c) flowers were exposed to a 6 ppm ethylene-enriched atmosphere for 6 h in a glass chamber of about 40 l, either on the cutting day or on day 6 after cutting.

Homogenate preparation

Cut flowers were homogenized with a Potter-Elvejhem homogenizer in 60 mM phosphate buffer–100 mM KCl (pH 7.0), filtered through four layers of cheesecloth and then centrifuged at 750 *g* for 10 min.

Ethylene production

Ethylene production was measured by incubating two petals in glass flasks sealed with rubber caps for 3 h. Aliquots of 1 ml of head space were injected in a KNK-3000-HGRC (Konik) gas chromatograph, using an alumina column and a flame ionization detector (Han *et al.*, 1991; Serrano *et al*, 1991).

Leakage of solutes

Two petals (0.3 g) were immersed in 20 ml of distilled water and the conductivity was measured after 3 h at room temperature (Eze *et al.*, 1986; Beja-Tal and Borochov, 1994).

α-Tocopherol content

The content of α-tocopherol (αT) in the homogenates was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6 V (Desai, 1984). Extraction of the samples was performed with 1 ml of methanol and 4 ml of hexane. After the samples were centrifuged at 1500 *g* for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Samples were dissolved in methanol: ethanol (1 : 1, v/v) and injected for HPLC analysis. *d,l*-αT from synthetic phytol (Sigma) was used as standard.

Glutathione analysis

Total glutathione concentration was assayed in acid-soluble extracts (0.2 g FW ml^{-1}) as described by Tietze (1969). The homogenate was prepared in TCA 3% (w/v) and after a brief centrifugation, the supernatant was diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Glutathione was determined by measuring the absorbance at 412 nm in the presence of 0.5 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 0.5 U ml^{-1} gluthathione reductase and 0.2 mM NADPH.

Reactive oxygen species generation

An *in vivo* assay was performed by placing one petal of a cut carnation flower in 8 ml of 40 mM Tris–HCl buffer (pH 7.0) in the presence of 15 μM 2′,7′-dichlorofluorescein (DCFH-DA, Molecular Probes Inc, Eugene, OR) at 30 °C. Supernatants were removed after 90 min and fluorescence monitored in a Hitachi spectrofluorometer with excitation at 488 nm and emission at 525 nm (Simontacchi *et al.*, 1993). To differentiate reactive oxygen species from other long-lived substances able to react with DCFH-DA, the petals were taken out after 30 min of incubation and the medium was supplemented with DCFH-DA and incubated for 30 min before the fluorescence was determined. This fluorescence value was considered as the contribution of long-lived substances to the total fluorescence and subtracted from all readings to assess the reactive oxygen species-dependent fluorescence. Corrections for autofluorescence were made by the inclusion, in each experiment, of parallel blanks (assay mixture without petals).

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Enzyme assays

The homogenates were added to 1% (w/v) polyvinylpolypyrrolidone (PVP). Glutathione reductase (GR) activity was measured according to Schaedle and Bassham (1977). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM $MgCl_2$ and up to 100 μl of homogenate (7 mg protein ml^{-1}). NADPH oxidation was followed at 340 nm.

Superoxide dismutase (SOD) activity was determined spectrophotometrically as the inhibition of xanthine oxidase-dependent reduction of nitroblue tetrazolium (Beauchamp and Fridovich, 1971). The reaction mixture contained 0.1 mM nitroblue tetrazolium, 0.1 mM EDTA, and 50 μM xanthine and xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.8). One unit of SOD is defined as the amount of enzyme that inhibits by 50% the control rate (0.025 units of absorbance at 550 nm min^{-1}) (McCord and Fridovich, 1969).

Catalase activity was measured according to Aebi (1984). The reaction mixture contained 15 mM H_2O_2, up to 100 μl of homogenate (7 mg protein ml^{-1}) with 0.2% (v/v) Triton X-100 in 50 mM potassium phosphate buffer (pH 7.0).

The protein content was assayed according to Bradford (1976) utilizing bovine serum albumin as standard.

