The role of \(N\)-lauroylethanolamine in the regulation of senescence of cut carnations (\textit{Dianthus caryophyllus})

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Received 10 April 2006; accepted 5 July 2006

**Summary**

\(N\)-acylethanolamines (NAEs) are a group of lipid mediators that play important roles in mammals, but not much is known about their precise function in plants. In this work, we analyzed the possible involvement of \(N\)-lauroylethanolamine [NAE(12:0)] in the regulation of cut-flower senescence. In cut carnation flowers of cv. Red Barbara, the pulse treatment with 5 \(\mu\)M NAE(12:0) slowed senescence by delaying the onset of initial wilting. Ion leakage, which is a reliable indicator of membrane integrity, was postponed in NAE(12:0)-treated flowers. The lipid peroxidation increased in carnation petals with time, in parallel to the development in activity of lipoxygenase and superoxide production rate, and these increases were both delayed by NAE(12:0) supplementation. The activities of four enzymes (superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase) that are implicated in antioxidant defense were also upregulated in the cut carnations that had been treated with NAE(12:0). These data indicate that NAE(12:0)-induced delays in cut-carnation senescence involve the protection of the integrity of membranes via suppressing oxidative damage and enhancing antioxidant defense. We propose that the stage from the end of blooming to the onset of wilting is a critical period for NAE(12:0) action.

**Keywords**

Antioxidant enzymes; \textit{Dianthus caryophyllus}; \(N\)-lauroylethanolamine; Oxidative damage; Senescence

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**Abbreviations:** ABA, abscisic acid; ASC, ascorbate; APX, ascorbate peroxidase; CAT, catalase; EDTA, ethylene diaminetetraacetic acid; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; LOX, lipoxygenase; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NAEs, \(N\)-acylethanolamines; NAE(12:0), \(N\)-lauroylethanolamine; NBT, nitro blue tetrazolium; \(O_2^{\cdot-}\), superoxide anion; ROS, reactive oxygen species; SOD, superoxide dismutase; PLD, phospholipase D.

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Introduction

N-acylethanolamines (NAEs) are fatty-acid amides, which are minor membrane lipid constituents of plant and animal cells. In animals, NAEs have been identified to be part of the endocannabinoid signaling system that regulates a variety of physiological functions (Chapman, 2004). Increasing evidence has indicated that some biological activities are attributed to NAEs in plants. It has been shown that NAEs are involved in plant defense signaling (Chapman et al., 1998; Tripathy et al., 1999), inhibit the activity of phospholipase D activity (Austin-Brown and Chapman, 2002), and disrupt normal seedling root development at elevated levels (Blancaflor et al., 2003). Recently, NAEs have been used to preserve the freshness of cut flowers and to delay the ripening of fruits (Chapman and Austin-Brown, 2001). However, the possible relationship between NAEs and post-harvest longevity has not yet been studied in flowers.

Loss of membrane integrity that results in an increase in membrane permeability is the final and irreversible phase of senescence (Marangoni et al., 1996), which is closely correlated with lipid modifications that are principally due to peroxidation (Paulin et al., 1986). The overall peroxidation process may be as follows: initially, phospholipases remove the polar heads of fatty acids, which are then more easily degraded. The peroxidation is then initiated and sustained by reactive oxygen species (ROS) and lipoxygenase (Paulin and Droillard, 1989; Marangoni et al., 1996). However, plant cells possess both enzymatic and non-enzymatic mechanisms that can overcome oxygen damage and delay the deleterious effects on membranes (Foyer et al., 1994).

Cut carnation flowers have been shown to be a convenient model system for the study of post-harvest physiological events during flower senescence (Paulin and Jamain, 1982). In the present investigation, we examined the possible relationship between NAEs and senescence and, moreover, attempted to follow the loss of membrane integrity via regulating oxidative damage and antioxidant defense levels during senescence that was delayed by the use of NAE(12:0).

Materials and methods

Plant material

Carnations, Dianthus caryophyllus L. cv. Red Barbara, were obtained from local commercial growers. Flowers were collected at the preopening stage (stage 1) as described below, and transported to the laboratory on the day of harvest. Flowers were trimmed to a peduncle length of \( \sim 12 \text{ cm} \) for subsequent treatments.

Chemicals

N-lauroylethanolamine [NAE(12:0)] was prepared by refluxing lauric acid with ethanolamine for 6 h and purified by recrystallizing in dichloromethane and acetone. Purity was greater than 99%, as determined by GC–MS.

Application of NAE(12:0)

Trimmed flowers were randomized and placed individually in test tubes (10 mL), then subjected to pulse treatment on the day of harvest (day 0 of the experiment). Pulse treatments were performed by holding the flowers in the vase solution (5 mL) containing 5 \( \mu \text{M} \) NAE(12:0) with a solvent of 0.1% (v/v) isopropanol and 0.001% (v/v) Tween-20 for 4 h. The concentration in preliminary experiments was found to be optimal for improving longevity in cut carnation flowers of cv. Red Barbara. Flowers were pulsed in solution with a solvent containing 0.1% isopropanol and 0.001% Tween-20, which served as controls, and no effect of isopropanol and Tween-20 on cut carnation flowers post-harvest was observed (data not shown). After pulse treatment, carnation flowers were transferred to deionized water for vase holding. Subsequently, the pulse treatment was performed again every 2 days until the experiment was complete. All flowers were held in an observation room at 21 ± 2 °C, 60 ± 10% relative humidity under white fluorescent light (20\( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) and 12-h photoperiod for 1 day.

Evaluation of development stages and longevity

To evaluate the senescence in response to NAE(12:0), four stages of development were arbitrarily distinguished according to Droillard et al. (1987). Stage 1, pre-opening: the petals form a right angle with the stem axis. Stage 2, opening: the petals form a 45° angle with the stem. Stage 3, beginning to withering: the petals have lost turgor and their edges are wrinkling. Stage 4, withering: the petals are wrinkled and discolored and there is a complete loss of turgor (Droillard et al., 1987). The post-harvest performance of flowers was evaluated twice each day, and the vase life was
monitored to the point when withering symptoms appeared (stage 4).

For the determination of development stages and longevity of cut carnation flowers of cv. Red Barbara, 30 flowers were used in each experiment. Statistical significance between mean values was assessed using one-way analysis of variance with SPSS-11.5 statistical software. A probability of \( p < 0.05 \) was considered to be significant.

### Ion leakage

The loss of membrane integrity was assayed by the efflux of electrolytes. The petals of each flower were immersed in 40 mL of 0.11M mannitol for 3.5 h at room temperature. The conductivity of the mannitol solution was then measured using a conductivity meter (Droillard et al, 1987).

### Lipid peroxidation

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA), as in Heath and Packer (1968). Fresh petals (0.5 g) were homogenized in 10 mL 5% (w/v) trichloroacetic acid. The homogenates were then centrifuged at 4000g for 10 min. A reaction mixture of equal volume of the supernatant (2 mL) and 0.67% (w/v) thiobarbituric acid (2 mL) was incubated at 95 \(^\circ\)C in a water bath for 25 min, then cooled immediately before centrifugation. The absorbance was monitored at both 532 and 600 nm. The MDA concentration was calculated using the extinction coefficient 155 mM\(^{-1}\) cm\(^{-1}\).

### Determination of superoxide anion

The production rate of superoxide anion (O\(_2^−\)) was measured, as described by Jiang and Zhang (2002), by monitoring the nitrite formation from hydroxylamine in the presence of O\(_2^−\). The absorbance was read at 530 nm. Corrections were made for the background absorbance in the presence of 50 units SOD. A standard curve with NO\(_2^-\) was used to calculate the production rate of O\(_2^−\) from the chemical reaction equation of O\(_2^−\) and hydroxylamine.

### Enzyme assays

Frozen petals (0.5 g) were crushed into fine powder in a pestle and mortar under liquid N\(_2\). Soluble proteins were extracted by homogenizing the powder in 10 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylene diaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone, with the addition of 1 mM ascorbate (ASC) in the case of ascorbate peroxidase assay. The homogenate was centrifuged at 15,000g for 20 min at 4 \(^\circ\)C and the supernatant was used for the following enzyme assays. Protein content was determined according to the method of Bradford (1976), with bovine serum albumin used as the standard.

Superoxide dismutase (EC 1.15.1.1) (SOD) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Jiang and Zhang (2001). The 3-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 \(\mu\)M NBT, 2 \(\mu\)M riboflavin, 0.1 mM EDTA and 100 \(\mu\)L enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 100 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of NBT, as monitored at 560 nm.