Statistical analyses

Data are expressed as mean ±SEM from 4–6 independent experiments. The effects of treatments were tested for significance using a single-factor analysis of variance (ANOVA). Significantly different means are separated using the Fisher PLSD test, Statview SE+, v 1.03 (Abacus Concepts Inc, Berkeley, CA).

Results

The pattern of ethylene production by carnations cut as young fully emerged flowers and held in water is illustrated in Fig. 1. Ethylene production by freshly cut flowers was either negligible or extremely low, but 8 d after cutting ethylene production began to increase and reached a peak by day 9 before beginning to decline again. The peak in ethylene production at day 9 coincided temporally with the onset of petal-inrolling, which is the first morphological symptom of carnation senescence. This pattern of ethylene production has been noted previously for senescing carnations (Mayak and Halevy, 1980; Thompson *et al.*, 1982; Paulin *et al.*, 1986) and, because it marks the onset of visual symptoms of flower senescence, it has been linked to the climacteric rise in ethylene production by ripening fruits (Mayak and Halevy, 1980).

To assess *in vivo* oxidative stress condition, an assay based upon DCFH-DA oxidation to a fluorescent compound was employed. The absence of fluorescence observed when the petals were incubated in the presence of DCFH-DA and then separated from the medium and homogenized, indicates that DCFH-DA is not concentrated into the petals. Intact carnation petals generate oxidants capable of diffusing and/or being released into the medium and reacting with DCFH-DA. Previous reports (Scott *et al.*, 1988; LeBel *et al.*, 1990) suggest that

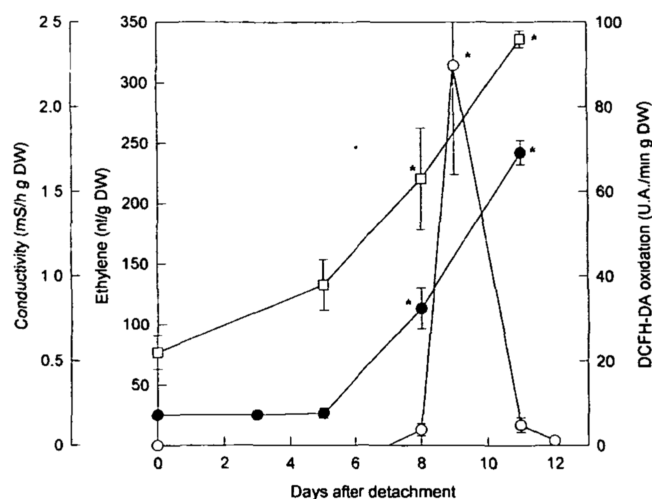

Fig. 1. Time-course of ethylene evolution (○), electrolyte leakage (●) and DCFH-DA oxidation (□) by carnation petals. (*) Significantly different from values at the day of cutting (ANOVA, $P \leq 0.05$). Data are expressed as mean ±SEM of 4–6 independent experiments.

the oxidants responsible for the fluorescence could be H_2O_2, $\cdot OH$ and O_2^-. The efflux of electrolytes (index of membrane integrity) increased 101% and DCFH-DA oxidation rate increased 53% from day 8 to day 11 after detachment (Fig. 1), suggesting that the ethylene rise, membrane damage and free radical generation could be related events, as previously hypothesized (Paulin *et al.*, 1986).

The burst of ethylene was not detected after 12 d in carnation flowers supplied with AOA. Paraquat aspersion to the cut flowers limited the ethylene burst to 19±16, 36±18 and 22±8 nl g^{-1} DW after 7, 8, and 9 d of cutting, respectively. Ethylene supplementation on the cutting day did not significantly affect ethylene burst, but ethylene addition at day 6 significantly increased its content during days 7–9 (Fig. 2).