Catalase (EC 1.11.1.6) (CAT) activity was determined by following the consumption of H\(_2\)O\(_2\) (extinction coefficient: 0.0394 mM\(^{-1}\) cm\(^{-1}\)) at 240 nm for 3 min. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H\(_2\)O\(_2\) and 200 \(\mu\)L enzyme extract in a 3-mL volume (Jiang and Zhang, 2001).

Glutathione reductase (EC 1.6.4.2) (GR) activity was determined by following the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (extinction coefficient: 6.2 mM\(^{-1}\) cm\(^{-1}\)) for 3 min in 1 mL of an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 200 \(\mu\)L enzyme extract. The reaction was initiated by adding NADPH (Jiang and Zhang, 2001).

Ascorbate peroxidase (EC 1.11.1.11) (APX) activity was determined by following the decrease in A\(_{290}\) (extinction coefficient: 2.8 mM cm\(^{-1}\)) for 1 min in 1 mL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ASC, 0.1 mM H\(_2\)O\(_2\) and 200 \(\mu\)L enzyme extract. The reaction was initiated by adding enzyme extract (Jiang and Zhang, 2001).

Lipoxygenase (EC 1.13.11.12) activity was spectrophotometrically assayed using linoleic acid as substrate, according to the method of Chérif et al. (1997) with some modifications. The reaction mixture consisted of 250 \(\mu\)M linoleic acid, 0.005% (v/v) Tween-20, 50 mM Glycin-NaOH buffer (pH 8.6) (Lynch et al., 1985), and 10 \(\mu\)L of enzyme extract. Activity was expressed in \(\Delta\)A min\(^{-1}\) mg protein\(^{-1}\).
Statistical analysis

For the physiological parameters, results are expressed as mean ± SE from 4 to 6 replicates. Statistical significance between mean values was assessed using one way analysis of variance with SPSS-11.5 statistical software. A probability of $p < 0.05$ was considered to be significant.

Results

Evaluation of longevity and development stages

NAE(12:0) treatment significantly increased the longevity of cut carnation flowers of cv. Red Barbara, with an extension of 19% compared with that of controls (Fig. 1). The increase in the wilting percentage started on the 10th day for the samples that were fed with NAE(12:0), which was 2 days later than the controls. The senescence of control flowers proceeded rapidly during day 10 to day 14, when significant differences in the wilting percentage were shown between controls and treated samples (Fig. 2). The duration of development in stage 2 in NAE(12:0)-treated flowers was 10.4 days, which was ~2 days longer than that of controls, and the duration of stage 1 and stage 3 development was 4.1 days and 1.8 days, respectively (but this was not significantly different compared with the controls (Table 1)).

Ion leakage

Ion leakage of petal tissue, an indicator of membrane permeability, gradually increased from day 0 to day 8 for both samples. However, NAE(12:0) application resulted in delayed and less marked increases compared with controls after day 8 (Fig. 3).

Oxidative damage

Lipid peroxidation of fatty acids results in the formation of several byproducts, such as MDA. The changes in the MDA content were slight from day 0 to day 8 for both samples, and then increased sharply after day 8. In contrast, the increase rate in the treated flowers was significantly slower than that of controls (Fig. 4A). MDA is the product of oxidative enzymes, the interesting changes in MDA
content thus prompted us to follow the dynamics of lipoxygenase (LOX) activity (Fig. 4B). As could be expected from the results on lipid peroxidation, the activity of LOX exhibited an increasing trend from day 0 to day 12, which was almost parallel to the yield of MDA, and less increase was also noted in NAE(12:0)-treated samples. A peak activity that was 3-fold higher than the initial activity was detected by day 12 for controls, and was then followed by a decrease. However, LOX activity increased constantly in NAE(12:0)-treated samples until day 14. The superoxide anion production rate followed the activity of the LOX pattern during the vase period (Fig. 4C). Treatment with NAE(12:0) significantly reduced the generation of $O_2^-$ by day 12, with a decrease in production rate of 34% compared with the controls.