Since during natural ageing of cut carnations significant changes in conductivity and DCFH-DA oxidation were detected over the study period, the effect of the treatments on those parameters was evaluated. At day 8 after cutting, petal conductivity was significantly decreased by AOA treatment and increased by either PQ or ethylene supplementation (Table 1). AOA treatment between days 8 and 11 after detachment decreased the significant increase measured in the dye oxidation rate after 8 and 11 d of cutting and blocked the net increase in DCFH-DA oxidation detected in control petals over the study period (Table 1). Paraquat treatment significantly enhanced both DCFH-DA oxidation rate and its net increase over the period studied. Ethylene supplementation on the day of cutting did not affect the net increase in the dye oxidation rate from days 8–11 (29 AU g^{-1} DW min^{-1}), but individual rates were increased as compared to those measured in untreated

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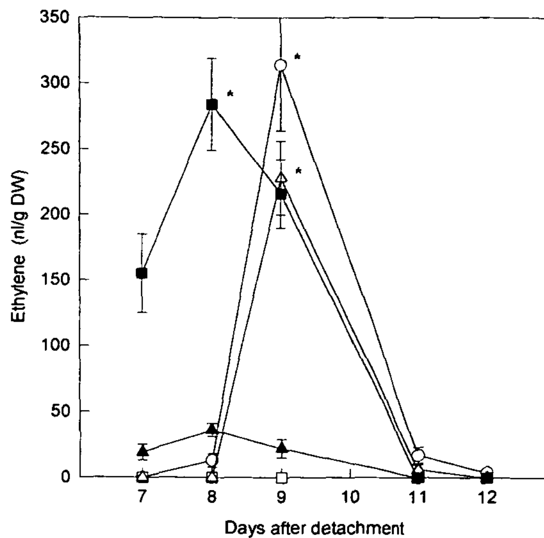

Fig. 2. Time-course of ethylene evolution by carnation petals supplied with basal solution (○), exogenous ethylene at day of cutting (▲), exogenous ethylene on day 6 after cutting (■), 1 mM AOA (□) and 1 mM PQ (△). (*) Significantly different from values on day 7 after cutting (ANOVA, $P \leq 0.05$). Data are expressed as mean ±SEM of 4–6 independent experiments.

flowers. Ethylene supplementation on day 6 significantly increased the DCFH-DA oxidation rate (Table 1).

On the day of cutting the activities of SOD, GR and catalase were 21 ± 4 U mg^{-1} protein, 234 ± 35 U mg^{-1} protein and 1.3 ± 0.2 pmol mg^{-1} protein, respectively. The activities of catalase and GR showed no significant changes between days 8 and 11 after detachment and these profiles were not affected by any of the treatments. SOD activity did not change between days 8 and 11 of detachment, neither in the control nor in the treated petals (Table 2).

The content of α-tocopherol in the petals decreased significantly from 79 ± 9 nmol g^{-1} DW on the day of cutting to 51 ± 8 and 41 ± 3 nmol g^{-1} DW after 8 and 11 d of detachment, respectively. The treatment with AOA prevented the decrease in the content of the antioxidant measured after 11 d of cutting, and ethylene exposure at day 6 decreased the α-tocopherol content by 43.9%

Table 1. *Effect of alteration in ethylene generation on conductivity and DCFH-DA oxidation by carnation petals*

Treatment	Conductivity ($\mu S\ h^{-1}\ g^{-1}$ DW)		DCFH-DA (AU $min^{-1}\ g^{-1}$ DW)	
	Day 8	Day 11	Day 8	Day 11
None	859 ± 142	1734 ± 71	63 ± 12	96 ± 2
+1 mM AOA	211 ± 28*	471 ± 74*	35 ± 7*	35 ± 10*
+1 mM PQ	1537 ± 145*	1415 ± 95	193 ± 3*	611 ± 137*
+6 ppm $ethyl_0$	1782 ± 140*	1800 ± 64	156 ± 76*	185 ± 52
+6 ppm $ethyl_6$	2048 ± 187*	1800 ± 54	457 ± 172*	241 ± 53*

*Significantly different from petals without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean ±SEM of 4–6 independent experiments.

after 11 d (Table 3). Paraquat treatment of the petals decreased the α-tocopherol content by 45% and 29% after 8 d and 11 d, respectively.

The content of total glutathione in the petals decreased from 1078 ± 71 nmol g^{-1} DW on the cutting day to 212 ± 19 nmol g^{-1} DW after 11 d of detachment. AOA treatment prevented the observed decrease in total glutathione content after 11 d of cutting. Paraquat treatment did not significantly affect glutathione content in the petals over the period of senescence studied. Ethylene supplementation on day 6 decreased glutathione content by 17% and 66% after 8 and 11 d of detachment, respectively (Table 3).

Discussion

A sudden rise in ethylene production is known to occur during the senescence of cut flowers (Nichols, 1966; Kende and Baumgartner, 1974) and symptoms of senescence are accelerated and amplified when flowers are placed in an atmosphere containing ethylene (Nichols, 1968; Thompson *et al.*, 1982). Through *in vitro* studies it has been suggested that the conversation of ACC to ethylene may involve a peroxidative reaction (Legge and Thompson, 1983). Studies performed with *Dianthus caryophyllus* L. indicated that senescence can be slowed by retarding peroxidation by neutralizing free radicals. Moreover, inhibition of the ethylene burst slows peroxidation and prolonged the life of cut carnations, suggesting a relationship between free radical generation and ethylene production (Mayak *et al.*, 1983). In this study, carnation petals exposed to ethylene on the day of cutting did not showed any significant difference in the kinetic of ethylene production, suggesting that the metabolic conditions of the petal could be critical in order to limit ethylene effect. The ethylene peak appeared earlier in petals exposed to ethylene 6 d after cutting and higher ethylene levels were maintained for longer periods in treated petals as compared to those not exposed to ethylene (Fig. 2). However, the oxidation of DCFH-DA (oxidative stress index) and conductivity (membrane damage index) (Table 1) were increased by both treatments as compared to control petals.

Previous findings (Paulin *et al.*, 1985; Borochov *et al.*, 1982; Sylvestre and Paulin, 1987) showed that feeding with AOA nearly completely inhibited the ethylene rise which also occurred much later. The lower rate of DCFH-DA oxidation and leakage of electrolytes as compared to control petals, measured after AOA treatment (Table 1) suggest that both oxidative stress and membrane disruption are ethylene-related parameters, since both of them could be controlled by limiting ethylene production.

Antioxidant defence systems in the cell include the activities of SOD, catalase, and GR. Several studies have indicated that SOD activity decreases progressively up to

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Table 2. *Effect of alteration in ethylene generation rate on the activities of antioxidant enzymes in carnation petals*

Treatment	Catalase (pmol mg^{-1} protein)		SOD (U mg^{-1} protein)		GR (U mg^{-1} protein)	
	Day 8	Day 11	Day 8	Day 11	Day 8	Day 11
None	1.1±0.3	1.2±0.2	33±7	21±3	283±5	170±54
+1 mM AOA	1.5±0.5	1.7±0.5	23±4	17±2	290±9	173±9
+1 mM PQ	0.7±0.1	1.5±0 9	18±3*	21±6	235±28	193±42
+6 ppm $ethyl_0$	0.7±0.2	1.7±0.9	18±2*	16±3	175±66	117±35
+6 ppm $ethyl_6$	1.0±0.1	1.4±0.2	26±1	14±2	218±44	149±24

* Significantly different from petals without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean ±SEM of 4–6 independent experiments.

Table 3. *Effect of alteration in ethylene generation rates on non-enzymatic antioxidants in carnation petals*

Treatment	α-Tocopherol (nmol g^{-1} DW)		Glutathione (nmol g^{-1} DW)	
	Day 8	Day 11	Day 8	Day 11
None	51±8	41±3	814±41	212±19
+1 mM AOA	54±7	90±8*	1528±275*	1646±126*
+1 mM PQ	28±4*	29±3*	905±87	301±89
+6 ppm $ethyl_0$	64±10	36±5	673±134	208±28
+6 ppm $ethyl_6$	54±1	23±5*	643±82	141±13*

* Significantly different from petals without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean ±SEM of 4–6 independent experiments.

complete wilting and catalase activity increases progressively in carnation (Droillard and Paulin, 1987). Woodson (1987) showed that senescence in carnation petals is linked to the expression of specific genes, and that ethylene may play a role in the regulation and transcription of these mRNAs. On the other hand, in *Chrysanthemum morifolium* RAM petals a consistent increase in the content of antioxidant enzymes during senescence was reported (Bartoli *et al.*, 1995), representing an induced protective reaction to an increased rate of generation of reactive species, independently of ethylene generation.