Antioxidant enzyme activities

The involvement of antioxidant enzymes in the regulation of free-radical metabolism was followed by measuring the changes in SOD, CAT, GR and APX activities. SOD activity increased markedly during the initial 4 days, and then decreased constantly throughout the remainder of the vase period. The activities of SOD were practically the same before day 8 for both samples, but significantly higher activities were observed in the NAE(12:0)-treated flowers on the 12th and 14th days (Fig. 5A). The pattern of changes in GR activity was similar to that of SOD (Fig. 5C). However, a different profile was found in CAT activity, which steadily increased from
day 0 to day 12 (Fig. 5B). The activity of CAT peaked on day 12, followed by a rapid decline in the controls, whereas a continued increase was observed for NAE(12:0)-treated samples until day 14, when the CAT activity in the treated samples was 7-fold higher than that of the controls. After a slight elevation, APX activity steadily dropped, and this trend continued to day 14 (Fig. 5D). The level of APX activity was extremely similar in both samples before day 8. In contrast, NAE(12:0)-treated samples maintained relatively higher levels of activity of APX than controls during the subsequent days, and showed significant differences compared with the controls by day 12 and day 14.

Discussion

Pulse treatment with NAE(12:0) slowed senescence and extended the longevity of cut carnation flowers of cv. Red Barbara (Fig. 1 and Fig. 2). These results are in agreement with previous reports that NAEs could preserve the freshness of cut flowers and delay the ripening of fruits (Chapman and Austin-Brown, 2001). These data indicate that NAEs have general effects on the post-harvest life and senescence of cut flowers and fruits. The duration of stage 2 flower development was prolonged for NAE(12:0)-treated flowers (Table 1), indicating that NAE(12:0) retarded the rate at which the initial wilting symptoms occurred. However, the profiles in stages 1 and 3 were not affected by NAE(12:0). In addition, significant differences for physiological parameters measured in the present study (including ion leakage, oxidative damage and antioxidant enzyme activities) between treated samples and controls were only observed during day 12 to day 14, which was the stage of end of blooming to onset of wilting. These data collectively indicate that the stage from the end of blooming to the onset of wilting might be a vital period for NAE(12:0) action. However, we do not exclude the possibility of the cumulative effects of NAE(12:0) on senescence before the initial wilting symptom occurred.

A predominant feature of senescence in many systems is the loss of differential permeability of cellular membranes leading to ion leakage (Borochov and Woodson, 1989). It has been shown that leakage rates increased progressively in the carnations during aging (Paulin and Droillard, 1989; Bartoli et al., 1996). The membrane damage resulting in ion leakage may be related to peroxidation of membrane lipids (Thompson, 1988). Lipid peroxidation makes the membranes rigid and is involved in the development of a gel phase in the lipid bilayer (Pauls and Thompson, 1980). In our experiment, peroxidation also increased with the same trend as ion leakage during the vase period.
Senescence in cut carnation

(Fig. 3 and Fig. 4A), which is in agreement with earlier reports (Paulin and Droillard, 1989; Bartoli et al., 1995). However, the loss of membrane function was not related to the accumulation of lipid hydroperoxides in the petals of Alstroemeria peruviana (Leverentz et al., 2002), so it is possible that the effect of lipid peroxidation on membrane integrity differs between Alstroemeria peruviana and carnation petals.

The peroxidation of membrane lipids may be related to the increasing LOX activity and ROS production (Leshem, 1988; Siedow, 1991). LOX is a ubiquitous enzyme in eukaryotic organisms, which catalyzes the hydroperoxidation of cis,cis 1,4-pentadiene motifs of polyunsaturated fatty acids, and results in the propagation of peroxidation (Siedow, 1991). Increases in the activity of LOX and the content of MDA have been shown in carnation (Paulin and Droillard, 1989) and daylily (Panavas and Rubinstein, 1998) petals. This is consistent with the present study that showed how LOX activity increased markedly up to the onset of wilting (day 12), followed by a decline on day 14 (withering stage) under the non-treated conditions (Fig. 4B). The drop in activity of LOX could be explained by membrane degradation. LOX has been considered to be a membrane-bound enzyme (Lynch et al., 1985). On the other hand, ROS are also thought to be involved in membrane deterioration (includes induction of lipid peroxidation and fatty-acid de-esterification) during aging of tissues (Kellogg and Fridovich, 1975). For instance, Pauls and Thompson (1980) showed that O$_2^-$ brought about de-esterification of phospholipids, and the fatty acids released by this breakdown were then peroxidized, which in turn affected membrane permeability. Under our experimental conditions, O$_2^-$ was generated in increasing quantities until the initial wilting appeared (Fig. 4C). The result may be compared with the results of Paulin and Droillard (1989), who found that microsomal membranes from carnations produced an increased quantity of O$_2^-$ between the full blooming stage and the onset of wilting. O$_2^-$ may be produced by a membrane-bound oxidase (Droillard et al., 1987). So, the low value observed by day 14 for control petals may be explained by significant changes in membrane structural characteristics of the advanced stages. However, plant cells possess both enzymatic and non-enzymatic mechanisms that can essentially neutralize ROS (Foyer et al., 1994), thereby in part controlling the level of lipid peroxidation. The well-defined enzymatic antioxidant defense systems in the cell include the activities of SOD, CAT, GR and APX.

Senescence can be slowed down by retarding peroxidation (Paulin and Droillard, 1989), and high levels of antioxidant enzymes are correlated with delayed senescence (Lacan and Baccou, 1998). The compromise of oxidative stress in aging is a common feature to cut flowers, and this factor could be a key to designing adequate methods to prevent or delay deterioration and to improve the conservation of cut flowers (Bartoli et al., 1997). Paulin et al. (1986) demonstrated that the vase life of carnations was increased by 100% by the use of 3,4,5-trichlorophenol (a free-radical scavenger). In the present study, the upregulated activities of the antioxidant enzymes were observed in the NAE(12:0)-treated samples during day 12 to day 14 (Fig. 5), accompanying the retarded lipid peroxidation and leakage rates (Fig. 3 and Fig. 4). These data indicate that NAE(12:0)-induced delays in the senescence of cut carnations involve the protection of the integrity of membranes via defending the cells from age-related oxidative damage. Although our results do not prove that NAE(12:0) directly upregulate enzyme activities that are implicated in antioxidant defense and that suppress oxidative stress associated with the initial stages of aging, it would explain, at least to some extent, the observed improvements in the longevity of cut carnation flowers.

Accumulating evidence indicates that NAEs might have an important lipid mediator role by regulating phospholipase D$_\alpha$ (PLD$_\alpha$) activity in vivo (Chapman, 2004). The supplement with low concentrations of NAEs to epidermal peels of Nicotiana tabacum and Commelina communis inhibited abscisic acid (ABA)-induced stomatal closure (Austin-Brown and Chapman, 2002), a process that has been shown, by transgenic approaches, to involve PLD$_\alpha$ (Sang et al., 2001). Application of NAEs to Arabidopsis induced defects in seedling development that were similar to the effects induced by 1-butanol, an antagonist of PLD-dependent phosphatidic-acid production (Blancaflor et al., 2003; Motes et al., 2005). These observations implicating NAEs in the modulation of PLD indicate that some of the physiological effects of NAEs could be related, at least in part, to the inhibition of PLD activity or PLD-mediated cellular processes. Membrane disruption has been proposed to be a key event in plant senescence, and PLD has been thought to play an important role in membrane deterioration. Fan et al. (1997) used PLD$_\alpha$-antisense-suppressed Arabidopsis plants to examine the role of PLD$_\alpha$ in plant senescence, which provided direct evidence of PLD$_\alpha$ involvement in ABA- and ethylene-promoted senescence in detached leaves. Another study that investigated the regulation of tomato fruit ripening and its...
relationship to PLD\textsubscript{x} activity drew a similar conclusion as that of Fan and colleagues (Oke et al., 2003). In addition, lysophosphatidylethanolamine that was inhibitory towards cabbage PLD\textsubscript{x} (Ryu et al., 1997) could retard senescence in attached and detached leaves and in fruits of tomato (Farag and Palta, 1993), and could enhance the vase life of snapdragons (Kaur and Palta, 1997). We do not yet know the mode of action of NAEs, but the circumstantial evidence mentioned above indicates that one possible target of NAE(12:0) in petal cells is PLD\textsubscript{x}. However, additional work will be needed to clarify the role of NAEs in plant senescence.

Acknowledgments

We are particularly grateful to Prof. Kent Chapman, The University of North Texas, for having offered beneficial suggestions for this study. We would like to thank Prof. Mingyi Jiang and Prof. Guangdong Wang, Nanjing Agricultural University, for their critical reading and English editing of this manuscript. This study was supported by the Chinese Ministry of Education Foundation (No. 20020307038).

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