In carnation petals, supplementation of ethylene on the day of cutting did not significantly alter the content of either antioxidant enzymes (except for SOD) or non-enzymatic antioxidants as compared to control petals on day 8 and 11, respectively. On the other hand, ethylene supplemented 6 d after cutting did not affect the content of antioxidant enzymes, but significantly decreased both α-tocopherol and glutathione contents (Table 2). These data are in agreement with the hypothesis that links ethylene generation and oxygen radicals, since the increase in ethylene level by the exposure to exogenous ethylene on day 6 of detachment paralleled non-enzymatic antioxidant consumption. Moreover, the decline in the content of non-enzymatic antioxidants is prevented by AOA. α-Tocopherol content increased by 120% in petals after 11 d of development as compared to petals not exposed to AOA and the drastic decrease in glutathione content after 11 d of detachment, was prevented by the treatment (Table 3). This profile suggests that the disappearance of the ethylene peak leads to decreased oxidant production, in agreement with the hypothesis stating that both parameters are closely related. Glutathione seems more sensitive than enzymatic antioxidants and α-tocopherol, since the increase in its content after AOA treatment was greater than that measured with α-tocopherol.

To study the possible relationship between ethylene peak and radical production further, the petals were treated with paraquat, a potent herbicide that produced marked changes in cell ultrastructure including disruption of membranes (Birchem *et al.*, 1979). It was previously reported that both paraquat and ethylene treatment resemble very closely the effects of natural senescence on membranes (Sar Chia *et al.*, 1981). The data presented here (Table 1) are in agreement with those reports since both treatments increased DCFH-DA oxidation and conductivity by intact cut carnation petals.

The decrease in SOD activity in petals exposed to PQ could be interpreted as a result of the damage by the excess of O_2^- generated. PQ treatment resembled the effects of ethylene supplementation in terms of α-tocopherol consumption and morphological deterioration of the petals, but showed a slight difference in relation to the effect on glutathione. On the other hand, since PQ enhanced oxidative stress and cellular damage through the generation of free radical species, it would be expected that ethylene generation would be increased by the treatment. However, as it is shown in Fig. 2, PQ supplementation suppressed ethylene production. These data suggest that the oxidants produced by paraquat could be different from those responsible for triggering ethylene synthesis.

Taken as a whole, the data presented here are experimental evidence of a close association between ethylene production and antioxidant capacity in cut carnations. A complex and specific antioxidant defence system is operative in petals. The comparison between these results in carnation petals and the data previously reported on the activity of antioxidants in *Chrysanthemum morifolium* RAM petals (Bartoli *et al.*, 1985), show that clear differences could be established between species in terms of different mechanisms of senescence. This study on antioxidant metabolism during ageing of cut carnation petals can be understood, not only as experimental evidence

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confirmating the hypothesis of a link between ethylene and radical generation in ageing, but as a key to the design of adequate methods to prevent or delay deterioration. Since the activity of the antioxidant enzymes seems not to be induced and the non-enzymatic antioxidants in carnation appear to decline over the critical ageing period studied, additional means of improving the conservation of cut flowers may be possible. The ability to generate transgenic plants provides a powerful tool to increase the level of stress tolerance by reinforcing the plant's defence systems with new genes. This technology, applied to the production of plants that control the expression of the native genes of the antioxidant enzymes, in conjunction with supplementation with lipid and water-soluble antioxidants, could be a way of substantially delaying senescence of cut carnations.

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